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Ectocytosis caused by sublytic autologous complement attack on human neutrophils

The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles

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During sublytic complement attack on human neutrophils, plasma-membrane vesicles are shed from the cell surface as a cell-protection mechanism. By using surface-iodinated neutrophils it was found that $< 2\%$ of surface label was recovered in shed vesicles under conditions where 40% of complement component C9 was shed. SDS/PAGE of ^{125}I-labelled shed vesicles and plasma membranes showed differences in iodination pattern, demonstrating the sorting of membrane proteins into the shed vesicles. Analysis of ^{32}P-labelled phospholipids after labelling of neutrophils with [^{32}P]P_i before sublytic complement attack showed the presence of phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and polyphosphoinositides in shed vesicles. Quantitative analysis using a [^{3}H]acetic anhydride-labelling method showed that the molar proportions of phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin were the same in shed vesicles as in plasma membranes. In contrast, the molar proportions of cholesterol and diacylglycerol relative to sphingomyelin were almost twice those found in plasma membranes. The data demonstrate the existence of protein and lipid sorting mechanisms during the formation of shed vesicles when neutrophils are subject to sublytic complement attack. The term 'ectocytosis' is proposed to describe triggered shedding of right-side-out membrane vesicles from the surface of eukaryotic cells.

INTRODUCTION

Removal of complement membrane-attack complexes (MACs) from the cell surface as a protective mechanism allowing mammalian cells to resist complement attack was first proposed when a rise in cytosolic Ca^{2+} was shown to be the earliest detectable intracellular event during sublytic complement attack (Campbell & Luzio, 1981). It was already known that Ca^{2+} ionophores can cause microvesiculation and membrane shedding from cells without causing lysis (Shukla *et al.*, 1978; Scott & Maercklein, 1979) and that membrane-vesicle formation occurs during complement attack on adipocytes (Richardson, 1979; Richardson & Luzio, 1980). Membrane shedding from mammalian cells without cell lysis has been widely observed both in response to a variety of agents added to the extracellular medium (Holdsworth & Coleman, 1976; Hoerl & Scott, 1978; Scott *et al.*, 1979) and also spontaneously from both normal and tumour cells (reviewed by Black, 1980). Shedding in response to sublytic MAC attack (Morgan *et al.*, 1984, Campbell & Morgan, 1985) is a triggered phenomenon requiring membrane insertion of the terminal complement component C9 and a subsequent rise in cytosolic Ca^{2+} concentration (Morgan & Campbell, 1985; Morgan *et al.*, 1986, 1987; Luzio *et al.*, 1987; Sims *et al.*, 1988; Wiedmer *et al.*, 1990). It is one of several resistance mechanisms [for reviews, see Muller-Eberhard (1988) and Morgan (1989)] that apply particularly when autologous complement is used on nucleated cells, including inhibition of MAC formation by cell-surface receptors and humoral proteins and MAC endocytosis (Carney *et al.*, 1986, 1990).

In the present paper we have investigated the sorting of endogenous plasma-membrane components into shed vesicles after sublytic MAC attack on human neutrophils (polymorphonuclear leukocytes). We have used the conditions previously employed by Morgan *et al.* (1987) and have shown that, during the triggered membrane shedding which occurs after C9 insertion into the MAC, there is evidence of sorting of endogenous cell-surface proteins and membrane lipids into the shed vesicles. We propose the term 'ectocytosis' to describe the triggered shedding of right-side-out membrane vesicles from the surface of eukaryotic cells in order to clearly distinguish this process from endocytosis and exocytosis, in both of which the sorting of membrane components and membrane fusion events also occur.

MATERIALS AND METHODS

Isolation of cells

Neutrophils were isolated from fresh human blood by the method of Böyum (1974), then washed twice in Krebs/Hepes buffer containing NaCl (120 mM), KCl (4.8 mM), KH_2PO_4 (1.2 mM), $CaCl_2$ (1.3 mM) and Hepes (25 mM), adjusted to pH 7.4 with NaOH; BSA (0.1%, w/v) was included in the buffer unless otherwise stated.

Complement reagents

C9 and C9-depleted serum (NHS-C9) were prepared from fresh human serum as described by Morgan *et al.* (1983) using anti-C9 monoclonal antibodies immobilized on immunoaffinity

Abbreviations used: complement proteins are named in accordance with the IUPAC–IUB (1968) recommendations; NHS-C9, normal human serum (NHS) depleted of C9; NA, antibody-sensitized neutrophils; NAC1-8, NA incubated in NHS-C9; p[NH]ppG, guanosine 5'-[$\beta\gamma$-imido]triphosphate; DTT, dithiothreitol; PtdOH, phosphatidic acid; PtdCho, phosphatidycholine; PtdEtn; phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; Sph, sphingomyelin; MAC, membrane-attack complex.

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columns. A sheep erythrocyte haemolytic assay (Lachmann & Hobart, 1978) was used to detect fractions containing NHS-C9 and to confirm that the haemolytic function of these fractions was restored by the addition of pure C9.

Plasma-membrane preparations

Neutrophils were disrupted by sonication as described by Bennett *et al.* (1982) and the sonicated material was centrifuged either on discontinuous sucrose gradients [2.2 M-sucrose (12 ml), 1.6 M-sucrose (8 ml) and 0.6 M-sucrose (4 ml), all in Hepes (pH 7.2)/1 mM-EDTA, as described by Bennett *et al.*, 1982] or on continuous 1–22% (w/v) Ficoll gradients in 0.25 M-sucrose/10 mM-Tes/1 mM-EDTA, pH 7.4, on a 5 ml cushion of 45% (w/v) Nycodenz containing 10 mM-Tes, pH 7.4 and 1 mM-EDTA (Branch *et al.*, 1987). Marker enzymes used to locate the subcellular fractions included 5′-nucleotidase and adenylate cyclase (plasma membranes), lactate dehydrogenase (cytosol), β-*N*-acetylglucosaminidase (lysosomes) and β-glucuronidase (azurophil granules). Measurement of 5′-nucleotidase (Luzio & Stanley, 1983), adenylate cyclase (Salomon *et al.*, 1974), lactate dehydrogenase (Bergmeyer, 1983), β-*N*-acetylglucosaminidase (Maguire *et al.*, 1983) and β-glucuronidase using 4-methylumbelliferyl β-D-glucuronide as substrate (Barrett & Heath, 1977), were as described in the cited references.

^{125}I cell-surface labelling

Neutrophils [(5–7) $\times 10^7$ cells] were prepared from fresh blood (50 ml) as above, washed three times in BSA-free Krebs/Hepes buffer, resuspended in fresh ice-cold BSA-free buffer (1 ml) and transferred to a polypropylene vial (on ice), coated with 200 μg of Iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycoluril) and containing 500 μCi of Na^{125}I (Baron *et al.*, 1986). A preliminary experiment showed that ^{125}I-labelling of trichloroacetic acid-precipitable material was maximal after 30 min. Accordingly, iodination of neutrophils was terminated after 30 min by removing the cells from the Iodogen tube, and washing twice with BSA-free Krebs/Hepes buffer containing KI (5 mM) substituted for KCl, twice with Krebs/Hepes buffer containing 0.1% ovalbumin, and twice with Krebs/Hepes containing 0.1% BSA, all at 4 °C.

Gel electrophoresis

Protein was precipitated from plasma membranes and vesicles with trichloroacetic acid (final concn. 10%, w/v), the pH of the pellets adjusted to 6.8 with Tris/HCl (20 mM), and the pellets then solubilized for 5 min at 100 °C in gel sample buffer, with final concentrations of SDS (5%, w/v), glycerol (3.3% v/v), Tris/HCl, pH 6.8 (20 mM), DTT (10 mM) and Bromophenol Blue (0.003%). The samples were Microfuged (10000 *g*) and the supernatants were applied to a SDS/10%-polyacrylamide gel (Laemmli, 1970). After electrophoresis, protein bands were revealed with Coomassie Blue, the gels dried and autoradiographed. Absorption of bands on the autoradiographs and their position relative to molecular-mass markers was quantified with a Joyce–Loebl Chromoscan 3 scanner using a linear scan setting.

Shed-vesicle preparation for protein studies

The ^{125}I-surface-labelled neutrophils [(2–5) $\times 10^7$/ml], prepared on ice as above, were sensitized on ice for 30 min with a 1:100 dilution of a hybridoma culture supernatant containing a mouse monoclonal antibody (TG1) directed against an antigen present on peripheral blood polymorphonuclear leucocytes (Beverley *et al.*, 1980), then washed three times with ice-cold Krebs/Hepes buffer. The neutrophils [(2–5) $\times 10^7$/ml] were incubated for 30 min at 37 °C with NHS at a dilution previously determined to be sublytic (1:10), then centrifuged at 200 *g* for 5 min. The supernatant, containing shed vesicles, was applied to a column of Sepharose 2B as in Morgan *et al.* (1987), and the vesicle fraction eluted in the void volume from the column, using Tris/HCl buffer (pH 7.4) (10 mM)/NaCl (150 mM)/EGTA (1 mM).

Shed-vesicle preparation for lipid studies

A preliminary experiment showed that, when complement-mediated vesiculation from neutrophils was carried out using NHS as the source of complement (as for protein studies), the Sepharose-column-purification step was not sufficient to remove all traces of plasma lipid from the vesicles. For quantitative lipid studies, we used a method (Morgan *et al.*, 1987) in which sensitized neutrophils (NA) were incubated with NHS-C9 at a concentration previously determined to be sublytic, and then washed at 4 °C with buffer to remove serum lipids. Vesicle shedding was then promoted by adding to the cells (now NAC1-8) an appropriate amount of pure C9. For preparation of ^{32}P-labelled vesicles from neutrophils the Krebs/Hepes buffer contained added glucose (5 mM) in order to maintain ATP levels and so support phospholipid synthesis, which is dependent on ATP. Neutrophils were sensitized by incubation for 15 min at 37 °C with antibody TG1 (1:100 dilution of hybridoma culture supernatant in Krebs/Hepes buffer), washed three times with buffer, then incubated for 45 min at 37 °C with [^{32}P]P$_i$ (300 μCi/ml; Amersham) in Krebs/Hepes. To this was added NHS-C9 at a sublytic concentration, and the incubation was continued at 37 °C for a further 15 min. The ^{32}P-labelled NAC1-8 were washed four times with ice-cold Krebs/Hepes buffer in the coldroom, pure C9 was added, at a concentration equivalent to the amount of NHS-C9 previously added, the preparation incubated at 37 °C for 1 h, spun at 200 *g* for 10 min, and ^{32}P-labelled shed vesicles collected in the supernatant.

Lipid analyses

Neutral lipids and phospholipids were extracted from plasma membranes and shed vesicles by the method of Bligh & Dyer (1959) or Folch *et al.* (1957) and polyphosphoinositides were extracted by the method of Hauser *et al.* (1971). Two-dimensional t.l.c. of ^{32}P-labelled phospholipids was as described by Stein & Smith (1982), and of ^{32}P-labelled polyphosphoinositides as described by Allan & Cockroft (1983), followed by autoradiography. The area of spots on autoradiographs was quantified with the Chromoscan 3, using a raster scan setting. Quantitative analysis of cholesterol, diacylglycerol, PtdEtn, PtdSer, PtdIns and Sph was by an acetylation method using [^{3}H]acetic anhydride [see the accompanying paper (Stein *et al.*, 1991)].

RESULTS

Human neutrophil plasma-membrane fractions for comparison with shed vesicles were prepared by centrifugation on sucrose or Ficoll density gradients. Initially, plasma membrane was isolated on a discontinuous sucrose gradient as described by Bennett *et al.* (1982) for rabbit neutrophils. 5′-Nucleotidase was used as a plasma-membrane marker, with a peak of activity observed in the eluted gradient fractions at a density of 1.130 ± 0.005 g/ml (mean $\pm$ S.E.M. for four separate preparations). A representative gradient profile is shown in Fig. 1(*a*): gradient fraction 25 contained 26% of recovered 5′-nucleotidase activity (with a specific activity 12 times that of the initial sonicated cells), 9% of the lactate dehydrogenase activity and 6.7% of the β-*N*-acetylglucosaminidase activity of the sonicated cells. In a separate experiment adenylate cyclase activity in the sucrose-gradient peak plasma-membrane fraction was 18.8 ± 10.5 pmol of cyclic AMP/min per mg (basal), 28.5 ± 1.0 pmol of cyclic AMP/min

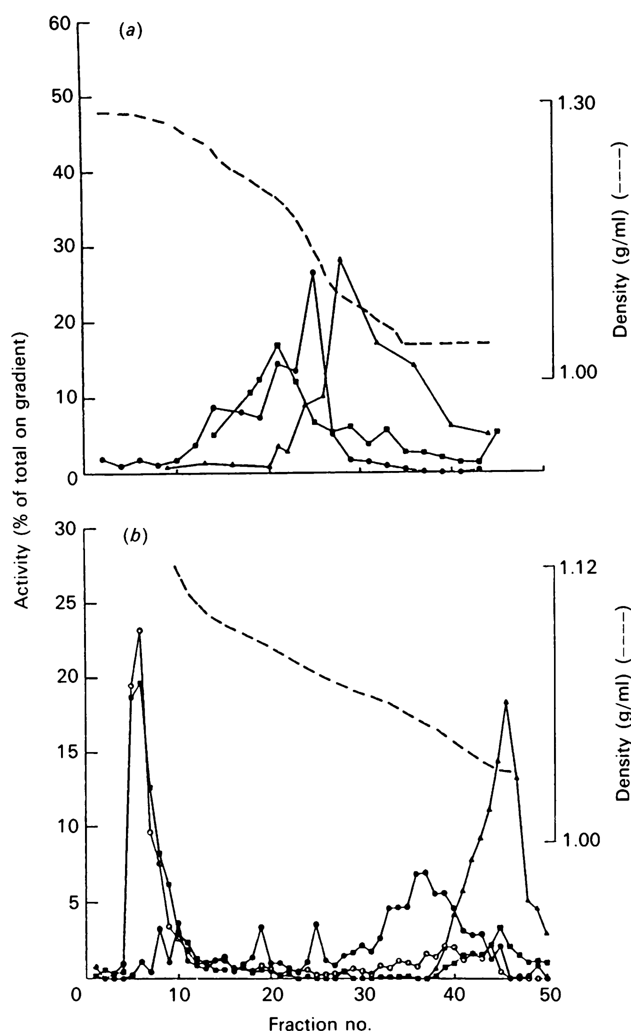

Fig. 1. Distribution of marker enzymes after isopycnic centrifugation

Sonicated neutrophils were centrifuged on either (*a*) sucrose or (*b*) Ficoll gradients as described in the Materials and methods section. Marker enzymes were 5′-nucleotidase (●), lactate dehydrogenase (▲), β-*N*-acetylglucosaminidase (■) and β-glucuronidase (○). Data from representative experiments are shown for both (*a*) and (*b*).

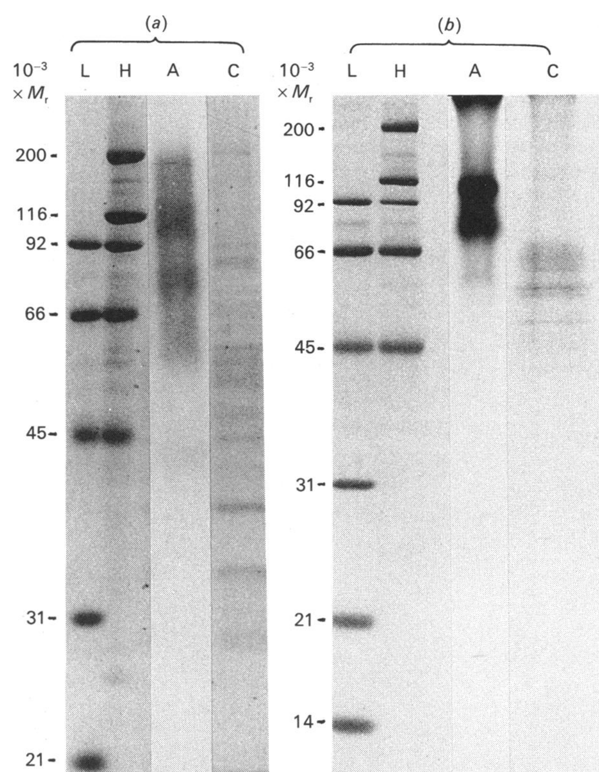

Fig. 2. Comparison of proteins in plasma membranes (*a*) and shed vesicles (*b*)

Solubilized proteins from plasma membranes and shed vesicles from ^{125}I-labelled neutrophils were separated by SDS/PAGE stained with Coomassie Blue (C), then the same gels autoradiographed (A). Low-M_r (L) and high-M_r standards (H) are shown. Data are from a representative neutrophil preparation.

per mg [guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG), 5×10^{-5} M] and 69.2±17.3 pmol of cyclic AMP/min per mg [+p[NH]ppG (5×10^{-5} M)+forskolin (10^{-4} M)].

A plasma-membrane preparation with lower contamination by intracellular enzymes was subsequently achieved by isopycnic centrifugation of sonicated neutrophils on a Ficoll density gradient in a vertical rotor. A peak of 5′-nucleotidase activity occurred in the eluted fractions at a density of 1.053±0.003 g/ml (mean±S.E.M. of four separate preparations). A representative separation (Fig. 1*b*) shows the peak of 5′-nucleotidase activity at gradient fractions 36 and 37. These fractions contained 14% of recovered 5′-nucleotidase activity, with specific activity 11 times that of the sonicated cells. Fractions 36 and 37 also contained < 3% of the lactate dehydrogenase activity, < 1% of the β-*N*-acetylglucosaminidase activity and 6–12% of the β-glucuronidase activity of the sonicated cells.

By using ^{125}I-surface-labelled neutrophils, shed vesicles were obtained by sublytic complement-mediated attack; plasma membranes were also prepared from the same cells in the absence of complement-mediated attack and proteins were precipitated with 10% (w/v) trichloroacetic acid. The percentage of total cell ^{125}I surface label recovered in the shed vesicles was 1.33±0.46 (mean±S.E.M. of three independent experiments). In a separate experiment carried out as described by Morgan *et al.* (1987), using unlabelled neutrophils and ^{125}I-labelled C9, 40% of the label initially bound to the cell surface was found in the shed vesicles. The endogenous proteins of plasma membranes and of shed vesicles prepared from ^{125}I-surface-labelled neutrophils were compared by SDS/PAGE. Both plasma-membrane fractions and shed vesicles contained ^{125}I-labelled proteins of similar M_r values (Fig. 2), though the distribution of radioactivity was different (see Table 1 for collated data from several experiments). There were also differences between the Coomassie-stained proteins of plasma membranes (Fig. 2*a*, lane C) and vesicles (Fig. 2*b*, lane C), with a more restricted range of bands (M_r 40000–70000) in the latter. Similar amounts of plasma membrane and shed vesicle protein were loaded on to the SDS/PAGE gels as measured by Coomassie Blue staining.

In initial experiments to investigate the possibility of phospholipid sorting into shed vesicles, neutrophil phospholipids were labelled by incubation of the cells with [^{32}P]P$_i$ for 1 h before initiation of complement-mediated vesicle shedding. This period of labelling was not long enough to result in equilibrium labelling, but did allow ^{32}P incorporation into phospholipid in sufficient amounts for subsequent detection by autoradiography. For each experiment, plasma membranes and vesicles were prepared simultaneously from the same batch of ^{32}P-labelled NAC1-8 and the ^{32}P-labelled phospholipids extracted and separated by t.l.c. Autoradiographs of the t.l.c. plates showed that ^{32}P-labelled PtdOH, PtdIns, PtdCho and PtdEtn were present in both plasma

Table 1. Distribution of ^{125}I-labelled proteins in autoradiographs of SDS/polyacrylamide gels from plasma membranes and vesicles

Autoradiographs were scanned and the percentage contribution in each M_r range was quantified (see the Materials and methods section). Results are from nine preparations of plasma membranes from four separate experiments and four preparations of vesicles from three separate experiments. Values are means ± S.E.M.

	Plasma membranes		Vesicles	
$10^{-3} \times M_r$	Radioactivity (% of total)	$10^{-3} \times$ Peak mean M_r	Radioactivity (% of total)	$10^{-3} \times$ Peak mean M_r
> 200	12.4 ± 0.9	–	18.2 ± 1.3	–
199–140	9.8 ± 1.2	176 ± 16	10.0 ± 0.7	158 ± 6
139–90	18.9 ± 1.4	114 ± 3	28.2 ± 3.1	130 ± 15
89–70	33.1 ± 2.1	81 ± 2	18.6 ± 5.8	78 ± 2
69–55	16.2 ± 1.2	63 ± 1	7.8 ± 0.9	58 ± 1
< 55	8.0 ± 1.0	–	17.1 ± 2.6	–

Table 2. Comparison of distribution of radioactivity in ^{32}P-labelled lipids in autoradiographs of t.l.c. separations of phospholipids from plasma membranes and shed vesicles

Autoradiographs of t.l.c. of extracted phospholipids from plasma membranes and shed vesicles from three separate neutrophil preparations were scanned by densitometry, the contribution of each spot quantified (see the Materials and methods section) and the percentage contribution for each phospholipid calculated separately for each autoradiograph. Results are presented as means for three experiments ± S.E.M.

	Percentage contribution	
Phospholipid	Plasma membranes	Vesicles
PtdOH	50.8 ± 8.5	33.0 ± 10.5
PtdIns	26.3 ± 5.3	28.9 ± 3.0
PtdCho	26.3 ± 5.3	25.6 ± 9.8
PtdEtn	9.1 ± 4.6	12.6 ± 5.0
Sph*	4.1	–

* Sph was detected on one radiograph only.

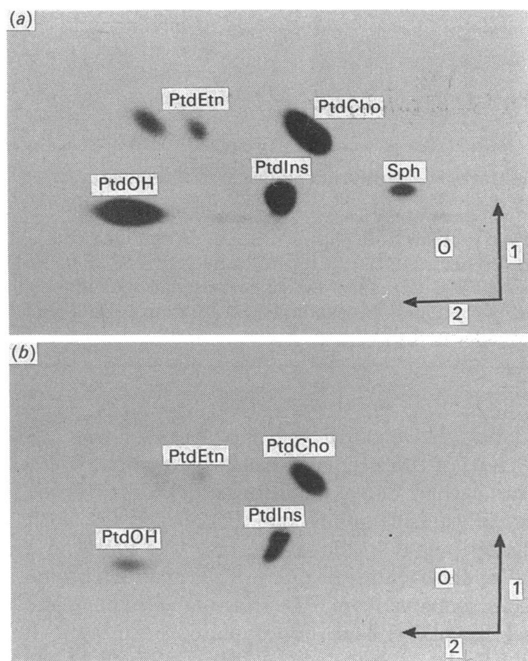

Fig. 3. Autoradiographs of ^{32}P-labelled phospholipids from plasma membranes (*a*) and shed vesicles (*b*)

Phospholipids were separated by t.l.c., and autoradiographs were prepared as described in the Materials and methods section. 'O' marks the origin; the dimensions were as follows: 1, chloroform/methanol/15 M-NH_3 (13:6:1, by vol.); 2, chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.).

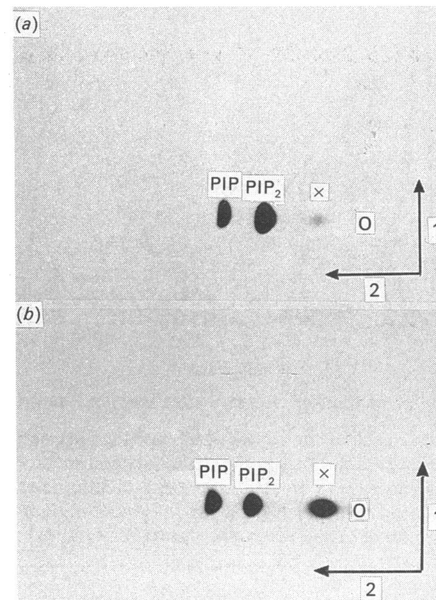

Fig. 4. Autoradiographs of ^{32}P-labelled polyphosphoinositides from plasma membranes (*a*) and shed vesicles (*b*)

Polyphosphoinositides were separated by t.l.c., and autoradiographs were prepared as described in the Materials and methods section. 'O' marks the origin and 'x' is unidentified lipid. The dimensions were as follows: 1, chloroform/methanol/15 M-NH_3 (13:6:1, by vol.); 2, chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol); abbreviations: PIP, PtdIns4*P*; PIP$_2$, PtdIns(4,5)P_2.

membranes and shed vesicles (Figs. 3*a* and 3*b*). ^{32}P-labelled Sph was detected as a minor component in one of the three plasma-membrane preparations (Fig. 3*a*), but was not detected in ^{32}P-labelled lipids of the shed vesicles prepared from the same cells in any of these experiments (Fig. 3*b*). The amount of radioactivity contributed by each phospholipid as a percentage of total radioactivity was quantified for each autoradiograph, and the data from three separate experiments were collated (Table 2). [^{32}P]PtdOH was the most radioactive phospholipid in both plasma membranes and shed vesicles, though the contribution of this species to the total radioactivity was always lower in shed-vesicle preparations than in plasma membranes. [^{32}P]PtdIns contributed similar amounts of radioactivity to plasma-membrane and vesicle preparations, whereas the amounts contributed by [^{32}P]PtdCho and [^{32}P]PtdEtn varied widely.

^{32}P-labelled polyphosphoinositides were extracted from plasma membranes and vesicles prepared simultaneously from the same batch of ^{32}P-labelled neutrophils. [^{32}P]-PtdIns4*P* and-PtdIns-(4,5)P_2 were detected on autoradiographs after separation by t.l.c. (Fig. 4). In one experiment, an unidentified ^{32}P-labelled lipid ('x' in Figs. 4*a* and 4*b*) was detected on autoradiographs from ^{32}P-labelled polyphosphoinositide-containing extracts from both

Table 3. Comparison of lipids of neutrophil plasma membranes and shed vesicles

Plasma membranes and shed vesicles were prepared as described in the Materials and methods section. The lipids were quantified by acetylation with [^{3}H]acetic anhydride (Stein *et al.*, 1991). Results are means ± S.E.M. for the numbers of observations given in parentheses.

	Composition (mol of lipid/10 mol of Sph)	
Lipid	Plasma membranes	Vesicles
Cholesterol	23.9 ± 2.6 (5)	40.5 ± 3.3 (8)
PtdEtn	17.3 ± 1.1 (5)	17.4 ± 2.3 (8)
PtdIns	5.9 ± 1.8 (3)	6.8 ± 2.3 (4)
PtdSer	4.1 ± 0.9 (3)	7.8 ± 2.6 (3)

plasma membrane and vesicles. The unidentified lipid was more polar than PtdIns(4,5)P_2, since it ran more slowly than PtdIns(4,5)P_2 on t.l.c. in an acid solvent system; it had the characteristics of a ^{32}P-labelled lysolipid formed by the action of a phospholipase to remove a fatty acid chain from PtdIns(4,5)P_2. For both plasma membranes and shed vesicles, quantification of the amount of radioactivity contributed by each polyphosphoinositide in two experiments showed that PtdIns(4,5)P_2 (including the lysolipid) contributed 45–70%, the remainder being PtdIns4P.

Since it proved impossible to analyse phospholipid sorting into shed vesicles after non-equilibrium labelling of neutrophils with [^{32}P]P_i, it was necessary to measure the amount of each lipid in plasma-membrane and vesicle preparations. The small amount of shed-vesicle preparation available required the development of a new method of quantification [the accompanying paper (Stein *et al.*, 1990)] capable of measuring 2–3 nmol of each lipid and based on acetylation with [^{3}H]acetic anthydride. The molar proportions of the phospholipids quantified (PtdEtn, PtdIns, PtdSer and Sph) were not significantly different from each other when plasma membranes and shed vesicles were compared (Table 3), but the molar proportion of cholesterol to Sph in shed vesicles was almost twice that found in plasma membranes. The diacylglycerol/cholesterol ratio was found to be 0.53 ± 0.14 (3) and 0.57 ± 0.29 (3) for plasma membranes and shed vesicles respectively from the same neutrophil preparations.

DISCUSSION

The present experiments have extended our knowledge of the membrane events occurring during shedding of membrane-attack-complex-enriched membrane vesicles in response to sublytic autologous complement attack on human neutrophils. In order to compare the macromolecular composition of shed vesicles with plasma membranes, it was necessary to develop a method for the purification of neutrophil plasma membranes. Sonication and sucrose-density-gradient centrifugation achieved a purification and yield of plasma membranes similar to that obtained for rabbit neutrophils (Bennett *et al.*, 1982), though the substitution of isopycnic-density-gradient centrifugation on a Ficoll gradient in a vertical rotor (Branch *et al.*, 1987) improved the purity with respect to intracellular marker enzymes. A sensitive radioassay for 5′-nucleotidase (Luzio & Stanley, 1983) was used to identify neutrophil plasma-membrane fractions, though the specific activity of this enzyme in sonicated human neutrophils was approx. 1% of that found in sonicated rat neutrophils (Newby, 1980), consistent with the previous difficulty in observing this enzyme in human neutrophils (Shirley *et al.*, 1976).

In agreement with a previous study (Morgan *et al.*, 1987) it was found that a large proportion (40%) of surface-bound C9 could be shed from neutrophils when only a small proportion (< 2%) of radioiodinated endogenous cell-surface proteins were released. This level of sorting is analogous to that observed during endocytosis where, for example, 50–80% of cell-surface low-density-lipoprotein receptors are found in coated pits, though these represent only 2% of the cell surface (Goldstein *et al.*, 1979). In the present study, SDS/PAGE of the shed vesicles revealed a restricted composition of membrane proteins by Coomassie Blue labelling relative to whole plasma membranes and provided evidence of differential segregation of endogenous cell-surface-iodinated proteins into the vesicles. The observation of endogenous membrane protein sorting into shed vesicles during sublytic complement attack on human neutrophils is consistent with the sorting observed during complement-induced vesiculation of the plasma membranes of other cell types and during membrane shedding from cell surfaces in response to agents stimulating a rise in cytosolic Ca^{2+} concentration (Shukla *et al.*, 1978; Richardson, 1979; Richardson & Luzio, 1980; Luzio *et al.*, 1987; Wiedmer *et al.*, 1990).

Experiments to measure the appearance of specific phospholipids in shed vesicles by identifying ^{32}P-labelled phospholipids after metabolic labelling of intact cells with [^{32}P]P_i demonstrated the presence of the phospholipid species PtdOH, PtdCho, PtdEtn, PtdIns, PtdIns4P and PtdIns(4,5)P_2 in both plasma membranes and shed vesicles, but no quantitative assessment was possible. We therefore established a quantitative microassay for membrane lipids [see the accompanying paper (Stein *et al.*, 1991)]. By using an [^{3}H]acetic anhydride labelling method, it was possible to quantify all lipid species containing free hydroxy or amino groups (i.e. not PtdOH or PtdCho, but including all the remaining major phospholipids plus cholesterol and diacylglycerol) in the small amounts of membrane obtained in the shed-vesicle preparations. A requirement for the comparison of the lipid composition of plasma membranes with shed vesicles was that the vesicles were not contaminated by lipids in the serum used as a complement source. Shedding was therefore initiated from neutrophils incubated with NHS-C9 (NAC1-8) and extensively washed by addition of purified C9. For true comparison, plasma membranes were isolated from washed NAC1-8. Although the analytical data showed no difference in phospholipid composition (PtdEtn, PtdIns, PtdSer and Sph) between the shed vesicles and plasma membranes, the molar proportions of cholesterol and diacylglycerol compared to Sph were almost doubled in the vesicles. This implies preferential sorting of cholesterol and diacylglycerol into the shed vesicles from the plasma membrane, though from the present data it is not possible to rule out production *de novo* of diacylglycerol at the site of membrane shedding. The increased cholesterol/sphingomyelin ratio in the shed vesicles is of interest in view of the claims that a preferential interaction between these two lipids and cholesterol sorting into sphingomyelin microdomains are responsible for their distribution on membrane traffic pathways (van Meer, 1989). The presence of cholesterol in a membrane has a condensing effect on membrane phospholipids [it has been reported that whereas the area of PtdCho alone in a bilayer is 0.64 nm^2 (64 Å^2), in a bilayer of equimolar cholesterol and lecithin this is reduced to 0.53 nm^2 (53 Å^2); Levine, 1973] and its increased molar ratio in the shed vesicles is consistent with their small size (0.1–1 μm diameter; Morgan *et al.*, 1987) and hence greater membrane curvature compared with the cell-surface plasma membrane (neutrophil diameter 8.5 μm, rising to 12 μm after sublytic complement attack; Morgan, 1988). The increased content of diacylglycerol in the shed vesicles is of interest in terms of the mechanisms by which they originate, since the production of diacylglycerol was a notable feature during

Ca^{2+}-ionophore-induced membrane shedding from erythrocytes (Allan & Michell, 1975). Diacylglycerol is one of two lipids (the other is ceramide) which have been postulated to initiate localized membrane curvature and membrane fusion (Allan & Michell, 1979). There is some evidence for ceramide production during complement attack on rat adipocytes (Richardson & Luzio, 1980).

The shedding of plasma-membrane vesicles from the cell surface without cell lysis has been termed 'exocytosis' by some authors (Allan & Michell, 1979; Morgan *et al.*, 1987). However, it needs to be clearly distinguished from the process normally called exocytosis (Alberts *et al.*, 1989), in which intracellular secretory vesicles fuse with the plasma membrane and open to the extracellular space. We propose that the term 'ectocytosis' be used to describe the triggered shedding of right-side-out plasma-membrane vesicles where sorting of endogenous membrane components occurs. In the present experimental system the shed vesicles have been shown to be right-side-out, since classical complement lesions have been observed by electron microscopy on their surface (Morgan *et al.*, 1987). We have now provided evidence for the sorting of endogenous plasma-membrane proteins and lipids into the shed vesicles. Although it is clear that the incorporation of complement component C9 into the MAC triggers the shedding process via a rise in cytosolic Ca^{2+} concentration (for references, see the Introduction), the signal(s) and mechanism(s) of vesicle formation and release are not as yet known. Since the problems of membrane-vesicle formation, alteration in membrane curvature and membrane fusion are common to secretory and endocytic pathways, a greater knowledge of the mechanisms of ectocytosis is likely to provide insights into membrane events on these other membrane traffic pathways.

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