Internalization of retinol-binding protein in parenchymal and stellate cells of rat liver

Haruki Senoo,[1] Espen Stang,* Astrid Nilsson, Grete M. Kindberg, Trond Berg, Norbert Roos,* Kaare R. Norum, and Rune Blomhoff[2]

Institute for Nutrition Research, School of Medicine, University of Oslo, P.O. Box 1046, Blindern, 0316 Oslo 3, Norway, and Electronmicroscopical Unit for Biological Sciences,* University of Oslo, P.O. Box 1066, Blindern, 0316 Oslo 3, Norway

Abstract We have studied uptake of retinol-binding protein (RBP) by rat liver cells. First, we compared the in vivo uptake in different liver cells of ^{125}I-labeled RBP with that of other well-known ligands. We found that the ligands studied were recognized differently by the various cell types in the liver, and that RBP was most efficiently taken up by parenchymal and stellate cells. We then studied the in vivo uptake of RBP in liver cells by immunocytochemistry at the electron microscopic level using ultrathin cryosections. Ten min after injection, RBP was localized to parenchymal cells and stellate cells. In these cells, RBP was detected on the cell surface and in vesicles near the cell surface. RBP was observed mainly in association with the membrane in these vesicles. Two hours after injection, RBP was localized not only on the cell surface and in vesicles close to the cell surface, but also in larger vesicles located deeper in the cytoplasm of these cells. RBP in larger vesicles was observed at a distance from the vesicular membrane. Finally, we compared the distribution of endocytosed RBP in liver parenchymal cells with that of asialo-orosomucoid, a ligand known to be internalized by receptor-mediated endocytosis. We detected both ligands on the cell surface and in small vesicles located close to the cell surface and in larger vesicles located deeper in the cytoplasm. Asialo-orosomucoid and RBP were seldom observed in the same small vesicles, but the larger vesicles contained both ligands. These data suggest that RBP is internalized in parenchymal and stellate cells of the liver by receptor-mediated endocytosis. —**Senoo, H., E. Stang, A. Nilsson, G. M. Kindberg, T. Berg, N. Roos, K. R. Norum, and R. Blomhoff.** Internalization of retinol-binding protein in parenchymal and stellate cells of rat liver. *J. Lipid Res.* 1990. **31:** 1229–1239.

Supplementary key words perisinusoidal stellate cells • fat-storing cells • Ito cells • RBP • receptor-mediated endocytosis

Retinol is transported in plasma bound to a specific plasma transport protein, retinol-binding protein (RBP) (1). The mechanism by which retinol is transferred from retinol-RBP to cells is not yet fully elucidated. Since a small amount of retinol may be in equilibrium with retinol-RBP in plasma, it has been speculated that it may partition into the plasma membrane without the involvement of a receptor. Support for this uptake mechanism has been reported by Fex and Johannesson (2) who incubated artificial phospholipid membranes with [^{3}H]retinol. At equilibrium, 10–30% of the retinol was transferred to membranes, and $t_{1/2}$ for retinol transfer was about 30 min. The same authors subsequently showed that retinol can rapidly "flip-flop" across phospholipid bilayer membranes ($t_{1/2}$ < 30 sec) (3). Similar observations have been made by Ho, Pownall, and Hollyfield (4).

Other observations indicate that a spontaneous, nonspecific transfer is not a major mechanism by which many cells obtain retinol. For example, since white blood cells do not store retinyl esters, and erythrocytes have much longer half life in blood than white blood cells, one would expect, based on a nonspecific spontaneous transfer, that erythrocytes contained at least as much retinol as the white blood cells. However, erythrocytes contain much less retinol (about 1/1000th) than white blood cells (5). In addition, Heller and Bok (6, 7), studied retinol-^{125}I-labeled RBP uptake in vitro and in vivo by bovine retina and retinal pigment epithelial cells. They reported that ^{125}I-labeled RBP was bound only to the choroidal surface of the retinol pigment epithelial cells, and that an excess of unlabeled RBP greatly reduced the binding. Binding to photoreceptor cells or any other retinal cells was not observed. McGuire, Orgebin-Crist, and Chytil (8) studied the interaction of ^{125}I-labeled RBP with rat testicular cells by autoradiography. They observed that RBP bound primarily to interstitial cells of rat testes. No interaction of retinol-RBP with seminiferous tubule cells was observed. A selective uptake of RBP was also observed by Gjøen et al. (9) who injected ^{125}I-labeled tyramine cellobiose-labeled RBP intravenously into rats, and determined the

Abbreviations: PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); RBP, retinol-binding protein; NCS, newborn calf serum; PBS, phosphate-buffered saline; MVB, multivesicular bodies.

[1]Permanent address: Department of Anatomy, School of Medicine, Tokyo Medical and Dental University, Tokyo 101, Japan.

[2]To whom reprint requests should be addressed.

recovery of radioactivity in liver, kidneys, intestine, spleen, heart, lungs, etc. at different times. After 1 h, ~20% and 10% of the injected dose was recovered in liver and kidneys, respectively, whereas other organs contained <3%. By isolating the liver cell types, it was estimated that parenchymal and stellate cells took up about equal amounts of RBP when calculated per g of liver, whereas Kupffer cells and endothelial cells in liver were much less active.

Several investigators have also studied the binding of RBP to cells in vitro. Evidence for a saturable plasma membrane receptor that recognizes RBP has been reported in experiments using isolated bovine retinal pigment epithelial cells (10), a crude suspension of chicken testicular cells (11), crude interstitial cells from rat testes (8), and the F9 embryonal carcinoma cell line (12). In two other studies using keratinocytes (13) and epithelial cells from the intestinal mucosa (14), no binding of ^{125}I-labeled RBP to cells could be demonstrated.

Recently, Sivaprasadarao and Findlay (15, 16) presented data that convincingly support the idea of a highly specific receptor for RBP on human placental brush border membranes. The putative receptor was sensitive to pH, trypsin, heat, and thiol-group reagents. Scatchard analysis of the equilibrium binding of ^{125}I-labeled RBP revealed both high (3×10^{-9} M) and low (9×10^{-8} M) affinity binding components. Interestingly, the bound RBP has a very high dissociation rate constant, i.e., most of the bound RBP is displaced from the putative receptor after 5 min. Since most uptake assays involve repeated centrifugations to reduce nonspecific cell-association, specific RBP binding may have escaped several of the previous attempts to identify the RBP receptor.

By cross-linking radiolabeled RBP (mol wt 21,000) with retina pigment epithelial membranes, Laurent et al. (17) observed a cross-linked complex with a molecular weight of 90,000–92,000 that could be blocked by excess unlabeled RBP. The data suggest that the RBP receptor has a molecular weight of about 70,000. A similar result was obtained by a ligand-blotting technique (17).

In conclusion, these studies support the existence of a membrane receptor for RBP. To study the mechanism for uptake of RBP in liver cells in more detail, we have now applied immunocytochemistry at the electron microscopic level. We have injected human RBP into rats and localized the human RBP in rat liver cells by the cryosection technique.

MATERIALS AND METHODS

Materials

Vectastain ABC reagents were from Vector Laboratories (Burlingame, CA). Sheep anti-human RBP was purchased from Biogenesis (Bournemouth, UK) and rabbit anti-human orosomucoid was purchased from DAKO Immunoglobulins, Glostrup, Denmark. Rabbit anti-sheep IgG and swine anti-rabbit IgG were obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). Protein A-colloidal gold (5–7 nm in diameter) (18) was generously donated by Dr. Jan W. Slot (Center for Electron Microscopy, School of Medicine, University of Utrecht, The Netherlands). Protein A-colloidal gold with a larger diameter (10 nm) was obtained from Janssen Biotech N. V. (Olen, Belgium), and protein A was obtained from Pharmacia (Uppsala, Sweden). RBP was isolated from human and rat plasma as described (9), and bovine testicular β-galactosidase was isolated as described by Distler and Jourdian (19). Invertase and orosomucoid were obtained from Sigma Chemical Co. (St. Louis, MO). Orosomucoid was desialylated by neuraminidase (20), and low density lipoproteins were isolated from human plasma and acetylated as described (21). The various proteins were labeled with ^{125}I by the sodium hypochlorite method (22). Plasma containing [^{3}H]retinol-labeled rat RBP was prepared as described previously (23). More than 95% of the [^{3}H]retinol-RBP was in a complex with transthyretin as determined by gel filtration (23).

Animals

Male Wistar rats weighing 270–350 g were used. The animals were maintained on an ordinary pelleted diet (No. 3155, AREX, Møllesentralen, Norway) which contained about 9.4 μmol of retinoids/kg (50% retinyl acetate and 50% retinyl palmitate). All animals were fasted for 12 h before the start of the experiments.

Preparation of liver cells

Total liver cell suspensions were prepared by a modified collagenase perfusion technique (9, 24). Parenchymal cells were isolated from the total liver cell suspension by differential centrifugation and by centrifugal elutriation. About 98% of the isolated parenchymal cells were viable as determined by the trypan blue exclusion test. About 40% of the parenchymal cells estimated to be present in intact liver were recovered in the isolated parenchymal cell suspension.

Pure fractions of endothelial cells, Kupffer cells, and stellate cells were isolated as described earlier (9, 24). Cells were identified by transmission electron microscopy (24). In addition, endothelial cells were identified after incubation with fluorescein-conjugated ovalbumin, Kupffer cells showed a positive cytochemical peroxidase reaction, and stellate cells had a strong vitamin A-specific autofluorescence (24). About 55%, 45%, and 8%, respectively, of the total number of endothelial cells, Kupffer cells, and stellate cells estimated to be present in liver were recovered in the final cell suspensions.

Western blot analysis

The specificity of sheep anti-human RBP was examined by Western blot analysis as described by Towbin, Staehelin, and Gordon (25). Briefly, rat and human plasma, and purified human and rat RBP were analyzed by 15% SDS-PAGE (26). Proteins in the gel were then transferred to nitrocellulose sheets by electroblotting, and immunoreactive protein was visualized with Vectastain ABC reagents and 4-chloro-1-naphthol.

Immunolabeling of cryosections and electron miscroscopy

Rats were anesthetized with pentobarbital and 0.5 mg human RBP dissolved in 500 μl phosphate-buffered saline (PBS) was injected into the femoral vein. In some experiments, 3.3 mg asialo-orosomucoid in 500 μl PBS was injected at the same time. At various times after injection, livers were prepared for ultrathin cryosectioning as described by Griffiths et al. (27) and Geuze, Slot, and Schwartz (28). The rat livers were fixed in situ by a modification of the perfusion fixation method (29) with 0.5% glutaraldehyde and 2% formaldehyde in 0.2 M PIPES, pH 7.4. Liver slices were further fixed by immersion in the same fixatives for 1 h, rinsed in 0.2 M PIPES, pH 7.4, and small pieces (1 mm^3) from the fixed slices were immersed in 2.3 M sucrose in 0.2 M PIPES for 1 h for cryoprotection, before freezing in liquid nitrogen. Livers of control rats were fixed and prepared by the same procedure. Ultrathin sections of these specimens were cut on an LKB Ultratome V with a cryonova unit at approximately −100°C using improved glass knives (30, 31). For electron microscopy, sections were put on Formvar-carbon-coated copper grids.

Indirect immunolabeling was performed at room temperature. The cryosections were first incubated with 10% newborn calf serum (NCS) for at least 15 min to block nonspecific binding of antibody, rinsed with PBS, and incubated for 30 min with sheep anti-human RBP diluted 1:100 in 5% NCS. Sections were then rinsed with PBS, incubated with rabbit anti-sheep IgG diluted 1:100 in 5% NCS for 30 min, rinsed, and, finally labeled with protein A-colloidal gold (5–7 nm in diameter) for 20 min. Rinsing buffer contained 0.016 M glycine for quenching of free aldehyde groups (32).

Cryosections obtained from rats injected with both human RBP and asialo-orosomucoid were used for double labeling (18, 33). In one series of experiments (results shown in Fig. 8), the sections were labeled in the following order: *1*) sheep anti-human RBP, *2*) rabbit anti-sheep IgG, *3*) protein A-colloidal gold (d = 5 nm), and then with *4*) rabbit anti-human orosomucoid, and *5*) protein A-colloidal gold (d = 10 nm). As a control for cross-reactivity, we also labeled some sections as follows: *1*) rabbit anti-human orosomucoid, *2*) swine anti-rabbit IgG, *3*) protein A-colloidal gold (d = 5 nm), and then *4*) sheep anti-human RBP, *5*) rabbit anti-sheep IgG, and *6*) protein-colloidal gold (d = 10 nm). The cryosections were washed with PBS containing unconjugated protein A (0.05 mg/ml) and 5% NCS between the two labeling sequences to block nonspecific binding of protein A-colloidal gold to free IgG.

After immunolabeling, cryosections were stained and embedded in the mixture of 0.3% uranyl acetate in 2% methyl cellulose (33), for 10 min on ice, and then observed in a JEOL 100-CX electron microscope at an accelerating voltage of 80 kV. In control experiments, sections were incubated with sheep anti-human RBP and the same volume of 0.2 mg/ml human RBP, instead of sheep anti-human RBP alone.

RESULTS

Plasma clearance of RBP

As we planned to use heterologous RBP in the present study, we first compared with plasma clearance of iodinated human RBP with that of [^{3}H]retinol-labeled rat RBP. Both RBP preparations were 70–80% saturated with retinol. Tracer amounts of human ^{125}I-labeled RBP and [^{3}H]retinol-RBP were injected into the right femoral vein of three rats, and blood samples were drawn from the left femoral vein after different periods of time. The plasma decay curves, which are presented in **Fig. 1,** show that iodinated human RBP and retinol-labeled rat RBP have similar initial clearance rates from plasma, suggesting that human holoRBP is metabolized similarly to rat holoRBP by the rats. The curves tend to separate after about 1 h, suggesting that retinol may be more extensively recycled than RBP.

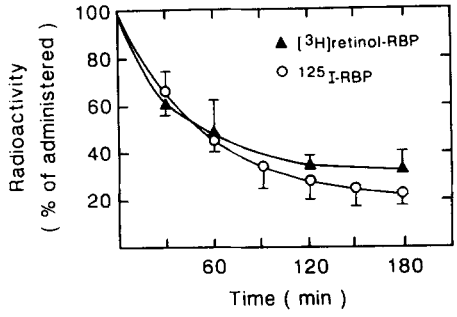

Fig. 1. Plasma clearance of RBP. Fifteen μg of ^{125}I-labeled human RBP (○) and [^{3}H]retinol-labeled rat RBP (▲) (5–50 × 10^5 cpm) was injected intravenously into the right femoral vein of three rats, and 100 μl blood was drawn from the left femoral vein after different periods of time. The data represent mean ± SD.

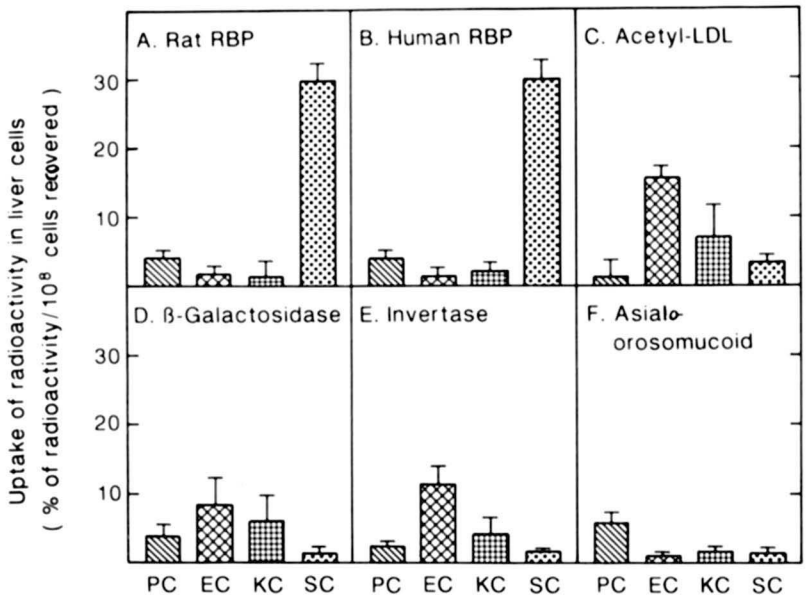

Fig. 2. Uptake of various ligands in liver cells. About 5 × 10^6 cpm of the various ^{125}I-labeled ligands were injected intravenously into rats, and liver cells were isolated after 10 min. The parenchymal cell fraction was contaminated with less than 0.1% endothelial cells, Kupffer cells, or stellate cells. The endothelial cell fractions were contaminated with 8% Kupffer cells and 2% stellate cells. The Kupffer cell fractions were contaminated with 6% endothelial cells and 4% stellate cells. The stellate cell fractions contained less than 4% contaminating endothelial and Kupffer cells. Data represent mean ± SD (n = 3), except the data for β-galactosidase and invertase which represent two rats.

Specific uptake of RBP in parenchymal and stellate cells

To further characterize the hepatic uptake of human RBP, we injected various ^{125}I-labeled ligands into rats and determined the uptake of the ligands in the different liver cells (**Fig. 2**). Three rats were used in each group, except for invertase and β-galactosidase where two rats were used. While endothelial cells were most active in the uptake of acetyl-LDL and the mannose-terminated glycoproteins, β-galactosidase and invertase, parenchymal cells accumulated most asialo-orosomucoid. Human and rat RBP were most efficiently taken up by the stellate cells. This experiment suggests that stellate cells have the largest number of RBP receptors per cell in liver. Since there are about 10 times as many parenchymal cells as stellate cells in liver, the data imply that about equal amounts of RBP are taken up by parenchymal cells and stellate cells per total liver. Similar results were obtained in a control experiment where 500 μg labeled human RBP was injected into rats, and cellular uptake in liver cells was determined.

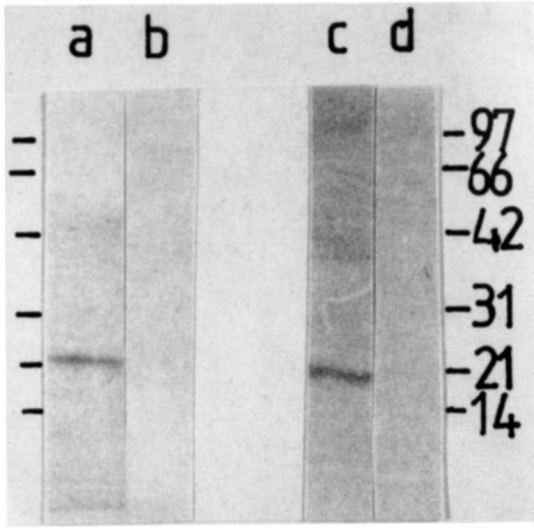

Fig. 3. Characterization of sheep anti-human RBP by Western blotting. Purified human (a) and rat (b) RBP (5 μg protein) and human (c) and rat (d) plasma (10 μg of protein) were analyzed by Western blot analysis. The proteins were transferred to nitrocellulose sheets and incubated with sheep anti-human RBP. The monospecific reaction at 21 kD was detected with Vectastain ABC reagents and 4-chloro-1-naphthol.

Identification of cells in cryosections of the liver

To study the cellular handling of RBP in rat liver by cryosectioning, it is essential to identify the individual cells. The shape, the size, and the lobular localization are important characteristics of the liver cells. Parenchymal cells are by far the largest cells in the liver. They are located as a single or double cell layer between two sinusoids. The basolateral plasma membrane of the parenchymal cells that are facing the space of Disse consist of characteristic microvilli. These cells also contain

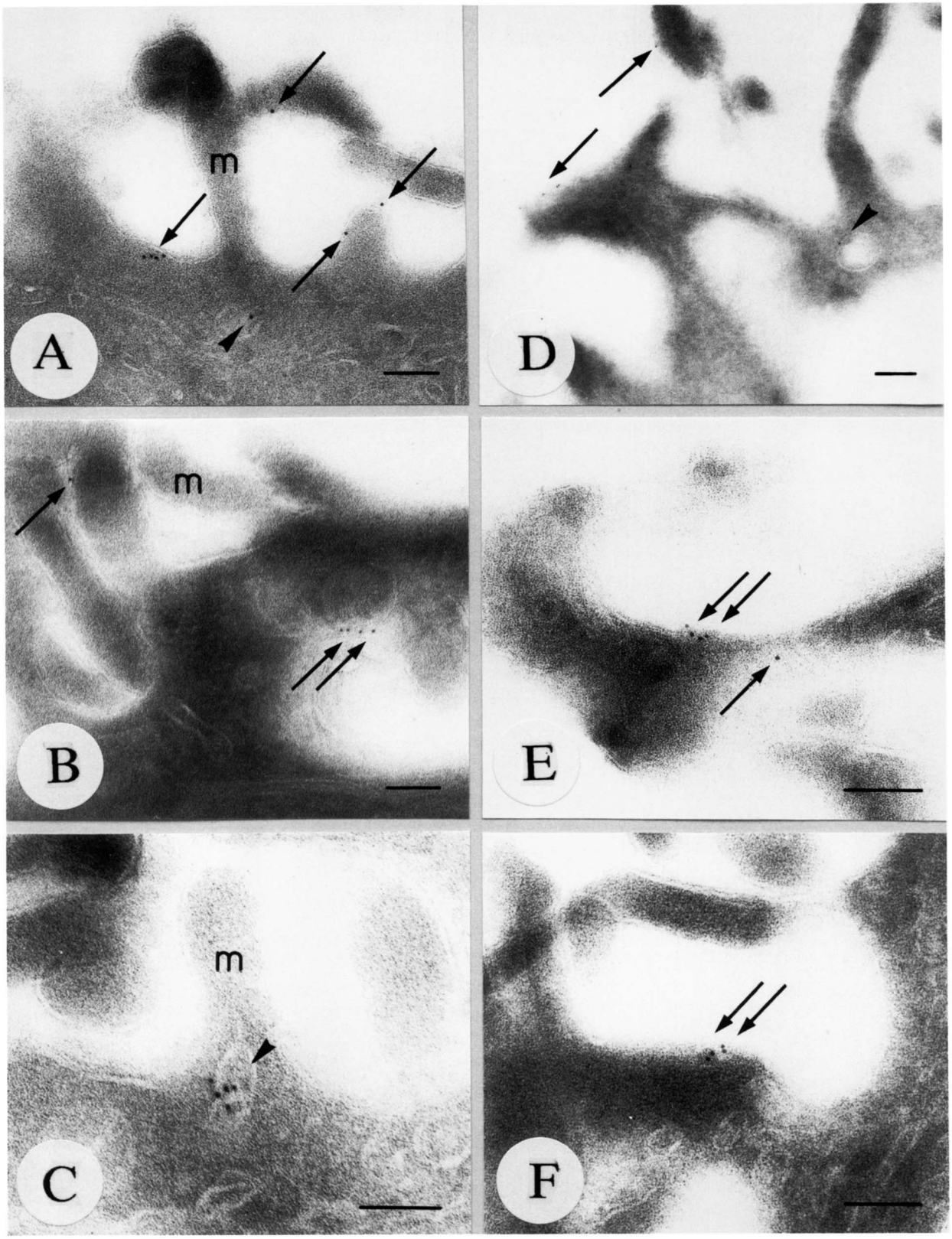

Fig. 4. Immunoelectronmicrographs of rat livers fixed 10 min after intravenous injection of 500 μg human RBP. Indirect immunolabeling was performed as described under Materials and Methods. Arrows and arrowheads indicate, respectively, gold particles distributed on the cell surface and in the cytoplasm near the cell surface of parenchymal (A-C) and stellate cells (D-F) (m, microvilli of parenchymal cells facing the perisinusoidal space). The bar represents 100 nm.

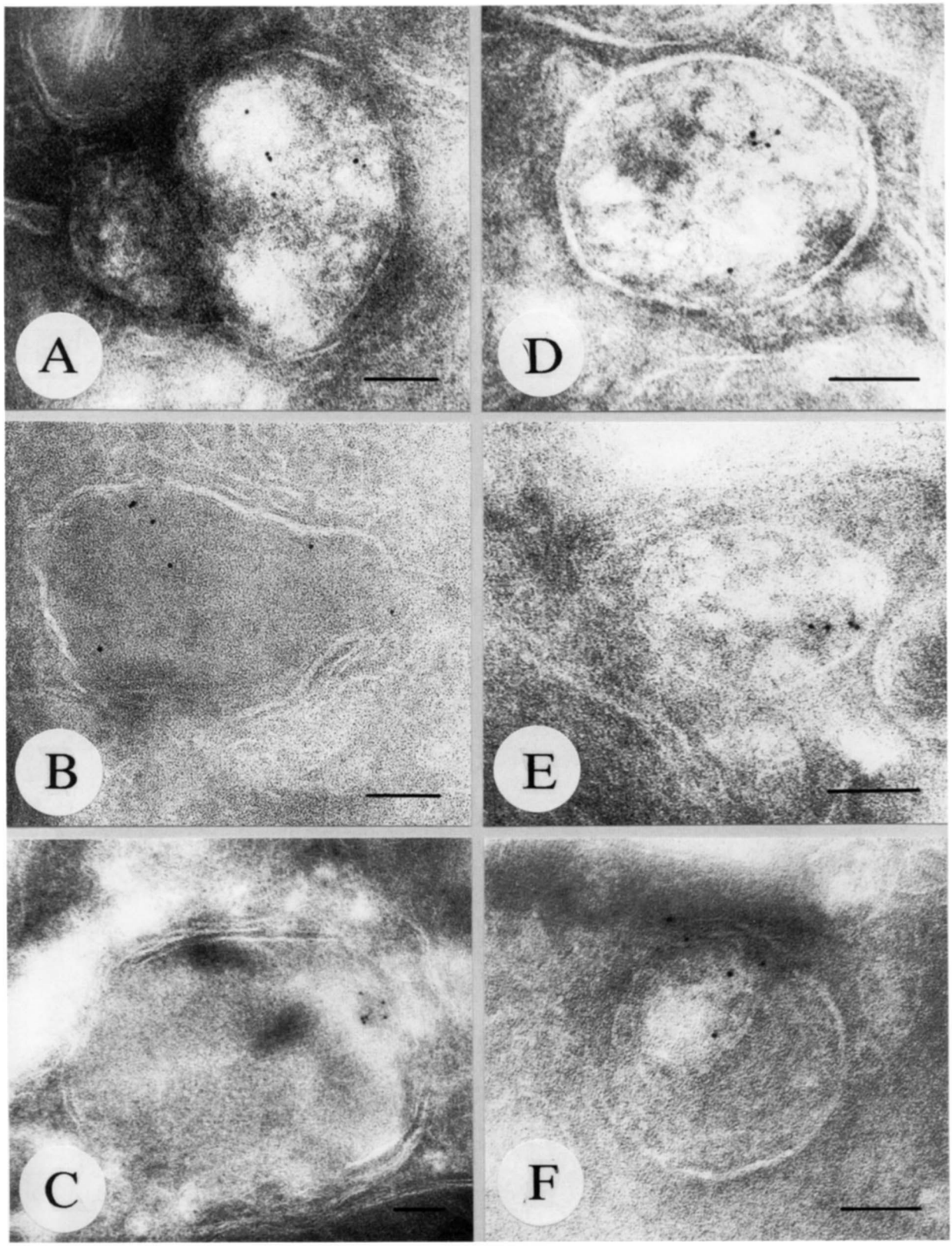

Fig. 5. Immunoelectronmicrographs of rat livers fixed 2 h after injection with 500 μg of human RBP. Indirect immunolabeling was performed as described under Materials and Methods. These vesicles were located deep within the cytoplasm of parenchymal cells (A-C) and stellate cells (D-F). The bar represents 100 nm.

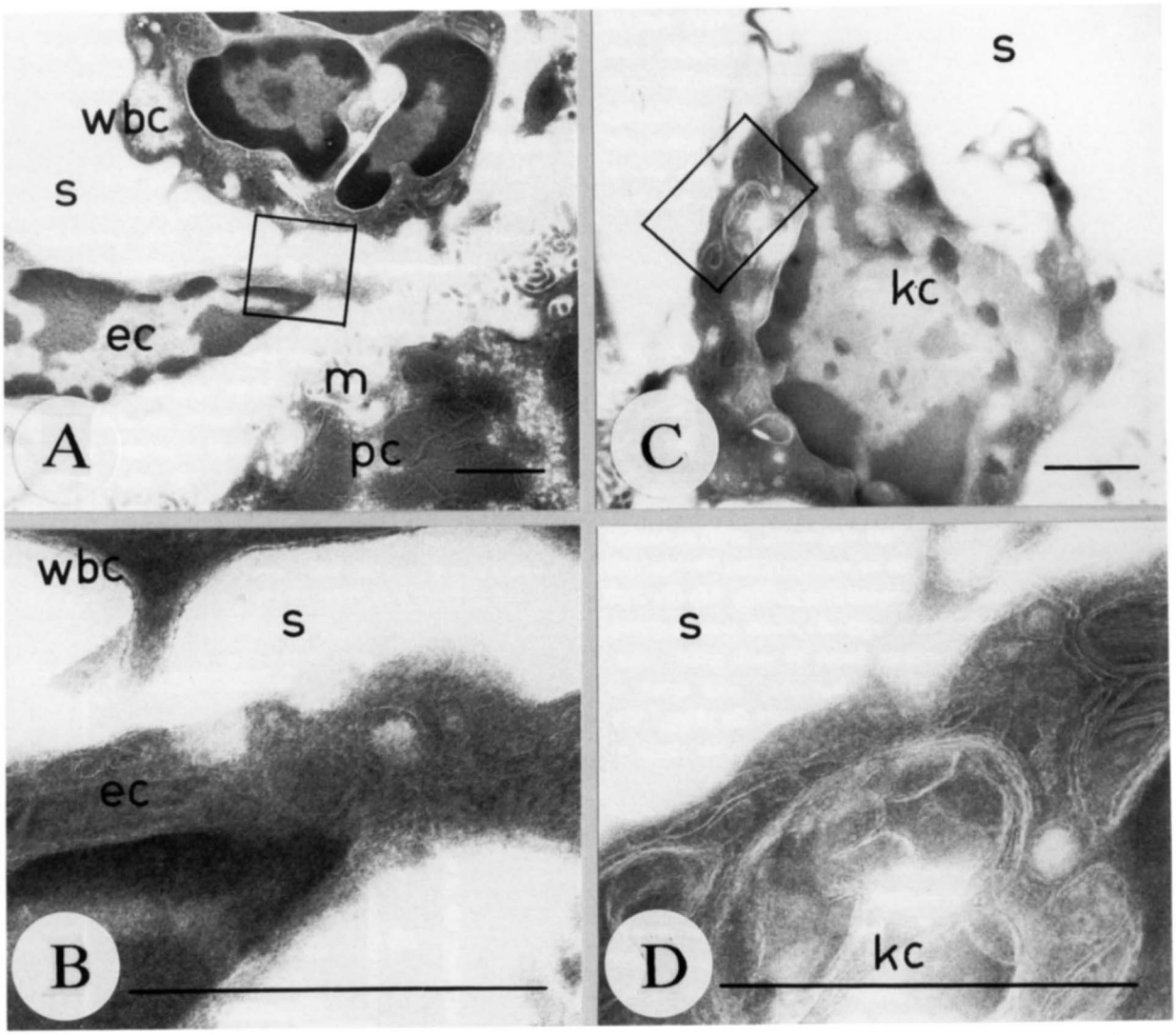

Fig. 6. Immunoelectronmicrographs of rat livers fixed 2 h after injection of 500 μg of human RBP. Indirect immunolabeling was performed as described under Materials and Methods. Panel A: endothelial cell; panel B: high magnification of a portion (rectangle) of A; panel C: Kupffer cell; panel D: high magnification of a portion (rectangle) of C (kc, Kupffer cells; ec, endothelial cells; pc, parenchymal cells; wbc, white blood cells; m, microvilli of parenchymal cells; s, sinusoid). The bar represents 1.0 μm.

structures resembling glycogen stores and bile canaliculi. The endothelial cells were identified as the cells that make up the wall of the sinusoids. The cells have a smooth surface, and the thin cell layer is thickened only around the nuclei. The stellate cells, which are located between the endothelial cells and the parenchymal cells in the space of Disse, contained huge lipid droplets. Structures resembling collagen were often seen close to the stellate cells. Their nucleus was indented by the lipid droplets and fine processes projected from the cells. The Kupffer cells are located within the sinusoids. The cells have an irregular surface and a part of the cells often penetrates the sinusoidal wall into the space of Disse.

Characterization of sheep anti-human RBP antibody

The sheep antibody against human RBP was characterized by Western blot analysis. As shown in **Fig. 3,** the antibody was monospecific for human RBP, and it did not cross-react with purified rat RBP or RBP in rat serum. Cryosections of liver from control rats showed no positive reaction against sheep anti-human RBP. Hence, positive reaction in the experiments described below represents cell-associated human RBP.

Distribution of cell-associated human RBP 10 min after injection

We then injected human RBP intravenously into two rats, and used the sheep antibody against human RBP to detect human RBP in cryosections. The sheep antibody was recognized by a rabbit anti-sheep IgG and protein A-colloidal gold.

Ten min after injection of human RBP, positive labeling of human RBP was observed in parenchymal (**Fig. 4A-C**) and in stellate cells (Fig. 4D-F). Gold particles

were seen at the cell surface (arrows) and in small vesicles close to the cell surface (arrowheads). The vesicles were 60–150 nm in diameter. Most of the gold particles were located close to the membrane of these vesicles, suggesting that the RBP was bound to a membrane receptor. In parenchymal cells, human RBP was localized only in the perisinusoidal part of the cell (Fig. 4A–C). No labeling was seen in Kupffer cells or in endothelial cells.

Distribution of human RBP in liver cells 2 h after injection

Two h after injection of human RBP into two rats, gold particles were still seen at the cell surface and in vesicles near the cell surface of parenchymal and stellate cells. The morphological characteristics of these vesicles were the same as for the labeled vesicles observed 10 min after injection.

In addition, at this time after injection, gold particles were also seen in larger vesicles or endosomes (34) located deeper in the cytoplasm of parenchymal (**Fig. 5A–C**) and stellate cells (Fig. 5D–F). This type of vesicle was negative in sections cut from specimens fixed 10 min after injection. They were 270–500 nm in diameter, and some of them resembled multivesicular bodies (MVB) (Figs. 5D, E). Almost none of the gold particles were seen close to the membrane of these vesicles.

In accordance with the uptake experiments presented in Fig. 2, most of the uptake was by parenchymal and stellate cells. No certain positive reaction above the background was recognized in endothelial cells (**Fig. 6A, B**) or Kupffer cells (Fig. 6C, D). Furthermore, liver cells in sections labeled with a mixture of sheep anti-human RBP and human RBP showed no positive reaction (see Materials and Methods), indicating that human RBP block binding of antibody to the section.

Counts of gold particles associated with different subcellular structures

The results of experiments in which we counted the number of gold particles associated with the different subcellular structures are shown in **Fig. 7.** Ten min after injection, most of the gold particles were identified on the cell surface or in small vesicles of both parenchymal and stellate cells. After 2 h, however, the relative distribution changed, in that many more of the gold particles were associated with larger vesicles or endosomes. In stellate cells about 30% of the gold particles were associated with MVB at that time.

Endocytosed asialo-orosomucoid and RBP are detected in the same intracellular vesicles in liver parenchymal cells

We then compared the distribution of human RBP in liver parenchymal cells with that of asialo-orosomucoid. Asialo-orosomucoid is a well-characterized ligand known to be internalized by receptor-medaited endocytosis (20, 28). We injected 3.3 mg asialo-orosomucoid and 0.5 mg human RBP into rats and studied the distribution of these ligands in cryosections of the liver after 10 (**Fig. 8A, B and D–F**) and 30 min (Fig. 8C).

In the experiments presented in Fig. 8A–C, we first incubated the sections with antibody against human RBP (small gold particles) and then with antibody against asialo-orosomucoid (large gold particles). In the sections presented in Fig. 8D–F, we changed the sequence of antibodies to ascertain that co-labeling in the same vesicles was not due to improper blocking of the first antibody.

Ten min after injection, both ligands were identified on the cell surface, in small vesicles located close to the plasma membrane (Fig. 8A) and in larger vesicles located deeper in the cytoplasm (Fig. 8B and D–F). Asialo-orosomucoid and human RBP were seldom observed in the same smaller vesicles (d = 60–150 nm). The larger vesicles (d = 270–500 nm) contained both asialo-

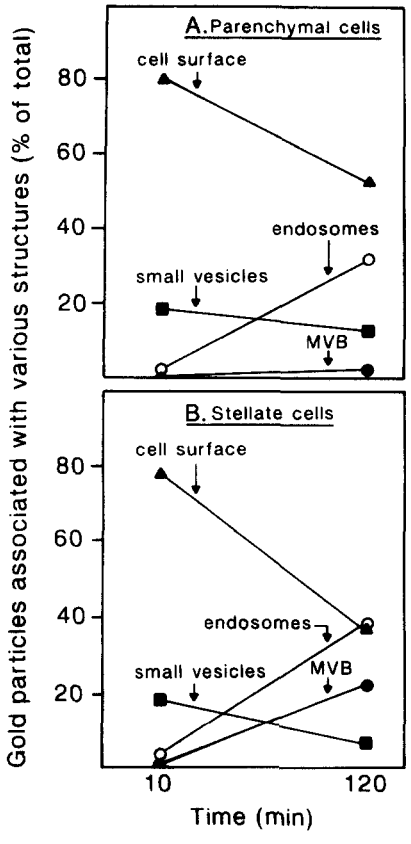

Fig. 7. Counts of gold particles associated with different structures. From several experiments similar to those presented in Figs. 4 and 5, gold particles associated with cell surface (▲), small vesicles close to the plasma membrane (■), larger vesicles or endosomes (○), and multivesicular bodies (MVB) (●) were counted. In parenchymal and stellate cells, respectively, 385 and 137 gold particles were counted in the 10-min experiment, and 435 and 382 gold particles were counted in the 120-min experiment.

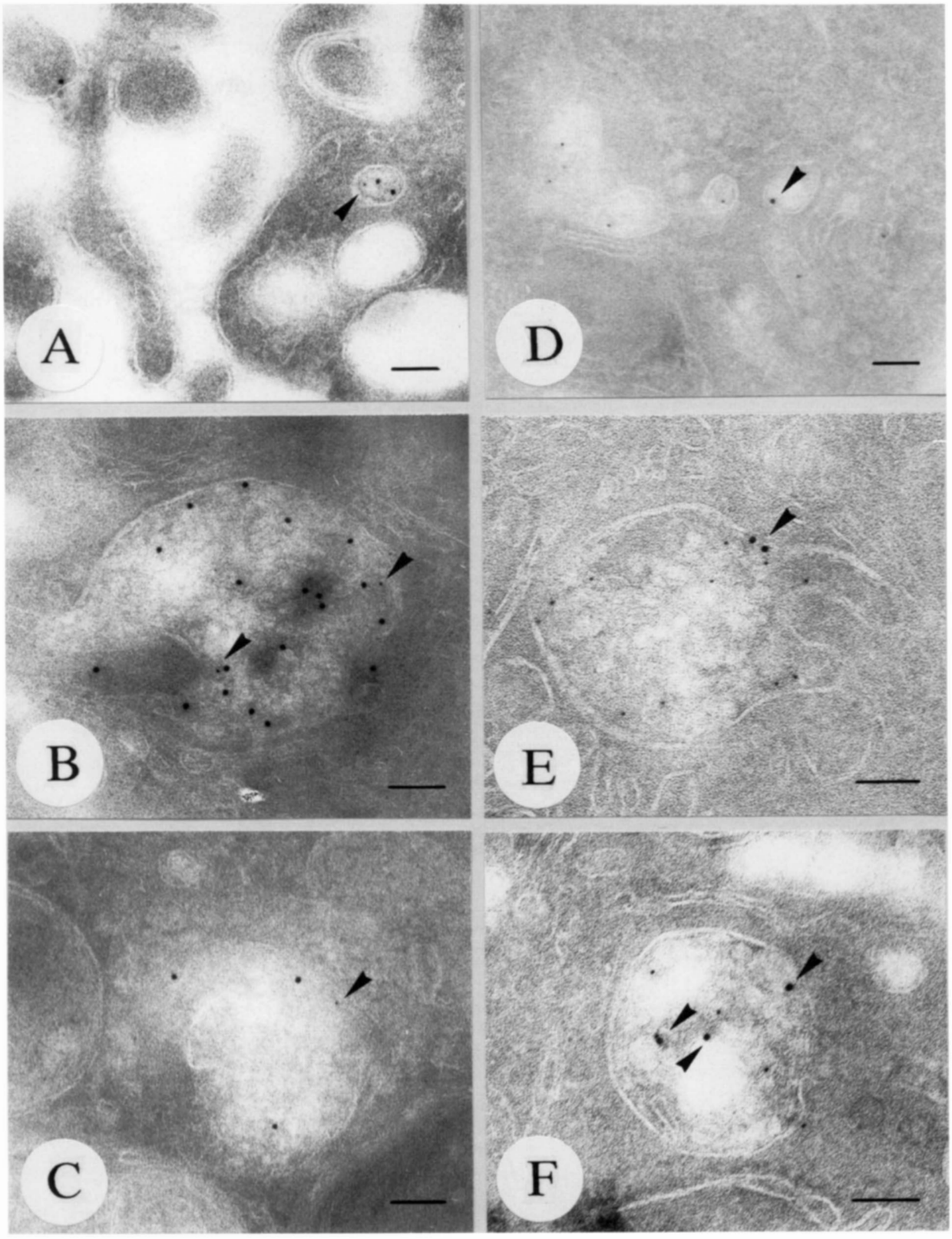

Fig. 8. Distribution of asialo-orosomucoid and RBP in parenchymal liver cells 10 min and 30 min after injection. Immunoelectronmicrographs of rat livers fixed 10 min (panels A, B and D–F) and 30 min (panel C) after injection with 0.5 mg of human RBP and 3.3 mg asialo-orosomucoid. The indirect double immunolabeling was performed as described under Materials and Methods. In the sections presented in panels A–C, human RBP was first immunolabeled, and then orosomucoid. In panels D–F, orosomucoid was first immunolabeled, and then human RBP. Small gold particles (d = 5 nm) in panels A–C, and large gold particles (d = 10 nm) in panels D–F, therefore, represent human RBP (arrowhead).

orosomucoid and human RBP. Typically, 2–4 gold particles specific for human RBP, and 5–20 gold particles specific for asialo-orosomucoid were observed in these vesicles. Thirty min after injection, much less asialo-orosomucoid was detected in the sections, suggesting that degradation had already started. Human RBP and asialo-orosomucoid were still detected together in vesicles with diameter 270–500 nm at this time (Fig. 8C).

DISCUSSION

In the present study, we have studied the in vivo uptake of RBP by rat liver cells. First, we compared the uptake in different liver cells of ^{125}I-labeled RBP with that of various well known ligands. While human and rat RBP were most actively taken up by stellate cells, acetyl-LDL and the mannose-terminated glycoproteins were mainly taken up by endothelial liver cells, and asialo-orosomucoid by parenchymal cells. Hence the ligands were recognized very differently by the various cell types in the liver, and the selective uptake of RBP by stellate cells was clearly different from the other ligands studied. This experiment suggests that stellate cells have the largest number of RBP receptors per cell in liver. Since there are about 10 times as many parenchymal cells as stellate cells in liver, the data imply that about equal amounts of RBP are taken up by parenchymal cells and stellate cells per total liver.

A selective uptake of RBP by parenchymal and stellate cells was also observed when we injected human RBP into rats and studied the uptake in rat livers by cryosectioning. This uptake of RBP by parenchymal and stellate cells is in accordance with the observation that only those two liver cell types contain a significant amount of retinol; only very small amounts of retinol are found in endothelial and Kupffer cells (35–39). These experiments support the suggestion that RBP plays an important role in the extensive recycling of retinol from plasma to the liver (40).

An important aspect of the present report is that we have studied the uptake of native, unmodified protein that was subsequently identified in the liver sections by a monospecific antibody (in contrast to gold conjugation of ligand before uptake studies or labeling of ligand with radioactive adducts such as ^{125}I-labeled tyramine-cellobiose).

Ten min after injection, RBP was found in close contact with the plasma membrane of parenchymal and stellate cells. The distribution of RBP suggests that a cell surface receptor specific to RBP exists along the perisinusoidal surface of parenchymal cells. RBP was also found in vesicles near the cell surface of parenchymal and stellate cells after 10 min. RBP was mainly observed attached to the membrane of these small vesicles, suggesting that it was bound to a receptor. Asialo-orosomucoid was only occasionally detected in the same initial vesicle as RBP. At later times, RBP was localized not only on the cell surface and in vesicles close to the cell surface, but also in larger vesicles located deeper in the cytoplasm of these cells. In these larger vesicles, RBP was most often observed at a distance from the vesicular membrane, suggesting that it had dissociated from its receptor.

Asialo-orosomucoid is a well-characterized ligand known to be internalized by receptor-mediated endocytosis. The observation that RBP and asialo-orosomucoid co-localized in these larger vesicles suggests that RBP is also taken up by receptor-mediated endocytosis.

Taken together, our present study supports the suggestion that a cell surface receptor for RBP is localized in parenchymal and stellate cells, and indicates that these cells internalize RBP by endocytosis.

This work was supported in part by grants from the Norwegian Cancer Society, Anders Jahres Foundation, the Norwegian Research Council for Science and the Humanities, and the Scandinavia-Japan Sasakawa Foundation.

Manuscript received 28 August 1989 and in revised form 21 February 1990.

REFERENCES

1. Goodman, D. S. 1984. Plasma retinol-binding protein. *In* The Retinoids. Vol 2. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press, New York. 41–88.
2. Fex, G., and G. Johannesson. 1987. Studies of the spontaneous transfer of retinol from the retinol:retinol-binding protein complex to unilamellar liposomes. *Biochim. Biophys. Acta.* **901:** 255–264.
3. Fex, G., and G. Johannesson. 1988. Retinol transfer across and between phospholipid bilayer membranes. *Biochim. Biophys. Acta.* **944:** 249–255.
4. Ho, M. T. P., H. J. Pownall, and J. G. Hollyfield. 1989. Spontaneous transfer of retinoic acid, retinyl acetate, and retinyl palmitate between single unilamellar vesicles. *J. Biol. Chem.* **264:** 17759–17763.
5. Mack, J. P., N. S. T. Lui, O. A. Roels, and O. R. Anderson. 1972. The occurrence of vitamin A in biological membranes. *Biochim. Biophys. Acta.* **288:** 203–219.
6. Heller, J., and D. Bok. 1976. A specific receptor for retinol binding protein as detected by the binding of human and bovine retinol binding protein to pigment epithelial cells. *Am. J. Ophthalmol.* **81:** 93–97.
7. Bok, D., and J. Heller. 1976. Transport of retinol from the blood to the retina: an autoradiographic study of the pigment epithelial cell surface receptor for plasma retinol-binding protein. *Exp. Eye Res.* **22:** 395–402.
8. McGuire, B. W., M-C. Orgebin-Crist, and F. Chytil. 1981. Autoradiographic localization of serum retinol-binding protein in rat testis. *Endocrinology.* **108:** 658–667.
9. Gjøen, T., T. Bjerkelund, H. K. Blomhoff, K. R. Norum, T. Berg, and R. Blomhoff. 1987. Liver takes up retinol-binding protein from plasma. *J. Biol. Chem.* **262:** 10926–10930.
10. Heller, J. 1975. Interaction of plasma retinol-binding protein with its receptor. Specific binding of bovine and human retinol-binding protein to pigment epithelium cells from bovine eyes. *J. Biol. Chem.* **250:** 3613–3619.

11. Bhat, M. K., and H. R. Cama. 1979. Gonadal cell surface receptor for plasma retinol-binding protein. A method for its radioassay and studies on its level during spermatogenesis. *Biochim. Biophys. Acta.* **587:** 273–281.
12. Eriksson, U., E. Hansson, M. Nilsson, K-H. Jønsson, J. Sundelin, and P. A. Peterson. 1986. Increased levels of several retinoid binding proteins resulting from retinoic acid-induced differentiation of F9 cells. *Cancer Res.* **46:** 717–722.
13. Creek, K. E., C. S. Silverman-Jones, and L. M. De Luca. 1989. Comparison of the uptake and metabolism of retinol delivered to primary mouse keratinocytes either free or bound to rat serum retinol-binding protein. *J. Invest. Dermatol.* **92:** 283–289.
14. Rask, L., and P. A. Peterson. 1976. In vitro uptake of vitamin A from the retinol-binding plasma protein to mucosal epithelial cells from the monkey's intestine. *J. Biol. Chem.* **251:** 6360–6366.
15. Sivaprasadarao, A., and J. B. C. Findlay. 1988. The interaction of retinol-binding protein with its plasma-membrane receptor. *Biochem. J.* **255:** 561–569.
16. Sivaprasadarao, A., and J. B. C. Findlay. 1988. The mechanism of uptake of retinol by plasma-membrane vesicles. *Biochem. J.* **255:** 571–579.
17. Laurent, B. C., C. O. Båvik, U. Eriksson, H. Melhus, M. H. L. Nilsson, J. Sundelin, L. Rask, and P. A. Peterson. 1987. Intra- and extracellular retinoid-binding proteins. *Chem. Scr.* **27:** 185–192.
18. Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* **38:** 87–93.
19. Distler, J. J., and G. W. Jourdian. 1978. β-Galactosidase from bovine testes. *Methods Enzymol.* **50:** 514–523.
20. Tolleshaug, H. 1981. Binding and internalization of asialoglycoproteins by isolated rat hepatocytes. *Int. J. Biochem.* **13:** 45–51.
21. Blomhoff, R., C. A. Drevon, W. Eskild, P. Helgerud, K. R. Norum, and T. Berg. 1984. Clearance of acetyl low density lipoprotein by rat liver endothelial cells. *J. Biol. Chem.* **259:** 8898–8903.
22. Redshaw, M. R., and S. S. Lynch. 1974. An improved method for the preparation of iodinated antigens for radioimmunoassay. *J. Endocrinol.* **60:** 527–528.
23. Blomhoff, R., K. R. Norum, and T. Berg. 1985. Hepatic uptake of [^{3}H]retinol bound to the serum retinol binding protein involves both parenchymal and perisinusoidal stellate cells. *J. Biol. Chem.* **260:** 13571–13575.
24. Blomhoff, R., T. Berg, and K. R. Norum. 1988. Distribution of retinol in rat liver cells: effect of age, sex and nutritional status. *Br. J. Nutr.* **60:** 233–239.
25. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
27. Griffiths, G., A. McDowall, R. Back, and J. Dubochet. 1984. On the preparation of cryosections for immunocytochemistry. *J. Ultrastruct. Res.* **89:** 65–78.
28. Geuze, H. J., J. W. Slot, and A. L. Schwartz. 1987. Membranes of sorting organelles display lateral heterogeneity in receptor distribution. *J. Cell Biol.* **104:** 1715–1723.
29. Wisse, E. 1970. An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids. *J. Ultrastruct. Res.* **31:** 125–150.
30. Stang, E. 1987. Modification of the LKB 7800 series knifemaker for symmetrical breaking of cryo-knives. *J. Microsc.* **149:** 77–79.
31. Stang, E., and Johansen, B. V. 1989. Improved glass knives for ultramicrotomy. A procedure for tungsten coating the knife edge. *J. Ultrastruct. Mol. Struct. Res.* **98:** 39–43.
32. Tokuyasu, K. T. 1984. Immuno-cryoultramicrotomy. *In* Immunolabeling for Electron Microscopy. J. M. Polak and I. M. Varndell, editors. Elsevier Science Publ. B. V., Amsterdam. 71–82.
33. Slot, J. W., and H. J. Geuze. 1984. Gold markers for single and double immunolabeling of ultrathin cryosections. *In* Immunolabeling for Electron Microscopy. J. M. Polak and I. M. Varndell, editors. Elsevier Science Publ. B. V., Amsterdam. 129–142.
34. Griffiths, G., and H. Hoppeler. 1986. Quantitation in immunocytochemistry: correlation of immunogold labeling to absolute number of membrane antigens. *J. Histochem. Cytochem.* **34:** 1389–1398.
35. Blomhoff, R., M. Rasmussen, A. Nilsson, K. R. Norum, T. Berg, W. S. Blaner, M. Kato, J. R. Mertz, D. S. Goodman, U. Eriksson, and P. A. Peterson. 1985. Hepatic retinol metabolism. Distribution of retinoids, enzymes, and binding proteins in isolated rat liver cells. *J. Biol. Chem.* **260:** 13560–13565.
36. Blomhoff, R. 1987. Hepatic retinol metabolism: role of the various cell types. *Nutr. Rev.* **45:** 257–263.
37. Wake, K. 1971. "Sternzellen" in the liver: perisinusoidal cells with special reference to storage of vitamin A. *Am. J. Anat.* **132:** 429–462.
38. Wake, K. 1974. Development of vitamin A-rich droplets in multivesicular bodies of rat liver stellate cells. *J. Cell Biol.* **63:** 683–691.
39. Wake, K. 1980. Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. *Int. Rev. Cytol.* **66:** 303–353.
40. Green, M. H., L. Uhl, and J. B. Green. 1985. A multicompartmental model of vitamin A kinetics in rats with marginal liver vitamin A stores. *J. Lipid Res.* **26:** 806–818.