Reticulocyte Maturation and Exosome Release: Transferrin Receptor Containing Exosomes Shows Multiple Plasma Membrane Functions

By R.M. Johnstone, A. Bianchini, and Kathy Teng

Vesicles (exosomes) released during sheep reticulocyte maturation contain a number of plasma membrane functions. Using an antibody coated, magnetic core bead, it has been shown unequivocally that vesicles that contain the transferrin receptor also contain other plasma membrane activities, such as the nucleoside transporter and acetylcholinesterase. Lysosomal activities, normally found in the maturation contain a number of plasma membrane functions.

It has long been observed that the mature mammalian red cell has reduced enzyme activities compared with its reticulocyte counterpart. Pan and Johnstone have proposed that the loss of plasma membrane proteins during maturation is achieved by a form of exocytosis and have shown that the sheep reticulocyte externalizes a variety of plasma membrane activities during maturation in vitro. Many of these activities can be recovered, at least in part, in a vesicular structure, which we have named the exosome.

Although exosomes have been detected during in vitro maturation of sheep cells, their presence has not yet been found in the circulation of anemic sheep or other animals. The exosome, ~50 nm in diameter, is membrane bound, containing phospholipids characteristic of the plasma membrane. The major recognizable protein in the exosome is the transferrin receptor (TFR), the disappearance of which from the mammalian red cell during maturation was established initially by Jandl and Katz and subsequently by others.

In addition to plasma membrane proteins, the exosome population from sheep reticulocytes shows the presence of lysosomal enzymes but is devoid of mitochondrial or cytoplasmic activities, such as succinate or lactate dehydrogenase. A ~70 Kd protein, identified as the clathrin uncoating ATPase, is present in exosomes in sufficient amounts to be detected by Coomassie blue staining.

The wide variety of plasma membrane enzymes and lysosomal enzymes in the 100,000 x g fraction prompted us to determine whether each exosome has a mixture of activities or is dedicated to a specific protein. Externalization is highly selective, and some membrane proteins, such as the anion exchanger, are never detected in the exosome population. The presence of several proteins in a single exosome could suggest that the proteins selected for externalization have a common feature that signals a maturation-dependent release. Therefore, it was first necessary to establish the distribution of activities in a single exosome population. To achieve this result, we have used a magnetic immunoaffinity support to remove selectively exosomes that bear the transferrin receptor. This communication reports the efficacy of this technique to isolate a specific population of subcellular organelles as well as providing evidence for the multiplicity of functions in a single population of exosomes. Furthermore, it is shown that exosome formation occurs in vivo in several mammalian species.

MATERIALS AND METHODS

Preparation of reticulocytes. Blood containing enriched reticulocytes was obtained from phlebotomized sheep as described by Benderoff et al and Pan et al. For preparation of anemic rats and rabbits with concomitant reticulocyte formation, phenylhydrazine treatment and phlebotomy were used. Blood from piglets less than 1-month-old was used directly.

Isolation of exosomes from the circulation. To determine the presence of exosomes in the circulation, heparinized blood was centrifuged twice at 7,700 x g for 8 minutes. The cell-free supernatant, diluted 1:1 with isotonic saline, was then centrifuged at 100,000 x g for 90 minutes over a 1 mL, 26% sucrose cushion. After complete removal of the supernatant, the tube was wiped dry and the small pellet stored frozen at ~70°C until required or suspended immediately in glutaraldehyde fixation buffer for electron microscopy of the pellet.

Formation and harvesting of vesicles in vitro. Washed, enriched sheep reticulocyte suspensions (70% to 90%) were cultured at 3% hematocrit in MEM containing 5 mmol/L adenosine and 10 mmol/L inosine as well as 4 mmol/L glutamine, penicillin, and streptomycin as described by Benderoff et al and Pan et al. After overnight incubation with gentle rotation at 37°C (or at the given temperature), the cells were removed by centrifugation and vesicles collected. The pellets were frozen and stored or suspended in the medium specified in the text and used immediately.

Preparation of immobilized aTFR. Dynal beads, containing covalently linked sheep, anti-mouse antibody were washed by centrifugation to remove the original medium and suspended in PBS. To the washed beads (70 μL) was added 20 μg of mouse IgG containing a monoclonal antibody against the human transferrin receptor (αTFR). In all cases a second sample was treated identically with non-immune-mouse IgG to examine the nonspecific binding. The IgG was allowed to bind to the beads in a rotating device at room temperature for 4 hours. Then excess unbound antibody was removed by centrifuging the beads (20 seconds at 12,000 x g in a microfuge). After removal of the supernatant, the beads were resuspended in PBS containing 20 μg/mL ovalbumin using a 200-fold excess of wash medium over bead volume. Following the ovalbumin wash, the procedure was repeated twice with PBS alone. To the IgG-containing beads was added a suspension of vesicles in...
PBS. After a number of trials, we found that if vesicles derived from ~3 x 10^6 cells (approximately 50 µg protein) were applied to 70 µL beads (2.8 x 10^5 beads) coated with αTFR, greater than 85% of the TFR in the suspension of vesicles could be recovered in the beads. If vesicles from more cells were used, part of the receptor remained in the supernatant. Vesicles were incubated overnight with rotation at 4°C with IgG-coated beads. On the following morning the magnetic beads were separated from the suspension with the Dynal magnet and both supernatant (S) and pellet (P) retained. The beads were washed twice with PBS and the wash added to the supernatant. The beads were suspended to the same volume as the pooled supernatants. Enzyme assays or SDS gel electrophoresis were carried out on the washed bead pellets and the supernatants derived therefrom.

EM of vesicles adsorbed to magnetic beads. To examine by EM the population of vesicles immobilized by the Dynal beads, the following procedure was adopted. Vesicles derived from ~15 mL of blood of phlebotomized rats (~30% circulating reticulocytes) were attached to the Dynal-bead specific antibody complex or nonimmune antibody complex. After removal of the unbound material by washing, the vesicle-Dynal bead complex was treated at room temperature with a solution of 4 mol/L urea, 0.2 mol/L glycine (pH 2.3) for 3 minutes and quickly neutralized with Na2PO4 (pH 12) to bring the pH to 7.0. The beads were removed by centrifugation and the supernatant was fixed in buffered glutaraldehyde and processed for thin sectioning.

Detection of TFR. To measure the presence of the transferrin receptor in the vesicles bound to Dynal beads, SDS gel buffer, containing mercaptoethanol, was added to the vesicle-containing bead pellet (P), heated for 5 minutes at 100°C, and the whole suspension including beads added to the SDS gels and electrophoresed according to Laemmli.13 The beads stayed in the stacking gel during electrophoresis. The supernatant (S) from the beads was processed for SDS gel electrophoresis in an identical way.

To measure 125I-transferrin (TF) binding to the beads (P) and supernatants (S) respectively, the ammonium sulphate precipitation procedure described by Klausner et al14 was used. TFR containing (P) and (S) were incubated with 125I-TF for 10 minutes at room temperature. Excess human TF (500-fold) was used to assess nonspecific binding. For filtration of the 125I-TF-TFR complex formed, the glass fiber filters were soaked in 30% (NH4)2SO4 with 0.8% bovine albumin to assure adherence of the magnetic beads to the filters.

Nucleoside Binding

[3H]-Nitrobenzylthioinosine (NBMPR) was used to measure the presence of the nucleoside transporter.15 The procedure for binding to vesicles was described before1 and is based on the method by Hammond and Martin.19 Both P and S obtained as above were assayed for activity.

Lysosomal Enzymes

To measure N-acetyl β-glucosaminidase, β-glucuronidase, and acid phosphatase, the umbelliferyl derivatives of N-acetyl β-glucosaminidase, β-glucuronidase, and the phosphate were used. The fluorescent product obtained after hydrolysis was measured as described by Peters et al,17 using an excitation wave length of 365 nm and emission at 460 nm.

Acetylcholine Esterase

The procedure used was described before and is an adaptation of the method by Eillman et al18 and both P and S were assayed. Because the beads are intensely colored, continuous measurements of acetylcholine hydrolysis with the suspension of beads was not possible. Instead, at intervals the suspending medium was withdrawn using the Dynal magnet to immobilize the beads, the absorbance of the suspending medium at 412 nm measured, and then returned to the beads for continued incubation at room temperature. The acetylcholinesterase in the supernatant was monitored by continuous assay of the change in absorbance at 412 nm.

Materials

Sheep transferrin was isolated following the procedure of Morgan et al19 and iodinated as described by Hunter and Greenwood.20 Human transferrin was purchased from Sigma Biochemicals (St Louis). The monoclonal antibody against the sheep transferrin receptor was a gift from J. Larrick of Genelabs, Redwood, CA. The antibody against rat TFR (DOX-26) was a gift from Dr A. Williams, MRC Cellular Immunology, Sir Thomas Dunn School of Pathology, University of Oxford. Mouse IgG was isolated from mouse serum by protein A chromatography. Pig blood from young animals was obtained courtesy of Dr C. Chavez, MacDonald College, McGill University. 3H-NBMPR was purchased from Moravec Biochemical Inc, Brea, CA and stored in methanol. Unlabeled NBMPR was purchased from Sigma Biochemicals. Acetylthiocholine and reagents for acetylcholinesterase, umbelliferyl N-acetyl β-glucosaminidase, umbelliferyl β-glucuronidase, and umbiliferone phosphate were purchased from Sigma Biochemical. Magnetic beads conjugated to an anti-mouse sheep antibody were obtained from Dynal Inc (Great Neck, NY).

RESULTS

Detection of Transferrin Receptor Containing Vesicles in the Circulation

Formation of vesicles (exosomes) during maturation of reticulocytes and their recovery in the medium has until now been detected only with sheep cells maturing in vitro.2,4 The precursor structures located in multivesicular bodies have also been seen in rat reticulocytes by Harding et al.21,22 as well as in sheep reticulocytes by Pan et al.4 That vesicle formation is restricted to neither the in vitro situation nor to the sheep is shown in Fig 1A-D. Blood from phlebotomized animals or phenylhydrazine-treated animals was processed to collect a 100,000 x g pellet from the cell-free plasma. It is evident that the plasma pellets from all four anemic species show the presence of exosomes. Significantly, however, exosomes can also be found in the plasma of normal, unbled sheep, although the number of vesicles detected is small relative to anemic animals (≤25%) (Fig 1E).

We have shown that the transferrin receptor can be found in exosomes from the circulation of a phlebotomized sheep2 as well as from exosomes formed during in vitro incubation of reticulocytes, suggesting that this is a normal route for receptor shedding. To assess whether other species release the transferrin receptor into the circulation, the 100,000 x g pellet, derived from plasma of phlebotomized anemic and normal rats was probed with a monoclonal antibody against the rat receptor. The data in Fig 2A show the presence of TFR in the high speed pellet from plasma of phlebotomized but not unbled rat plasma. Control experiments (Fig 2B) show that the antibody against the rat transferrin receptor does not recognize other plasma membrane proteins and that nonimmune serum even in great excess (60 µg IgG) does not bring down the transferrin receptor. To verify that the rat
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Fig 1. (A–D) Presence of exosomes in the circulation of anemic animals. Heparinized blood (50 mL) from phlebotomized sheep and rabbits was centrifuged at 7,700 × g for 8 minutes. The cell-free supernatant was recentrifuged in an identical way. Following these centrifugations, the supernatant was diluted with an equal volume of isotonic saline and centrifuged at 100,000 × g for 90 minutes. The pellet was collected, fixed in glutaraldehyde, and processed for EM as described. Blood from 7- to 14-day-old piglets (~10 mL) was treated as above. Ten to 15 milliliters of blood was used. More cellular breakdown products are evident in the plasma from phenylhydrazine–treated animals, but ~50 nm vesicles are evident. (A) Rabbit. (B) Sheep. (C) Rat. (D) Pig. Bar = 200 nm. (E) Presence of exosomes in the circulation of normal sheep. Fifty milliliters of blood from a normal, unbled sheep was processed as described in Fig 1A–D. The number of vesicles in the normal circulation were not quantified relative to phlebotomized animals, but sections with vesicles were much less frequent (~25%) of bled animals. No vesicles were detected with normal blood from rabbit or rat. Bar = 200 nm.

vesicles contain TFR, the vesicles obtained from the circulation of a phlebotomized rat were attached to Dynal beads containing immobilized rat αTFR. Nonimmune IgG was used as control. After binding the vesicles to the beads and removal of unbound material by washing, the beads were treated with urea/glycine to release any bound vesicles. The data show the presence of vesicles released from (rat) αTFR containing beads (Fig 3A). No vesicles were detectable in the nonimmune rat controls. Additional studies have also shown that after fixation for EM the vesicles bind rat αTFR. Fixed sections of vesicles derived from the circulation of phlebotomized rats were treated either with rat αTFR or nonimmune serum. The sections were rinsed and then treated with colloidal gold conjugated to protein A. The data in Fig 3B show gold grains dotting exosomes pretreated with immune serum. An equal number of sections (6) treated with immune and nonimmune sera was examined, and a total number of 500 vesicles from each treatment was counted. Despite the problems of postembedding treatment with protein A, with immune serum the number of gold grains associated with vesicles exceeded the nonimmune (338 ± 150 grains) and was higher in each of the six sections. Lack of antibodies that react with the TFR of other species precluded extension of this observation to additional species at this time. Nonetheless, these data suggest that vesicle formation may occur naturally in many mammalian species and that these vesicles carry obsolete transferrin receptors.

The formation and release of exosomes bearing the transferrin receptor is an event dependent on metabolic activity as illustrated in Fig 4. Sheep reticulocytes were incubated for 18 hours under three conditions: (1) at 4°C, (2) at 37°C with rotenone and 2-deoxyglucose to deplete ATP, and (3) in normal medium at 37°C. It is evident that considerably less...
TFR is detected in the 100,000 × g pellet from cells incubated under adverse metabolic conditions (compare lane 4 with lanes 1 and 2 in Fig 4). It has already been shown that reticulocytes incubated overnight at 4°C shed less protein and lose little transferrin binding activity in contrast with the loss seen in cells incubated at 37°C.

Selective separation of a vesicle population containing TFR. Although multiple functions can be recovered in the exosome fraction formed during in vitro incubation of sheep reticulocytes, our earlier studies failed to address the distribution of these activities within the population. Does a single vesicle contain all activities known to be released? To answer this question, we attempted to separate sheep vesicles bearing the TFR from the total vesicle population with an immobilized αTFR. We have found that magnetic core beads coated with the αTFR can nearly completely remove TFR-containing exosomes from the total vesicle suspension (Fig 5, lane 5). In contrast, beads coated with nonimmune IgG (Cγ) (Fig 5, lane 4) bring down little TFR. Conversely, it is also clear that the supernatant from Iγ (Iγ) contains little TFR (Fig 5, lane 3), whereas, the Cγ, the supernatant from the nonimmune IgG, retains most of the TFR (Fig. 5, lane 2 compared with lane 1 [total vesicle protein]).

In Fig 5 it is also worth noting that a ~70 kDa peptide is removed from the exosome suspension with the beads coated with αTFR. We have already demonstrated that this protein, the clathrin uncoating ATP-ase, is externalized with the transferrin receptor. The peptide at ~50 Kd found in both Iγ and Cγ appears to be a portion of the covalently bound IgG, released the magnetic beads themselves during processing and/or gel electrophoresis.

As reported earlier by Johnstone et al., some hemoglobin (Hb) is found with the vesicle population. On the basis of insensitivity to trypsin digestion, we concluded that the Hb was occluded, perhaps trapped inside vesicles. Using the magnetic beads it was determined that the majority of Hb stays in the Iγ and that Iγ is relatively free from Hb.

To substantiate the conclusion that the population of vesicles associated with Iγ is enriched in transferrin receptors, both the bead and supernatant fractions were assayed for 125I-TF binding. The data in Table 1 show that the immune beads remove all the 125I-TF binding activity. No specific 125I-binding remains in the immune supernatant. This result should be contrasted with the data using nonimmune IgG-coated beads where all detectable TF binding remains in the supernatant.

Multiple Functions Are Found in TFR Containing Vesicles

To determine whether other functions found in the exosomes are associated with the TFR containing population, enzyme or binding assays were performed on immune and nonimmune IgG-coated beads (P) and the respective supernatants (S).

Acetylcholinesterase and nucleoside transport and binding are plasma membrane functions in red cells known to diminish on maturation. We have previously shown that the acetylcholinesterase activity and nucleoside analogue binding (NBMPR) can be detected in exosomes. The results in Table 2 show that a substantial fraction of the acetylcholinesterase and 3H-NBMPR binding, are detected in the same population of vesicles as the TFR. For both acetylcholinesterase and 3H-NBMPR binding, approximately one half of the activity associates with the immune beads, with the remainder being in the immune supernatant. In contrast, almost 100% of both these activities remained in the nonimmune supernatant, with no significant activity being associated with the beads.

Because lysosomal enzymes have also been found in the 100,000 × g pellet, we determined the distribution of three purported lysosomal enzymes, N-acetyl-β-glucosaminidase, acid phosphatase (pH 5.2), and β-glucuronidase. The results show that no β-glucosaminidase or β-glucuronidase is associated with the immune beads; almost all the recovered activity remained in the immune supernatant (Table 3).
Fig 3. Vesicles from the circulation of phlebotomized rats contain TFR. (A) Binding of vesicles from phlebotomized rat plasma to an immobilized anti-rat transferrin receptor. Vesicles from the circulation of phlebotomized rats were attached as described in Materials and Methods to Dynal beads carrying an antitransferrin receptor antibody against the rat receptor. After elution of the vesicles with 4 mol/L urea/0.2 mol/L glycine, the vesicles were processed for EM. No vesicles were detected in nonimmune controls. Bar = 200 nm. (B) Detection of αTFR binding to vesicles from the circulation of phlebotomized rats. Vesicles were collected and fixed for EM, and the cut sections were incubated with αTFR. After washing to remove unbound antibody, the sections were treated with protein A conjugated to colloidal gold. Note the association of grains with vesicles. Bar = 200 nm.

With these enzymes there was no difference in distribution of activity with immune and nonimmune beads, suggesting that these activities are in a different population of vesicles, unassociated with the transferrin receptor.

Although acid phosphatase activity is normally considered a lysosomal marker, its distribution was unexpected and different from glucosaminidase activity. Phosphatase activity was partly removed by immune beads, characteristic of the plasma membrane functions noted in Table 2. The distribution of phosphatase activity between pellet and supernatant did not change if the assays were conducted at pH 6 rather than 5.2. Little activity remained at pH 9.0. Because rabbit red cell plasma membranes are known to possess fluoride sensitive acid phosphatase activity, we verified that plasma membranes of mature red cells from sheep contain acid phosphatase, the activity being ~15% of that in the reticulocyte membranes (~800 fmol/min/µg protein). The specific activity in exosomes is approximately fourfold higher than mature membranes and is fluoride-sensitive. Thus, the acid phosphatase activity in TFR containing exosomes from sheep may originate, at least in part, from plasma membrane enzymes.

Attempts to measure cathepsin activity (both B and C), often associated with lysosomal membranes, was unsuccessful. Neither the reticulocyte plasma membranes nor the exosomes had significant cathepsin B or C activity under our conditions.

DISCUSSION

In the present work we have made use of a novel approach to selectively remove a population of subcellular organelles. Antibody-coated magnetic beads have been used to isolate cells and endosomes with specific antigenic markers by Ugelstad et al. and Gruenberg and Howell. We have...
Fig 5. Separation of transferrin receptor containing exosomes. Exosomes derived from an overnight culture of sheep reticulocytes were applied to αTFR-coated beads or beads coated with nonimmune IgG. After separation of beads from supernatant and a quick wash of the beads with PBS, SDS was added to both the bead fraction and the supernatant fraction and the material was subjected to SDS polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue. An equal aliquot of the exosome suspension (total) was dissolved directly in SDS buffer without being treated with the magnetic beads. Lane 1, total exosomes. Lane 2, Csup. − supernatant from nonimmune IgG-coated beads. Lane 3, Csup. − supernatant from αTFR-coated beads. Lane 4, Cpel. − bead pellet from nonimmune IgG-coated beads. Lane 5, Cpel. − bead pellet from αTFR-coated beads.

Table 1. Separation of 125I-Transferrin Binding Activity From the Total Exosome Population

<table>
<thead>
<tr>
<th>Condition</th>
<th>Transferrin Bound (pmol/10^10 cell equivalents)</th>
<th>% of Total Binding (range of 5 experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total vesicles</td>
<td>10.1</td>
<td>—</td>
</tr>
<tr>
<td>Nonimmune supernatant</td>
<td>8.7</td>
<td>80–90</td>
</tr>
<tr>
<td>Immune supernatant</td>
<td>0.5</td>
<td>4–10</td>
</tr>
<tr>
<td>Nonimmune beads</td>
<td>0.3</td>
<td>1–3</td>
</tr>
<tr>
<td>Immune beads</td>
<td>8.2</td>
<td>80–90</td>
</tr>
</tbody>
</table>

Vesicles derived from overnight cultures of sheep reticulocytes were used. 125I-transferrin binding was carried out as described for total binding by Klausner et al. After loading the vesicles on the magnetic beads overnight at 4 °C, the beads were separated from the supernatant, washed, and transferrin binding measured using 10 μg/mL of 125I sheep transferrin. Five hundred-fold excess of human transferrin was used to assess the nonspecific binding. Values corrected for nonspecific binding are given. Transferrin binding ranged from 3 to 10 pmol in vesicles derived from incubating 10^10 cells containing over 70% reticulocytes.

Table 2. Acetylcholinesterase and 3H-NBMPR Binding in Transferrin Receptor Containing Exosomes

<table>
<thead>
<tr>
<th>Assay</th>
<th>Condition</th>
<th>Activity (nmol/10^10 cells/min) (typical experiment)</th>
<th>% of Total (mean ± SD) (no. of experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholinesterase</td>
<td>Total</td>
<td>6.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>6.0</td>
<td>84 ± 5 (4)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>2.9</td>
<td>47 ± 15 (4)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune beads</td>
<td>0.3</td>
<td>5 ± 0.5 (4)</td>
</tr>
<tr>
<td></td>
<td>Immune beads</td>
<td>3.8</td>
<td>49 ± 7 (4)</td>
</tr>
<tr>
<td>3H-NBMPR binding</td>
<td>Total</td>
<td>902</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>921</td>
<td>88 ± 5 (3)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>658</td>
<td>59 ± 4 (3)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune beads</td>
<td>197</td>
<td>9 ± 9 (3)</td>
</tr>
<tr>
<td></td>
<td>Immune beads</td>
<td>555</td>
<td>43 ± 5 (3)</td>
</tr>
</tbody>
</table>

Vesicles collected from overnight sheep reticulocyte cultures were used. The assays were conducted as described by Johnstone et al. To calculate the percent binding in the NBMPR assay, the calculations are based on the sums of the activity in the supernatant and corresponding pellet, which were always greater than the measured total binding.
characteristic of plasma membrane activities but may arise in the exosomes via release from the multivesicular bodies).

Orr and Johnston have shown that lysosomal enzymes are released with a different time frame from that obtained for the release of the transferrin receptor during reticulocyte maturation. Such an observation had in fact suggested the possibility that the lysosomal and plasma membrane activities might be segregated in different populations of exosomes collected by centrifugation at 100,000 × g. The present study provides direct evidence for this conclusion as well.

Although the mechanism for selecting specific proteins for externalization is not known, the data presented suggest that these proteins may contain a common feature in their cytoplasmic termini, which targets them for selective removal as the cells age. Moreover, because different species are known to lose different functions during maturation (eg, pig, not humans, loses all glucose transporters) there presumably exist species-specific differences in the cytoplasmic portions of these transmembrane proteins that may selectively target them for externalization during the maturation process.

Changes in the cytoplasmic portions of obsolescent proteins might target them for recognition by the 70-Kd clathrin uncoating ATPase, which is reported to bind to unfolded parts of proteins. This 70-Kd protein is present in high concentration in red cells. It is interesting to note that vesicles isolated by the coated, iron-core beads also contain the ~70-Kd protein (Fig 5). The close association of ~70-Kd with the transferrin receptor in externalized vesicles earlier led us to suggest a role for the 70-Kd protein in targeting specific proteins for externalization. It will be of interest to determine whether an immobilized antibody against another released membrane protein will also bring down some ~70-Kd peptide.

Although our data show that exosomes isolated by immune beads contain all the available transferrin receptor, it is not surprising that only a portion (~50%) of the other plasma membrane activities are co-isolated. It would be unlikely for every transferrin receptor-containing exosome to contain 100% of all other activities. It is more reasonable to expect a statistical distribution of functions, some vesicles being enriched in one function relative to another. Indeed, an immunocytochemical study showed that not all released vesicles contained TFR. One might reasonably expect that using an antibody against acetylcholinesterase, for example, only a fraction of transferrin binding activity would be pulled out. Significantly, however, the presence of multiple functions in one specific exosome population argues for a common recognition and processing system.

Using this nondestructive approach, we have been able to provide compelling evidence that each vesicle formed during reticulocyte maturation contains several plasma membrane functions. We have also demonstrated that little of the Hb or the externalized lysosomal functions are included in this population of the TFR containing exosomes. This simple technique has the potential to answer similar questions about intracellular segregation of functions in different populations of vesicles.

In conclusion, we have also presented evidence that exosomes are found in the circulation of anemic animals and that (at least in two species) the transferrin receptor is shown to be associated with the exosomes. These observations suggest that exosome formation, with concomitant release of the transferrin receptor, is a natural phenomenon.

ACKNOWLEDGMENT

The authors thank Francine Nault for her careful and dedicated technical assistance, Kathy Teng for all the electron microscopic work, Margaret Licorish for superior secretarial help, and J. Larrick, Genelabs, CA, and A. Williams, Department of Pathology, Oxford University, England, for the provision of monoclonal antibodies against the human and rat transferrin receptors, respectively.

Table 3. N-Acetyl-β-Glucosaminidase, β-Glucuronidase, and Acid Phosphatase in Transferrin Receptor Containing Exosomes

<table>
<thead>
<tr>
<th>Assay</th>
<th>Condition</th>
<th>Activity (pmol/10⁶ cells) (typical experiment)</th>
<th>% of Total (mean ± SD) (no. of experiments)</th>
</tr>
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<tbody>
<tr>
<td>N-acetyl-β-glucosaminidase</td>
<td>Total</td>
<td>336.0</td>
<td>96 ± 10 (4)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>246.0</td>
<td>90 ± 15 (4)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>280.0</td>
<td>4 ± 2 (4)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune beads</td>
<td>25.0</td>
<td>4 ± 3 (4)</td>
</tr>
<tr>
<td></td>
<td>Immune beads</td>
<td>23.0</td>
<td></td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>Total</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>21.0</td>
<td>90 (3)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>19.5</td>
<td>82 (3)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune beads</td>
<td>0.0</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>Immune beads</td>
<td>0.2</td>
<td>(3)</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Total</td>
<td>2,860.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>2,650.0</td>
<td>94 ± 5 (5)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>1,350.0</td>
<td>50 ± 12 (5)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune beads</td>
<td>150.0</td>
<td>8 ± 2 (5)</td>
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<tr>
<td></td>
<td>Immune beads</td>
<td>1,150.0</td>
<td>43 ± 8 (5)</td>
</tr>
</tbody>
</table>

Exosomes collected after 40 hours of culture of sheep reticulocytes were used for the glucosaminidase and phosphatase assays. Vesicles from an overnight culture were used for β-glucuronidase. In all cases the umbelliferyl derivative of the substance was used as substrate at 0.2 mmol/L and the sample was incubated for 30 minutes at 37°C in an acetate buffer at pH 5.2. The extent hydrolysis was determined fluorimetrically.
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Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions

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