The Isolation and Characterization of 60 nm Vesicles ('Nanovesicles') Produced During Ionophore A23187-Induced Budding of Human Erythrocytes

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In addition to the microvesicles released during the treatment of human erythrocytes with Ca^{2+} and ionophore A23187, a new subpopulation of still smaller dense vesicles ('nanovesicles') has now been identified. Nanovesicles are about 60 nm in diameter, have an acetylcholinesterase activity higher than that of microvesicles and appear to be relatively enriched in sphingomyelin and correspondingly depleted of phosphatidylethanolamine. They have a polypeptide composition quite different from those of erythrocyte membranes or microvesicles, consisting largely of components of mol.wts. 60 000 and 26 000 in addition to haemoglobin. These two major polypeptides do not appear to represent contaminating cytoplasmic proteins or proteolytic subfragments of a larger protein.

It has been shown that human erythrocytes, either when treated with Ca^{2+} and ionophore A23187 or during aging *in vitro*, respond by budding to release microvesicles (Allan *et al.*, 1976; Rumsby *et al.*, 1977; Lutz *et al.*, 1977). These microvesicles are about 150 nm in diameter and consist of erythrocyte cytoplasm enclosed by a plasma membrane that differs in certain important respects from the parent erythrocyte membrane. In particular, the microvesicles contain no spectrin (bands 1 and 2) or actin (band 5)*, which are prominent components of the normal erythrocyte membrane. Shukla and his colleagues (Shukla *et al.*, 1978) have extended these observations to show that of the two major membrane polypeptides that do remain in the microvesicles (bands 3 and 4.1), one of these (band 4.1) appears to reside in a curious membranous appendage ('tail') to each microvesicle that is often observed in electron micrographs (Allan *et al.*, 1976; Shukla *et al.*, 1978) and that can be detached by sonication. Shukla *et al.* (1978) and Allan & Mitchell (1979b) have suggested that segregation of polypeptide 4.1 into this 'tail' region may be significant in the membrane fusion process that leads

to release of microvesicles. The potential importance of this 'tail' structure as a relic of the membrane fusion event led us to investigate whether or not such a structure could be released spontaneously during ionophore A23187-induced budding so that it might be isolated in a more native form than by the sonication procedure adopted by Shukla. The work described in the present paper provides evidence for spontaneous release of dense vesicles even smaller than microvesicles that may be derived from those regions of the erythrocyte membrane that undergo fusion during the budding process. Surprisingly, in view of the results of Shukla *et al.* (1978) these 'nanovesicles' contain polypeptides 4.5 (mol.wt. 60 000) and 8 (mol.wt. 26 000) as the major species, with only traces of polypeptides 3 and 4.1.

Materials and Methods

Production and isolation of microvesicles and nanovesicles

Outdated citrate-treated human blood was obtained from University College Hospital, and the erythrocytes were sedimented and washed four times with 0.15 M-NaCl with removal of the buffy coat.

About 100 ml of packed erythrocytes was resuspended in 200 ml of 0.15 M-NaCl containing 14 mM-Hepes/NaOH buffer, pH 7.4, and the cells were then incubated at 37°C in the presence of

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

* The classification of Steck (1974) is used throughout.

1 mM-CaCl₂ and 5 μ M-ionophore A23187 (Eli Lilly and Co., Indianapolis, IN, U.S.A.). After 1 h the erythrocytes were sedimented at 500 *g* for 5 min and the cloudy supernatant was removed. This supernatant was then centrifuged at 16 000 *g*_{av.} for 20 min in a Sorvall RC5B machine to give a red pellet of microvesicles (see Allan *et al.*, 1976). The supernatant was removed and any pink flocculent material ('ghosts') overlaying the red pellet was removed by aspiration and discarded.

The pellet of microvesicles was resuspended in Hepes-buffered saline (0.15 M-NaCl) and spun at 500 *g* for 5 min to remove any contaminating cells. The supernatant was removed and spun at 16 000 *g*_{av.} for 20 min. The resulting supernatant was discarded together with any 'ghosts' still appearing on top of the pelleted microvesicles.

The microvesicles were finally resuspended at a protein concentration of approx. 20 mg/ml in 0.15 M-NaCl.

The original supernatant from the first 16 000 *g*_{av.} centrifugation was centrifuged at 100 000 *g*_{av.} for 60 min in a Beckman L2 65 machine. A very small pink pellet was obtained that was resuspended in about 10 ml of 0.154 M-NaCl. This suspension was then centrifuged at 16 000 *g*_{av.} for 20 min to remove any microvesicles and 'ghosts' still present.

The resulting supernatant was then centrifuged at 100 000 *g*_{av.} for 60 min and the final pellet of nanovesicles was resuspended in 0.2 ml of 0.154 M-NaCl.

In some experiments, fresh blood obtained from either D.A. or P.T. was used. Results with fresh blood were essentially the same as those with outdated stored blood.

Extraction of lipids

Lipids were extracted from the various samples by addition of 3.75 vol. of chloroform/methanol (1:2, v/v) and the resulting single-phase system was partitioned by addition of 1.25 vol. each of chloroform and 2 M-KCl. The lower (chloroform) phase from each sample was evaporated to dryness under reduced pressure and dissolved in 50 μ l of chloroform before analysis by t.l.c.

Chromatography of lipids

Phospholipids were separated by t.l.c. by the procedure of Skipski *et al.* (1964), but without addition of bicarbonate to the silica gel slurry. Neutral lipids were separated by t.l.c. on silica gel HR (Merck) plates in benzene/diethyl ether (5:4, v/v) containing a drop of concentrated ammonia solution.

Individual lipids were excised and quantified by the methods described below.

Phospholipids were quantified by a modification of the procedure of Bartlett (1959). After digestion

with 0.5 ml of 72% HClO₄ at 180°C for 2 h, the sample was cooled and 3 ml of water was added. The samples were then mixed with 0.5 ml each of 2.5% ammonium molybdate and 10% ascorbic acid. This mixture was then incubated at 60°C for 20 min and the resulting blue colour read at 830 nm on a Cecil CE 272 linear readout u.v. spectrophotometer.

Cholesterol was determined by the procedure of Brown *et al.* (1954).

Diacylglycerol was measured as described previously (Allan *et al.*, 1978).

Protein was determined as described by Lowry *et al.* (1951). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out on 7.5% gels (6 mm in diameter), as described by Weber & Osborn (1969). Samples containing about 100 μ g of protein were heated at 100°C for 5 min in 2% sodium dodecyl sulphate before application to the gels. Polypeptides were stained with Coomassie Brilliant Blue and glycoproteins were identified by periodate/Schiff staining (Zacharias *et al.*, 1969). Estimation of polypeptide molecular weights was achieved by comparison with molecular-weight standards run on duplicate gels. These standards were in the Combithek kit supplied by Boehringer G.m.b.H., Mannheim, Germany, and comprised RNA polymerase (subunit mol.wts. 165 000, 155 000 and 39 000), bovine serum albumin (mol.wt. 68 000) and trypsin inhibitor (mol.wt. 21 000).

In some cases, stained gels were scanned with a Joyce-Loebl scanning spectrophotometer and a quantitative measure of the relative amounts of protein in each band was obtained by integration.

Acetylcholinesterase (EC 3.1.1.7) was assayed by Ellman's method (Ellman *et al.*, 1961).

Catalase (EC 1.11.1.6) was assayed by a spectrophotometric method (Werner & Heider, 1963) and by a rapid-titration procedure (Takahara *et al.*, 1960).

¹²⁵I-labelling of erythrocytes

Intact erythrocytes were iodinated with ¹²⁵I (The Radiochemical Centre, Amersham, Bucks., U.K.) using iodogen as described by Markwell & Fox (1978), and microvesicles and nanovesicles were isolated from the iodinated cells as described above. Samples of cell membranes, microvesicles and nanovesicles were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After staining with Coomassie Brilliant Blue visible bands on the gels were excised and counted for radioactivity in a gamma counter.

Electron microscopy

Specimens for electron microscopy were fixed in 6.25% glutaraldehyde in 0.14 M-cacodylate buffer (pH 7.4) for 0.5 h at 4°C, washed in buffer and post-fixed for 2 h in 1% OsO₄ in 0.1 M-veronal

EXPLANATION OF PLATE 1

Electron micrographs of (a) microvesicles and (b) nanovesicles from ionophore A23187-treated erythrocytes
Microvesicle 'tails' are arrowed in (a). Inset in (a) is a thicker section of the same sample, which shows the 'tails' more clearly. Magnification $\times 65\,000$.

acetate buffer (pH 7.4). After dehydration in ethanol the material was embedded in Araldite or Spurr resin, and sections were stained in methanolic uranyl acetate followed by Sato's lead stain.

Results

Electron microscopy of microvesicles and nanovesicles (Plate 1)

The electron-microscopic appearance of microvesicles (Plate 1a) was similar to that described before; most were about 150–200 nm in diameter and many showed the presence of a 'tail' structure (arrowed in Plate 1a). However, in some preparations, there was evidence for the existence of extended vermiform structures as long as 500 nm, which appeared to be in the process of breaking up into smaller spheres. The nanovesicles (Plate 1b) were much smaller than the microvesicles; their average diameter was about 60 nm, but there were some indications of the presence of linear aggregates of the smallest unit. As with intact erythrocytes it was not easy to resolve the plasma membrane of any of these vesicular particles from their dense internal contents (mainly haemoglobin). However, no structures corresponding to the microvesicle 'tails' were observed on the nanovesicles.

Polypeptide composition of membranes, microvesicles and nanovesicles

Fig. 1 shows the sodium dodecyl sulphate/polyacrylamide-gel pattern of membranes, microvesicles and nanovesicles from erythrocytes treated with ionophore A23187. As demonstrated previously, microvesicles are largely devoid of spectrin (bands 1 and 2) and actin (band 5), but two other major membrane polypeptides (3 and 4.1) are well represented in the microvesicles. Traces of a polypeptide running in the band 4.5 region were seen in some preparations. The remaining bands in the microvesicles are probably cytoplasmic proteins including haemoglobin (Allan *et al.*, 1976). The polypeptide pattern of the nanovesicles is considerably different both from erythrocyte membranes and from microvesicles: the major polypeptides are 4.5 (mol.wt. 60 000) and 8 (mol.wt. 26 000), which each account for about 30–40% of the total Coomassie-staining material; haemoglobin accounts for most of the remainder. With larger amounts of material on the gels, traces of polypeptides 3 and 4.1 could be seen, but spectrin and actin were usually undetectable. No glycoproteins were observed in the nanovesicles when gels were stained with periodate/Schiff reagent under conditions where the normal three major glycoproteins (PAS-1, -2 and -3) were clearly visible in gels of erythrocyte membranes.

The only pronounced change seen in the mem-

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of (a) membranes from untreated cells, (b) membranes from cells treated with ionophore A23187 and Ca^{2+} for 1 h, (c) microvesicles and (d) nanovesicles. Abbreviation: Hb, haemoglobin.

branes of residual cells after treatment with ionophore A23187 was an increase in Coomassie-staining material that did not enter the gel (band O). Gel scans revealed a slight decrease in spectrin in the residual cell membranes compared with the membranes of untreated cells.

The composition and enzymic activity of nanovesicles in relation to cell membranes and microvesicles

For membranes and microvesicles the results shown in Table 1 are broadly similar to those in previous reports (Allan *et al.*, 1976; Lutz *et al.*, 1977; Shukla *et al.*, 1978). The yield of microvesicles was somewhat less in the present experiments, but this is accounted for by the shorter incubations now used to induce vesiculation. Measurements of total protein in each fraction can be somewhat misleading, since both microvesicles and nanovesicles contained encapsulated haemoglobin; a more meaningful quantity is the ratio of membrane protein to lipid, and this was calculated by measurement of peak areas in gel scans (see the Materials and Methods section) to assess the relative

amounts of membrane proteins and haemoglobin. For microvesicles, cytoplasmic proteins (mainly haemoglobin) accounted for 85–90% of the total protein, whereas for the nanovesicles, cytoplasmic proteins were about 20% of the total. It is clear from Table 1 that relative to lipids, microvesicles and especially nanovesicles are depleted in membrane protein compared with cell membranes.

Although the lipid compositions of the various fractions were similar, two significant alterations were seen. The first, which has been noted before (Allan & Michell, 1975; Allan *et al.*, 1976), was an increase in 1,2-diacylglycerol content in membranes from ionophore A23187-treated cells and in the two vesicle fractions. In contrast with previous results (Allan *et al.*, 1976), it did not appear that there was a selective increase in the diacylglycerol content of the microvesicles compared with the membranes of treated cells. The second change was a marked increase in the sphingomyelin content of the nanovesicles, which was reflected in a decreased content of phosphatidylethanolamine.

As seen previously (Allan *et al.*, 1976; Shukla *et al.*, 1978), acetylcholinesterase activity was increased in microvesicles relative to cell membranes. This was true also for the nanovesicles, which have about double the activity of the microvesicles in proportion to their lipid contents, but a much higher activity on the basis of protein content. Catalase activity in microvesicles and nanovesicles was lower than that in whole cells and could be explained in terms of their contents of encapsulated erythrocyte cytoplasm.

Time course of production of microvesicles and nanovesicles (Fig. 2)

The production of nanovesicles appeared to approximately parallel the release of microvesicles: both species increased with incubation time up to about 1 h, but thereafter release was very much slower. Cell lysis, on the other hand, was low initially, but increased markedly after 1 h incubation. Samples of microvesicles and nanovesicles taken after 5, 15 and 30 min incubation had lipid and polypeptide compositions indistinguishable from samples isolated after incubation for 1 h. Control incubations either in the absence of ionophore A23187 or with EGTA substituted for Ca^{2+} yielded no visible microvesicles or nanovesicles. Inclusion of proteinase inhibitors (1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one or 7-amino-1-chloro-3-L-tosylamidoheptan-2-one) together with ionophore A23187 and Ca^{2+} had no effect on the release of either species of vesicle. No vesicles of either type were observed in whole untreated blood or when cells were lysed osmotically in the presence or absence of Ca^{2+} . In experiments where erythrocytes were iodinated with ^{125}I and then incubated with

Fig. 2. Time course of release of vesicles from erythrocytes treated with ionophore A23187 and Ca^{2+} . Unfilled symbols correspond to incubations in the absence of ionophore.

ionophore A23187 and Ca^{2+} , microvesicles were released containing radioactive polypeptides 3 and 4.1. However, no significant radioactivity was detected in bands 4.5 or 8 of the nanovesicles, or in any other part of their gel pattern. Radioactivity in erythrocyte membranes was confined largely to polypeptides 3 and 4.1; no measurable activity was seen in the regions of bands 4.5 and 8.

Discussion

A variety of interrelated metabolic and morphological changes occur in human erythrocytes in response to the increase in intracellular Ca^{2+} concentration produced by treatment with ionophore A23187 (Allan & Michell, 1979a). One of the most profound of these changes is the conversion of the normal discocyte into a spherocytic cell that is able to release membrane material in the form of small vesicles containing encapsulated erythrocyte cytoplasm. In the present paper, it is shown that two classes of vesicle are released (termed microvesicles and nanovesicles) that can be differentiated on the basis of size and polypeptide composition. From the electron micrographs (Plate 1) there appeared to be a clear distinction in size and morphology between the microvesicles and nanovesicles, suggesting that the two types represented discrete populations. This impression was confirmed by the results of polyacrylamide-gel electrophoresis (Fig. 1), which showed that microvesicles and nanovesicles contained few polypeptides in common other than haemoglobin. Neither fraction contained any spectrin or actin, which are prominent polypeptides in normal erythrocyte membranes.

Some other apparent differences between the

microvesicles and nanovesicles are a consequence of their different sizes and especially of the extremely small dimensions of the nanovesicles where the thickness of the membrane (approx. 8 nm) represents a significant proportion of the total vesicle radius (approx. 30 nm). Thus the differences in lipid-to-protein ratio (Table 1) between the two classes of vesicles can be largely accounted for in terms of their volume/surface ratios. This factor is well illustrated in the measurements of acetylcholinesterase activity (Table 1), which appears to be very much increased in the nanovesicles when measured on a protein basis, but which is seen to be much more similar to the activity in the microvesicles when compared with a standard amount of membrane lipid. Both vesicle fractions are, however, enriched in acetylcholinesterase relative to intact cells and 'ghosts'; it appears that approx. 40% of the total cellular acetylcholinesterase is released in vesicles during treatment with ionophore A23187 and Ca^{2+} .

There may be some importance attached to the repeated observation of a small increase in the amount of sphingomyelin and a small decrease in the amount of phosphatidylethanolamine in the nanovesicles compared with cells and microvesicles (Table 1). The extreme curvature of the membrane of the nanovesicles would lead to an excess of outer leaflet area over inner leaflet and thus possibly to an increase in the amounts of those lipids normally present in the outer leaflet (e.g. sphingomyelin) relative to those in the inner leaflet (e.g. phosphatidyl-

ethanolamine). It is not clear why a similar effect does not appear to apply to phosphatidylcholine and phosphatidylserine, although in the case of these lipids the effect would be more difficult to detect either because the distribution is less polarized between the two leaflets (phosphatidylcholine) or because the smaller total amount of lipid (phosphatidylserine) makes reliable measurement less easy. The implication of these findings is that in the budding process that leads to release of nanovesicles, inner leaflet lipid, especially phosphatidylethanolamine, is squeezed out of those regions of the membranes that have a very low radius of curvature.

Our failure to confirm a previous report (Allan *et al.*, 1976) of a much higher 1,2-diacylglycerol content in the microvesicles compared with that in ionophore-treated cells (see Table 1) argues against the idea that this lipid is directly involved in the membrane fusion event that leads to vesiculation. However, our results do confirm a general increase of 1,2-diacylglycerol in the treated cell membranes and in the two vesicle populations, compared with untreated cells. This increase, which is thought to be at the expense of polyphosphoinositide (phosphatidylinositol phosphate + phosphatidylinositol bisphosphate) in the inner leaflet of the membrane bilayer (Allan & Michell, 1978), could be sufficient to produce a significant change in the relative areas of the two leaflets and thus force a transition from the original discocyte to an echinocytic shape (Allan *et al.*, 1978; Beck, 1978).

The origin and significance of polypeptides 4.5

Table 1. *Composition and enzymic activity of nanovesicles compared with cell membranes and microvesicles*
Results are expressed as means $\pm$ S.D. for values from several different experiments. The number of experiments is shown in parentheses.

	Membranes from untreated cells	Membranes from ionophore A23187-treated cells	Microvesicles	Nanovesicles
Cell protein (%)	2 (2)	2 (2)	0.35 ± 0.06 (4)	0.0036 ± 0.0009 (4)
Cell phospholipid (%)	100	89 (3)	10.5 ± 0.2 (4)	0.49 ± 0.32 (4)
Cell cholesterol (%)	100	90 (3)	9.4 ± 0.6 (4)	0.43 ± 0.23 (4)
Cholesterol/phospholipid (molar ratio)	0.77 ± 0.07 (5)	0.71 ± 0.02 (5)	0.70 ± 0.03 (5)	0.76 ± 0.10 (5)
Cholesterol/diacylglycerol (molar ratio)	1067 ± 450 (3)	249 ± 40 (4)	303 ± 50 (5)	234 ± 50 (5)
Phospholipid/protein ($\mu\text{mol}/\text{mg}$)	0.75 (4)	—	0.48 ± 0.10 (4)	4.1 ± 1.4 (4)
Phospholipid composition (mol %)				
Sphingomyelin	27 ± 3 (5)	26 ± 1 (5)	27 ± 3 (5)	$32 \pm 2^*$ (5)
Phosphatidylcholine	30 ± 2 (5)	30 ± 1 (5)	29 ± 1 (5)	29 ± 4 (5)
Phosphatidylserine	12 ± 2 (5)	14 ± 2 (5)	14 ± 3 (5)	13 ± 3 (5)
Phosphatidylethanolamine	31 ± 1 (5)	30 ± 2 (5)	30 ± 2 (5)	$26 \pm 1^*$ (5)
Acetylcholinesterase activity				
($\mu\text{mol}/\text{min}$ per mg of protein)	3.0 ± 0.5 (5)	1.6 (2)	4.2 ± 0.5 (5)	63 ± 5 (3)
($\mu\text{mol}/\text{min}$ per μmol of phospholipid)	4.0 ± 0.8 (5)	2.1 (2)	8.9 ± 1 (5)	15.4 ± 1 (3)
Catalase ($\mu\text{mol}/\text{min}$ per mg of protein)	$139 \pm 7^\dagger$ (3)		77 ± 3 (3)	83 ± 20 (3)

* Significantly different from membranes at $P < 0.025$.

† This value is for whole cells, not membranes.

and 8, the characteristic protein components of the nanovesicles, remain unknown. Corresponding polypeptides were present in the electrophoretic pattern of membranes from whole cells (although this was only apparent for band 8 when other components were overloaded) but no marked differences in these were seen in membranes derived from treated cells compared with those from normal cells; this is perhaps not surprising considering that the nanovesicles represent a very small proportion of the total cells. An increase in the amounts of bands 4.5 and 8 has been reported by other groups in cells incubated without glucose (Palek *et al.*, 1978) or with ionophore A23187 (Allen & Cadman, 1979) or with oleoylglycerol and Ca^{2+} (Quirk *et al.*, 1978) and it has been suggested that band 4.5 may represent catalase adsorbed to membranes in the presence of Ca^{2+} (Allen *et al.*, 1977; Allen & Cadman, 1979). However, in the nanovesicles we have been unable to demonstrate amounts of catalase activity that could be at all consistent with the idea that this enzyme is the major polypeptide. The activity in the nanovesicles is actually less than that present in whole cells, which had similar activities to those found by other workers (Pennell, 1964). Furthermore, polypeptides 4.5 and 8 seem to be specific for the nanovesicles; they are not apparent in the microvesicles a fact that is difficult to explain if they are adsorbed cytoplasmic components. Neither of the major polypeptides of the nanovesicles corresponds to acetylcholinesterase, which migrates with a molecular weight of about 80000 on sodium dodecyl sulphate/polyacrylamide gels. Another possibility is that polypeptides 4.5 and 8 are proteolytic subfragments of a larger membrane protein (Allen & Cadman, 1979; Quirk *et al.*, 1978). Their combined molecular weights would be about 80000–90000, which is in the range of the molecular weights of bands 3 and 4.1. However, although these latter polypeptides (but not spectrin) were well labelled with ^{125}I when intact cells were incubated with this isotope, no label was detected in either of the major polypeptides of the nanovesicles, suggesting that polypeptides 4.5 and 8 were not derived from polypeptides 3 or 4.1. Consistent with this finding, inhibitors of proteinase activity (7-amino-1-chloro-3-L-tosylamidoheptan-2-one and 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one) had no effect on the production of microvesicles and nanovesicles or their polypeptide composition. Our results therefore do not lend support to those studies which appear to demonstrate a Ca^{2+} -dependent proteolytic activity degrading the major erythrocyte polypeptides. It should be noted, however, that these studies involved the use of higher ionophore A23187 or Ca^{2+} concentrations than we have used (Allen & Cadman, 1979; Quirk *et al.*, 1978).

We therefore believe that polypeptides 4.5 and 8

are characteristic components of the membrane of the nanovesicles, although at present we can only speculate as to their role in the ionophore-induced budding process. In initiating this study, we had hoped to be able to isolate a fraction representing a fragment of the 'neck' region joining the budding microvesicle to the main body of the erythrocyte, perhaps corresponding to the microvesicle 'tail', which has been seen previously (Shukla *et al.*, 1978). The nanovesicles described in the present paper could correspond to fragments of the neck region, although it is surprising that they contain no polypeptide 4.1, which is the only polypeptide in the 'tails' as isolated by Shukla *et al.* (1978). However, there are indications from electron microscopy of the presence of extended 'neck' structures in budding erythrocytes ('myelin forms'; Bessis, 1973), which may fragment into smaller particles independent of the microvesicles that represent the terminal region of the buds. These smaller particles could be the nanovesicles referred to in the present paper; if so, their isolation and characterization would represent a further stage in the microdissection of the membrane close to the focus of membrane fusion.

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