Preparation of a low-density species of endocytic vesicle containing immunoglobulin A

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Immunoglobulin A is transported across hepatocytes in specialized vesicles. A population of endocytic vesicles of approx. 140 nm diameter, containing immunoglobulin A, has now been separated from all other major cytoplasmic organelles, including plasma membrane and lysosomes, by sequential centrifugation on Ficoll/sucrose and Metrizamide gradients.

IgA is carried intact across hepatocytes from blood to bile in endocytic vesicles (Mullock & Hinton, 1981). The vesicles move rapidly from the sinusoidal surface of the cell to the region of the hepatocyte near the bile-canalicular membrane where lysosomes are seen clustered. The speed of transit of IgA across hepatocytes (IgA appears in bile within 30 min of intravenous injection, and transport is completed with 3 h) shows that the transport of IgA cannot simply be explained by lysosomal discharge, for this latter process is slow, of the order of 5% of lysosomes/day (Godfrey *et al.*, 1981). Two alternative explanations seem possible. Firstly, two distinct types of vesicles may be formed, one which fuses with lysosomes, and a second type, carrying IgA, which avoids fusion with lysosomes. Secondly, there may be only a single type of vesicle, all of which fuse with lysosomes, release a part of their contents, then dissociate from the lysosomes and fuse with the biliary membrane (Limet *et al.*, 1982). Autoradiographic studies generally support the first mechanism, for while transport is actively proceeding little label is associated with lysosomes, whereas, at times when transfer of IgA into bile is effectively complete, only a relatively small amount of label remains in lysosomes, suggesting that IgA, if it does enter lysosomes, is not released (Birbeck *et al.*, 1979). However, although these studies support the first mechanism, they do not definitely exclude the second. A clear-cut distinction is only possible if the endocytic vesicles can be separated

from other cell organelles and their contents can be identified.

In previous studies, both we and other authors have examined the properties of the IgA-carrying endocytic vesicles on rate and isopycnic sedimentation in sucrose gradients (Mullock *et al.*, 1979; Smith *et al.*, 1981; Limet *et al.*, 1982). The results show that, although the properties of the endocytic vesicles naturally present in the cells differ from those of plasma-membrane vesicles formed during homogenization, it is not possible to separate the endocytic vesicles from other cell organelles. There are, however, marked differences in the relative densities at which different cell organelles band in different density-gradient media. We now describe a sequential separation on Ficoll/sucrose gradients and on Metrizamide gradients which gives a relatively pure preparation of at least one species of IgA-carrying endocytic vesicle, which are quite distinct from lysosomes.

Materials and methods

Metrizamide was purchased from Nyegaard and Co. (Oslo, Norway). Rat polymeric IgA was prepared as described by Orlans *et al.* (1978), labelled with ^{125}I (Salacinski *et al.*, 1979) and chromatographed to remove free iodine. Livers of male hooded rats of the University of Surrey strain were perfused via the aorta with cold 0.25 M-sucrose/10 mM-Tes, pH 7.4, and then homogenized in 15 ml of 0.25 M-sucrose/10 mM-Tes/1 mM-MgCl₂ (pH 7.4)/g in a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 1100 g and 4.5 ml samples of the supernatant were layered over linear 1–22% Ficoll gradients containing

Abbreviations used: IgA, immunoglobulin A; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid.

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0.25 M-sucrose, 10 mM-Tes and 1 mM-EDTA (pH 7.4) and centrifuged overnight (at least 16 h) in a 3×23 ml swing-out rotor at 90000 g. Fractions (1 ml) were collected by upward displacement with 25% Ficoll containing 0.25 M-sucrose. Fractions to be resedimented on Metrizamide gradients were pooled and diluted with 10 mM-Tes/1 mM-EDTA, pH 7.4, until n was 1.354 or less; 4.5 ml portions were then layered over linear 10–30% Metrizamide gradients containing 10 mM-Tes and 1 mM-EDTA, and centrifuged at 90000 g overnight.

5'-Nucleotidase, glucose 6-phosphatase, acid phosphatase, succinate dehydrogenase, β -galactosidase and *N*-acetyl- β -glucosaminidase were determined as described by Hinton & Norris (1972), and UDP-galactosyltransferase was measured as described by Beaufay *et al.* (1974). Sedimentable IgA was counted for radioactivity after dilution of the appropriate fractions with NaCl to give a final concentration of 0.15 M-NaCl and centrifugation for 120 min at 115000 g. For electron microscopy the pellet so obtained was fixed in 4% (v/v) glutaraldehyde, counterfixed with 2% (w/v) osmic acid and embedded in Epon 812 (TAAB Laboratories).

Results

Isopycnic centrifugation on Ficoll gradients of the mitochondrial supernatants from the livers of rats which had received an injection of ^{125}I -labelled IgA in the jugular vein 30 min before death resulted in a single peak of sedimentable IgA in the middle of the gradient (Fig. 1a). There was also some IgA in the pellet below the gradient. The peak of sedimentable IgA did not coincide with the main peak of 5'-nucleotidase activity, which on a sucrose gradient would be assumed to indicate the position of the sinusoidal plasma membrane on the gradient, although a shoulder in the 5'-nucleotidase peak was often discernible at the density of the sedimentable IgA peak. Deliberate labelling of the sinusoidal plasma membrane by addition of ^{125}I -labelled IgA to a rat liver homogenate before fractionation (Fig. 1b) showed that the peak of sinusoidal-plasma-membrane labelling came at a less-dense position, which coincided with the main 5'-nucleotidase peak; very little labelling was observed in the density range corresponding to the vesicles labelled *in vivo*. The pellet contained very little label after such addition. Examination of other classical marker enzymes showed that the concentrations of the mitochondrial marker succinate dehydrogenase and the lysosomal markers acid phosphatase (Fig. 1c), β -galactosidase and *N*-acetylglucosaminidase were very low in the density range containing the vesicles, being mainly found in the pellet. However, the peak of glucose 6-phosphatase (Fig. 1c), indicating smooth-endoplasmic-reticulum membrane, coincided almost

exactly with the position of the endocytic-vesicle peak. Addition of digitonin to the appropriate fractions and re-centrifugation on a sucrose gradient showed that under these conditions the modal density of the sedimentable IgA and 5'-nucleotidase-containing vesicles was increased to become 1.155 g/ml, whereas that of the glucose 6-phosphatase peak remained at about 1.140. However, the displacement was insufficient to permit separation of the endocytic vesicles from the smooth endoplasmic reticulum. Graham (1983) has shown that smooth endoplasmic reticulum behaves as if relatively dense on Metrizamide gradients. The peak fractions from the Ficoll gradient (n 1.361–1.365, usually fractions 12–15) were therefore layered over a Metrizamide gradient and re-centrifuged, whereupon the glucose 6-phosphatase peak moved to the dense end of the gradient, whereas the endocytic-vesicle fraction remained in the early part (Fig. 1d). The material under the IgA-containing peak contained some 5'-nucleotidase and a small amount of acid phosphatase. A small, very sharp, peak of UDP-galactosyltransferase activity also occurred at precisely the density range of the IgA peak. About 25% of the UDP-galactosyltransferase activity of the whole homogenate was transferred to the Metrizamide gradient; of this about one-third occurred in a peak at 1.07–1.08 g/ml. The lysosomal enzymes β -galactosidase and *N*-acetylglucosaminidase, which are typical of lysosomal contents (Dobrota *et al.*, 1979), were distributed in general like acid phosphatase, but did not show the small peak of activity at density 1.07–1.08 g/ml on the Metrizamide gradient seen for acid phosphatase in Fig. 1(d).

Electron microscopy (Fig. 2) of the material sedimented from the peak of the endocytic-vesicle-containing fractions (density 1.07–1.08 g/ml) showed a uniform population of small vesicles with distinct contents and bilayer membranes.

Discussion

Autoradiographic studies (Birbeck *et al.*, 1979) indicate that after injection *in vivo* IgA is associated both with the surface membrane and with intracellular particles. Any 'right-way-out' vesicles formed from the sinusoidal surface will be capable of binding IgA *in vitro*. Any endocytic vesicles (formed *in vivo*), on the other hand, will have any unoccupied receptors on the interior and hence will not be capable of binding IgA *in vitro*. There will therefore always be at least two populations of vesicles associated with IgA after labelling *in vivo*. The population derived from the sinusoidal plasma membrane can be identified by its ability to bind IgA *in vitro*. In the present work, this sinusoidal-plasma-membrane vesicle population is thus identi-

Fig. 1. Distribution of ^{125}I -labelled IgA and marker enzymes after isopycnic banding of rat liver fractions. ^{125}I -labelled IgA was either injected intravenously 30 min before the animal was killed (a and d) or added to the liver homogenate (b). In (a–c) the 1500 g -10 min supernatant was loaded on to a linear 1–22% Ficoll gradient containing 0.25 M-sucrose, 10 mM-Tes and 1 mM-EDTA (pH 7.4). Recoveries of 5'-nucleotidase, acid phosphatase, glucose 6-phosphatase and succinate dehydrogenase were 74%, 91%, 70% and 73% respectively. In (d) fractions of refractive index 1.361–1.366 from a separation similar to that shown in (a) were pooled, diluted with 10 mM-Tes/1 mM-EDTA and loaded on to a linear 10–30% Metrizamide gradient containing 10 mM-Tes and 1 mM-EDTA (pH 7.4). Recoveries were all very close to 100%. Sedimentable IgA was estimated in pellets prepared by pooling pairs of adjacent fractions, diluting with NaCl solution to give a final concentration of 0.15 M and centrifuging for 90 min at 115 000 g . ■, Sedimentable IgA; □, 5'-nucleotidase; △, acid phosphatase; ●, glucose 6-phosphatase; ····, refractive index (20°C) (a–c) or density (20°C) (d).

Fig. 2. Electron micrographs of the pellet collected from the peak of sedimentable IgA radioactivity on a Metrizamide gradient as shown in Fig. 1(d)

Radiolabelled IgA was injected into the rat 30 min before it was killed, and the liver homogenate was sequentially fractionated on Ficoll/sucrose and Metrizamide gradients. The bar represents 0.5 μ m.

fied as that population which coincides with the main activity peak of the plasma-membrane marker 5'-nucleotidase on the Ficoll/sucrose gradients. Centrifugation on such gradients therefore enabled most of the sinusoidal-plasma-membrane vesicles to be removed from the slightly denser population of vesicles that were not labelled by IgA added *in vitro* and which must therefore represent endocytic vesicles.

The final endocytic-vesicle preparation (density 1.07–1.08 g/ml) contains about 0.7% of the protein, 0.5% of the 5'-nucleotidase, 0.5% of the acid phosphatase and 0.4% of the glucose 6-phosphatase of the whole homogenate in association with 6% of the total IgA. Although the instability of the enzyme made accurate assessment of the proportion of the homogenate UDP-galactosyltransferase activity difficult, it seems that up to 7% of the total activity may be associated with the IgA-containing peak; certainly a sharp peak of UDP-galactosyltransferase activity is always seen at density 1.07–

1.08 g/ml. In contrast, β -galactosidase and *N*-acetylglucosaminidase showed no such association. The data above indicate that only 12% of the protein can be derived from the endoplasmic reticulum, assuming that glucose 6-phosphatase is a valid marker (Leighton *et al.*, 1968). Since β -galactosidase and *N*-acetylglucosaminidase show no specific association with the peak, it cannot be lysosomal, in spite of the small amount of associated acid phosphatase. The presence of 5'-nucleotidase and the density shifting by digitonin, suggesting a cholesterol-rich membrane, both indicate a membrane related to the plasma membrane. UDP-galactosyltransferase, the classical Golgi marker, is undoubtedly present in the preparation, but electron micrographs show no sign of dictyosomes or lipoprotein-containing vesicles. It therefore seems likely that the preparation contains endocytic vesicles, which have formed from the plasma membrane and retain some of its properties, but which, in some cases, may well have interacted with part of the Golgi apparatus.

The vesicles described are obviously an important component of the IgA-carrying system. However, other organelles also contain some IgA. As mentioned by Mullock *et al.* (1979), some radiolabelled IgA, although less than 20% of the total, undoubtedly appears in lysosomes. However, since no endogenous IgA could then be detected in lysosomes, we concluded that the 125 I-labelled IgA entered lysosomes probably as the result of damage to the protein during radiolabelling, rather than as part of the normal course of events as suggested by Limet *et al.* (1982). In the fractionation methods used here, lysosomes will largely appear in the pellet below the Ficoll gradient. This pellet characteristically contained about 14% of the total radiolabel of the homogenate, as compared with about 17% in the peak fractions taken for further fractionation on Metrizamide gradients. The radioactivity in the pellet was not due to non-specific binding, since only 1% of label added *in vitro* appeared in the pellet. However, in addition to lysosomes, the pellet might contain the denser but non-lysosomal IgA-carrying vesicles described in sucrose gradients by Smith *et al.* (1981). Some IgA-carrying organelles are also undoubtedly lost in the initial 1100 g pellet, which was not washed. Although after addition of 125 I-labelled IgA *in vitro* all the radioactivity was recovered in the 1100 g supernatant, after IgA administration *in vivo* only about 72% of the homogenate label appeared in the 1100 g supernatant. Some of this loss will result from trapping of the currently described endocytic vesicles and of lysosomes, but it is possible that another IgA-containing organelle exists in this fraction.

Whatever other IgA-containing fractions may exist, however, the endocytic vesicles now purified contain a sizeable proportion of the endocytosed

IgA and differ markedly in their properties from previously isolated organelle fractions such as lysosomes. Preliminary experiments show that the distribution of asialofetuin-containing vesicles is similar to that of the IgA-containing vesicles, although double-labelling experiments will be required to see if the properties of the two types of vesicles are identical. IgA-containing vesicles are destined to fuse with the bile-canalicular plasma membrane, whereas those of asialofetuin-containing vesicles should fuse with lysosomes. Bulk preparations of the endocytic-vesicle fraction, by using zonal rotors to scale up the methods here described, will enable such fusions to be carried out *in vitro* for the first time, so that the factors governing them can be investigated.

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