

Fc receptor mediated endocytosis of small soluble immunoglobulin G immune complexes in Kupffer and endothelial cells from rat liver

T. Løvdal^{1,*}, E. Andersen¹, A. Brech² and T. Berg¹

¹Division of Molecular Cell Biology, Department of Biology, University of Oslo, PO Box 1050, Blindern, 0316 Oslo, Norway

²Electron Microscopical Unit for Biological Sciences, Department of Biology, University of Oslo, PO Box 1062, Blindern, 0316 Oslo, Norway

*Author for correspondence (e-mail: torunnl@bio.uio.no)

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SUMMARY

Soluble circulating immunoglobulin G immune complexes are mainly eliminated by the liver, predominantly by uptake in the Kupffer cells, but also the liver endothelial cells seem to be of importance. In the present study we have followed the intracellular turnover of immune complexes after Fc γ receptor mediated endocytosis in cultured rat liver endothelial cells and Kupffer cells by means of isopycnic centrifugation, DAB cross-linking and morphological techniques. For the biochemical experiments the antigen, dinitrophenylated bovine serum albumin (BSA), was labeled with radioiodinated tyramine cellobiose that cannot cross biological membranes and therefore traps labeled degradation products at the site of formation. The endocytic pathway followed by immune complexes was compared with that followed by scavenger receptor ligands, such as formaldehyde treated BSA and dinitrophenylated BSA, and the mannose receptor ligand ovalbumin.

Both Kupffer cells and liver endothelial cells took up and degraded the immune complexes, but there was a clear delay in the degradation of immune complexes as compared to degradation of ligands taken up via scavenger

receptors. The kinetics of the endocytosis of scavenger receptor ligand was unaffected by simultaneous uptake of immune complexes. Experiments using both biochemical and morphological techniques indicated that the delayed degradation was due to a late arrival of the immune complexes at the lysosomes, which partly was explained by retroendocytosis of immune complexes. Electron microscopy studies revealed that the immune complexes were retained in the early endosomes that remained accessible to other endocytic markers such as ovalbumin. In addition, the immune complexes were seen in multivesicular compartments apparently devoid of other endocytic markers. Finally, the immune complexes were degraded in the same lysosomes as the ligands of scavenger receptors. Thus, immune complexes seem to follow an endocytic pathway that is kinetically or maybe morphologically different from that followed by scavenger and mannose receptor ligands.

Key words: Fc receptor, Endocytosis, Intracellular sorting, Kupffer cell, Liver endothelial cell, Rat

INTRODUCTION

Fc γ receptors (Fc γ Rs) are glycoproteins that recognize the Fc domain of immunoglobulin G (IgG). Integral membrane Fc γ Rs can mediate phagocytosis of large IgG coated particles as well as pinocytosis of soluble IgG immune complexes (IgG-ICs; for review see Daeron, 1997; Aderem and Underhill, 1999). Soluble circulating IgG-ICs are mainly eliminated by the liver (Arend and Mannik, 1971; Bogers et al., 1991; Johansson et al., 1996) predominantly by uptake in the Kupffer cells (KC), but also the liver endothelial cells (LEC) seem to be of importance (Skogh et al., 1985; Bogers et al., 1991; Johansson et al., 1996). In the present study we have examined the intracellular turnover of small soluble IgG-ICs after Fc γ R mediated endocytosis in cultured rat LEC and KC.

Small soluble IgG-ICs are endocytosed via clathrin coated pits (Hedin et al., 1984; Ukkonen et al., 1986; Miettinen et al.,

1989; Honing et al., 1991; Amigorena et al., 1992a). After formation of clathrin coated vesicles, the clathrin coat is shed and uncoated 'primary endosomes' are formed. These are small vesicles that transport their cargo to early endosomes, of which two populations exist, termed sorting endosomes and recycling endosomes. From the early endosome one of five possible routes may be followed: (1) the receptor-ligand complex may be directed to lysosomes for degradation; (2) the ligand may dissociate from the receptor and follow the pathway to the lysosomes; or (3) it may be transported to the Golgi apparatus, whereas the receptor is recycled back to the plasma membrane; (4) the receptor-ligand complex may be transported to the opposite side of the cell (transcytosis); or (5) the receptor-ligand complex may be recycled back to the plasma membrane (retroendocytosis; for review see Mukherjee et al., 1997). Transport from early endosomes to lysosomes occurs via the late endosomes. Based on their morphology in the

electron microscope late endosomes in some cells have been named multivesicular bodies because they appear to have internal vesicular profiles (Hirsch et al., 1968; Haigler et al., 1979; McKanna et al., 1979; Dunn and Hubbard, 1984; Croze et al., 1989; Felder et al., 1990; van Deurs et al., 1993) probably made by inward invagination of, and fission from the limiting membrane (Hirsch et al., 1968; McKanna et al., 1979; van Deurs et al., 1993). The internal vesicles of multivesicular bodies seem to have an important role in the catabolism of lipids (for review see Sandhoff and Kolter, 1996; Kobayashi et al., 1998; Möbius et al., 1999) and certain receptors, including the epidermal growth factor receptor (EGF-R) have been localized to the internal vesicles (Haigler et al., 1979; McKanna et al., 1979; Felder et al., 1990; Hopkins et al., 1990). The multivesicular bodies containing EGF/EGF-R complexes mature and fuse directly with lysosomes in which the complexes are degraded (Futter et al., 1996). However, in some systems multivesicular bodies are considered to be carrier vesicles moving from early to late endosomes (Gruenberg et al., 1989).

Similar to EGF-Rs, Fc γ Rs following cross-linking by multivalent ligands have been reported to be transported to and degraded in the lysosomes (Mellman and Plutner, 1984; Ukkonen et al., 1986), whereas binding of monovalent antibodies to Fc γ Rs leads to recycling of the receptor-ligand complex back to the cell surface (Mellman et al., 1984; Harrison et al., 1994).

In the present study we have examined the endocytic pathway followed by small soluble IgG-ICs in rat LEC and KC by means of isopycnic centrifugation, DAB cross-linking and morphological techniques. The endocytic pathway followed by IgG-ICs was compared to the endocytic pathway followed by scavenger receptor ligands, such as formaldehyde treated bovine serum albumin (f-BSA) and dinitrophenylated BSA (DNP-BSA), or by the mannose receptor ligand ovalbumin. We obtained evidence to suggest that following Fc γ R mediated endocytosis IgG-ICs mainly follow the same intracellular pathway and kinetics as scavenger and mannose receptor ligands to the early endosomes. However, there is a clear delay in the degradation of IgG-ICs as compared to these ligands, caused by a slower transport to a degradative compartment. The IgG-ICs were retained in the early endosomes, which is partly explained by recycling of the IgG-ICs to the plasma membrane. In addition, IgG-ICs were seen in multivesicular compartments apparently devoid of other endocytic markers. Finally, degradation products from all markers end up in common lysosomes. Thus, IgG-ICs seem to follow an endocytic pathway that is kinetically or maybe morphologically different from that followed by scavenger and mannose receptor ligands. A short account for this study has appeared previously (Andersen et al., 1997).

MATERIALS AND METHODS

Animals and reagents

Male Wistar rats weighing 200-300 g, were obtained from Møllegaard and Bomholt (Ry, Denmark). The rats were kept at 21 \pm 3°C and fed with pellets from B&K Universal (Humberside, England). BSA, 2,4-DNP, horseradish peroxidase (HRP) type VI, 1-fluoro-2,4-dinitrobenzene (Sanger's reagent), diaminobenzidine (DAB, free

base), phenylmethylsulfonyl fluoride, unconjugated and biotin-conjugated goat anti-rabbit antibodies and streptavidin conjugated to 10 nm colloidal gold particles were purchased from Sigma Chemical company (St Louis, MO). Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenyl-glycouril) was from Pierce Chemical Company (Rockford, IL). The monoclonal antibody RT1B OX-6 was purchased from Pharmingen (San Diego, CA). Digitonin, 2,4-dinitrobenzenesulfonic acid (sodium salt) and dry dimethyl formamide were obtained from Aldrich (Stockholm, Sweden). Keyhole limpet haemocyanin and FlouroSave were obtained from Calbiochemical-Novabiochemical international (La Jolla, CA), and Maxidens from Nycomed Amersham (Oslo, Norway). Dowex (1-x8) anion exchange resin was purchased from Bio-Rad Laboratories (Hercules, CA), Eagle minimal essential medium, glutamine and penicillin/streptomycin were obtained from Bio Whittaker, INC. (Walkersville, MD). Alexa 488 Protein Labeling Kit and Texas Red sulfonyl chloride from Molecular Probes (Eugene, OR) were used. Citrate filter number 3 was obtained from Gøteborg's Thermometer factory (Sweden), and columns and column materials from Pharmacia Biotech (Uppsala, Sweden). Nylon gauze was from Sefar AG (Thal, Switzerland). Na¹²⁵I was purchased from Isopharma (Kjeller, Norway) and tyramine cellobiose (TC) was a gift from Dr Helge Tolleshaug (Nycomed, Oslo, Norway). All additional chemicals were of analytical grade.

Preparation of the antigen

According to Eisen (1964) BSA was conjugated with DNP groups as follows: 100 mg 2,4-dinitrobenzenesulfonic acid, 100 mg BSA and 100 mg potassium carbonate were dissolved in 5 ml distilled water and incubated at 37°C for 60 or 90 minutes protected from light under continuous agitation. DNP conjugated to BSA was separated from free dinitrobenzenesulfonic acid on a Sephadex G-25 PD-10 column and dialyzed in distilled water overnight at 4°C. The preparation was lyophilized and stored at 4°C. At the time of use it was dissolved in 20 mM phosphate buffered saline (PBS), pH 7.4. The average number of DNP groups per BSA molecule was determined spectrophotometrically at 360 nm (Beckman DU-62 Spectrophotometer, Fullerton, CA) using 1.74×10^4 as the molar extinction coefficient of DNP and was found to be 8 or 11 after incubation for 60 or 90 minutes, respectively.

Preparation of the antibodies

Eurogentec (Ougree, Belgium) prepared anti-DNP immune serum by immunizing rabbits with DNP-conjugated keyhole limpet haemocyanin. Anti-DNP antibodies were purified by affinity chromatography essentially as described by Ljunghusen et al. (1990). The affinity column was prepared as follows: 4 g 2,4-dinitrobenzenesulfonic acid and 4 g potassium carbonate were dissolved in 160 and 40 ml distilled water, respectively, before the solutions were mixed. 4 g lysine-Sepharose 4B was added to 30-50 ml distilled water, allowed to swell for 15-30 minutes at room temperature before the supernatant was removed and the lysine-Sepharose washed in 80 ml distilled water via citrate filter. DNP was conjugated to lysine-Sepharose 4B overnight at 4°C protected from light. The DNP-lysine-Sepharose beads obtained were washed with distilled water via citrate filter until no color could be detected followed by 50 ml PBS with pH 11, 50 ml PBS with pH 2.5 and equilibrated in PBS with pH 7.4 before packed into a XK 16/20 column. 30-40 ml immune serum was passed through the column either at 4°C or room temperature and unbound plasma constituents were eluted with PBS until no absorbance was detected at 280 nm. Anti-DNP antibodies were eluted with 0.10 M 2,4-DNP and the eluate was immediately passed over a 50 ml Dowex 1-X8 column to detach DNP from the antibodies. IgG was concentrated by affinity chromatography on a HiTrap protein A column and the IgG concentration determined spectrophotometrically at 280 nm using 1.3 as the molar extinction coefficient. IgG was stored at -20°C.

Radiolabeling

The antigen, DNP-BSA, was labeled with ^{125}I -TC according to Pittman (1983). Glass tubes were coated with 20–500 μg iodogen introduced in chloroform, which evaporated. 150 nmol TC in 15 μl PBS and 40 MBq Na^{125}I were incubated for 30 minutes at room temperature in a iodogen coated tube, before the mixture was transferred to a tube containing 10 μl 0.1 M sodium bisulphite (omitted during radiolabeling of DNP-BSA used in the experiments presented in Fig. 1) and 5 μl 0.1 M potassium iodate to stop further reaction. 30 μl 10 mM cyanuric chloride in acetonitrile was added 3 minutes before addition of 1 mg DNP-BSA diluted 1:1 in 0.2 M sodium carbonate, pH 9.5. After 1.5–2 hours at room temperature free Na^{125}I was separated from iodinated protein on a Sephadex G25 PD-10 column. The radiolabeled protein was kept at -20°C . TC cannot cross biological membranes and therefore traps the labeled degradation products at the site of formation.

Goat anti-rabbit IgG antibody (130 μg) was diluted with PBS and iodinated with 40 MBq Na^{125}I in a iodogen coated glass tube in a final volume of 100 μl . The radioiodination was carried out for 30 minutes at room temperature and arrested by passage over a Sephadex G-25 PD-10 column. The radiolabeled goat anti-rabbit IgG antibodies were stored at 4°C .

Preparation and characterization of IgG-immune complexes

IgG-ICs were formed between radiolabeled DNP-BSA and rabbit anti-DNP IgG antibodies at varying molar antibody excess (1:1, 1:5, 1:10, 1:15 and 1:20) in PBS. The antigen and antibody were incubated at 37°C for 45 minutes under continuous agitation. ^{125}I -TC-DNP-BSA-IgG was characterized by centrifugation in linear sucrose gradients (5–45% sucrose with 10 mM *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid) (HEPES) and 1 mM EDTA) at 4°C for 10 hours at 30,000 rpm in a Beckman L8-80 ultracentrifuge equipped with a SW 41Ti rotor. Fractions (22 \times 0.5 ml) were collected as described below (under 'isopycnic centrifugation'). The concentration of rabbit anti-DNP IgG antibodies resulting in the formation of small to medium sized immune complexes (9–19 S), and at the same time gave the smallest amount of uncomplexed antigen (maximum 30%) was used for further preparations of IgG-ICs.

Preparation of colloidal gold protein conjugates

Colloidal gold particles, 5 and 20 nm in diameter, were prepared according to Slot and Geuze (1985). Solutions containing 5 and 20 nm colloidal gold particles were complexed with f-BSA and ovalbumin, respectively, according to established procedures (Horisberger and Rosset, 1977; Roth et al., 1978). Aggregates were removed from the 5 nm gold-f-BSA solution by centrifugation for 25 minutes at 16,000 rpm in a J2-21 Beckman centrifuge equipped with a fixed angle JA-20 rotor. Gold-protein conjugates were then recovered by centrifugation of the supernatant for 60 minutes at 30,000 rpm in a Beckman L8-80 ultracentrifuge equipped with a 60Ti fixed angle rotor. Ovalbumin-gold conjugates were sedimented by centrifugation for 25 minutes at 16,000 rpm in a J2-21 Beckman centrifuge equipped with a JA-20 rotor. BSA was added to the resulting soft pellets to a final concentration of 0.1% and the conjugates dialyzed in 2 l PBS at 4°C overnight and kept at 4°C .

Preparation of HRP-ovalbumin conjugates

Ovalbumin was conjugated to HRP as described by Nakane and Kawaoi (1974), except that 0.16 M instead of 0.32 M ethylene glycol was used.

Preparation of fluorescent protein conjugates

DNP-BSA was conjugated to Alexa 488 according to the procedure in the Protein Labeling Kit from Molecular Probes. For conjugation of BSA to Texas Red, 100 μl Texas Red sulfonyl chloride in dry dimethylformamide (10 mg/ml) was added dropwise to 1 ml BSA in

0.15 M sodium bicarbonate, pH 9.5 (10 mg/ml) while vortexing. Conjugation was carried out for 60 minutes at 4°C and stopped by adding 0.1 ml freshly prepared 1.5 M hydroxylamine, pH 8.0, and incubated for an additional 30 minutes (Molecular Probes). Protein was separated from unconjugated dye on a Sephadex G-25 PD10 column. Following conjugation to Texas Red, BSA was treated with formaldehyde as described by Eskild and Berg (1984).

Purification of fibronectin from human plasma

Fibronectin was purified from human plasma on a Gelatin-Sepharose 4B affinity column according to the procedure from Pharmacia Biotech. The column was equilibrated with 125 ml PBS containing 5 mM EDTA and 2 mM phenylmethylsulfonyl fluoride (binding buffer), before 600 ml human plasma containing 5 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride was applied. Washing with binding buffer was performed until no protein was detected by measuring absorbance at 280 nm, and fibronectin was then eluted with 4 M urea in binding buffer. Fractions containing fibronectin were dialyzed at 4°C against 3 \times 3 liter PBS with 2 mM EDTA and 0.02% sodium azide, the last time overnight. Precipitates, if present, were removed by centrifugation. The protein content was determined and fibronectin was either kept at 4°C in the presence of 0.02% sodium azide, or at -20°C in the presence of 60 μM iodacetamide.

Preparation and culture of liver cells

Rats were anaesthetized by ether. Isolated rat liver cells were prepared essentially as described by Seglen (1976). The isolated cells prepared by collagenase perfusion were suspended in cold PBS containing 1% BSA and filtered through a double layer of nylon gauze (100 and 250 μm). Parenchymal cells were sedimented by centrifugation for 2 minutes at 400 rpm in a Kubota 8800 centrifuge equipped with an RS-3000/6 rotor. The pellet was resuspended in PBS containing 1% BSA and the centrifugation repeated. Non-parenchymal cells were sedimented from the supernatants by centrifugation for 5 minutes at 1800 rpm in the Kubota 8800 centrifuge and resuspended in Eagle minimal essential medium containing 2 mM L-glutamine, 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin. Contaminating parenchymal cells were removed by repeated centrifugation in a conical tube for 2 minutes at 400 rpm in the Kubota 8800 centrifuge until no visible pellet was obtained. Alternatively, contaminating parenchymal cells were removed by centrifugation in Nycoprep as described by Johansson et al. (1996). The centrifugation steps were carried out at 4°C . The non-parenchymal cell fraction was allowed to adhere either onto surfaces coated with 2.5% glutaraldehyde fixed BSA for 30 minutes (Laakso and Smedsrød, 1987), or onto surfaces coated with human fibronectin (0.3 mg/ml) for 1.5 to 2 hours (Smedsrød et al., 1982) at 37°C in 5% CO_2 to obtain cultures of KC or LEC, respectively. The non-adherent cells were removed by washing with PBS. BSA was fixed with 2.5% glutaraldehyde for at least 1 hour at room temperature.

For all experiments cells were grown in Petri dishes (6 cm in diameter), except for the confocal microscopy experiments where cells were grown on glass coverslips (1 cm^2) and 2 or 0.5 ml of the different ligands were added, respectively. The concentration of all ligands added was 10 nM unless otherwise stated. For all incubations at 37°C in 5% CO_2 the cells were grown in Eagle minimal essential medium containing 2 mM L-glutamine, 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin, but following adherence of KC 10% heat inactivated fetal calf serum was added to the medium. For ligand binding on ice cells were kept in suspension buffer containing pyruvic acid (Seglen, 1976) and 1% BSA.

Isopycnic centrifugation

Cultured cells were homogenized in PBS by sonication (MSE Soniprep 150 Ultrasonic Disintegrator) at maximum force for 4 seconds. Sonication disrupts the plasma membrane as indicated by Trypan Blue exclusion, but not internal membranes to the same extent determined by release of the lysosomal enzyme β -glucuronidase

measured according to Gianetto and de Duve (1955). After removal of the nuclei by centrifugation for 2 minutes at 4000 rpm in a Heraeus Sepatech Biofuge 17RS equipped with a 2147 rotor, 0.25 M sucrose containing 10 mM HEPES and 1 mM EDTA was added (final volume 4.5 ml), and 4 ml of the postnuclear fraction was layered on top of a linear sucrose gradient (21–54% sucrose containing 10 mM HEPES and 1 mM EDTA). The centrifugation steps were carried out at 4°C. Following centrifugation for 5 hours at 20 000 rpm in a Beckman L8-80 Ultracentrifuge equipped with a SW 28 rotor, the gradients were divided into 18 × 2 ml fractions by upward displacement using Maxidens (1.9 g/ml) as displacement fluid. The density of each fraction was calculated from its refractive indices (η) using the formula: $\text{density} = 0.0468 + 3.0115 \times (\eta - 1) - 0.4647 \times (\eta - 1)^2$.

Ligand degradation was determined by measuring radioactivity soluble in 12.5% trichloroacetic acid. BSA was added as a carrier to a final concentration of 0.5%.

Protein assays

Protein concentration was determined using the Protein assay or the DC Protein assay from Bio-Rad and measured spectrophotometrically in an ICN Flow Titertek Multiscan Plus at 590 or 750 nm, respectively. The protein used for the standard curve was BSA.

Confocal laser-scanning microscopy

For confocal laser-scanning microscopy cells grown on glass cover slips were fixed in 2% paraformaldehyde in distilled water for 10–15 minutes at room temperature, before they were mounted upside down in a droplet of FluoroSave. Images were acquired with a Leica TCS-NT (DM IRBE) digital scanning confocal microscope equipped with a 100/1.40 NA oil immersion objective (Leica, Germany). The images were exported to Adobe Photoshop (Adobe Systems Inc.), processed for presentation, and printed on a Color Laser Jet 4500 N printer (Hewlett Packard).

DAB cross-linking in whole cells and solubilization

Following endocytosis of HRP-ovalbumin and ^{125}I -TC-DNP-BSA or ^{125}I -TC-DNP-BSA-IgG, LEC were cultured in 1 ml HEPES-saline buffer containing 10 mM HEPES and 140 mM sodium chloride, pH 7.4. DAB cross-linking was done as described by Thilo et al. (1995). The cells were then treated with 1.5 ml 0.1% Triton X-100 for 15 minutes at 37°C, before being scraped into 2 × 1.5 ml 0.1% Triton X-100. The samples (3 ml) were centrifuged for 10 minutes at 13 000 rpm in a Beckman L8-80 Ultracentrifuge equipped with a SW 50.1 rotor at room temperature. Radioactivity in the supernatants and in the pellets following solubilization in 1 ml HEPES-saline buffer was determined.

Electron microscopy

For electron microscopy the cultured liver cells were scraped in 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), pelleted in Eppendorf tubes by centrifugation for 12 minutes at 13,000 rpm in a bench top centrifuge and topped up with fresh fixation medium. For embedding the cells were treated with 10% polyvinylpyrrolidone/2.3 M sucrose overnight before they were mounted and frozen in liquid nitrogen. Ultrathin cryosections were cut at -110°C on a Leica microtome. Sections were transferred to Formvar/carbon-coated grids and labeled with biotinylated goat anti-rabbit IgG, followed by Streptavidin conjugated to 10 nm colloidal gold particles. Major histocompatibility complex (MHC) class II was detected by labeling with the monoclonal antibody RT1B OX-6 followed by goat anti-mouse IgG conjugated to 10 nm colloidal gold particles. Sections were observed in a Phillips CM100 electron microscope.

RESULTS

Effect of unlabeled f-BSA on the uptake of ^{125}I -TC-DNP-BSA or ^{125}I -TC-DNP-BSA-IgG

Conjugation of DNP to lysine residues of BSA (Eisen, 1964)

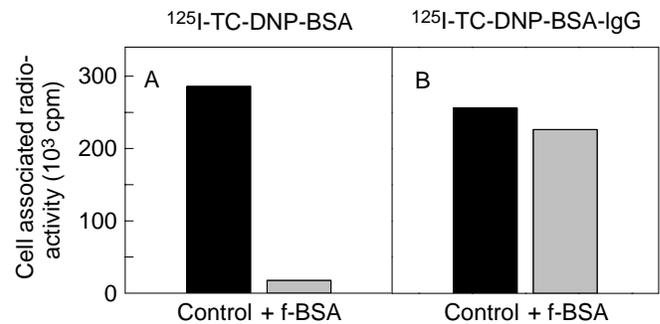


Fig. 1. Effect of unlabeled f-BSA on the uptake of ^{125}I -TC-DNP-BSA or ^{125}I -TC-DNP-BSA-IgG. Monolayer cultures of LEC were incubated on ice for 60 minutes with (gray columns) or without (black columns) f-BSA present. ^{125}I -TC-DNP-BSA (A) or ^{125}I -TC-DNP-BSA-IgG (B) was added and the cells incubated at 37°C for 60 minutes. The cells were treated with pronase on ice for 60 minutes to remove surface bound ligand and scraped in sodium dodecyl sulfate and sodium hydroxide. Cell-associated radioactivity (cpm) was measured and adjusted according to the protein content. The mean radioactivity of two separate experiments performed in duplicate is presented.

prevents ionization of the amino groups and consequently increases the net negative charge (Rhodes and Aasted, 1973; Skogh et al., 1983), which turns the molecule into a ligand for the scavenger receptor (Goldstein et al., 1979; Brown et al., 1980). F-BSA is another ligand for the scavenger receptor (Eskild and Berg, 1984). For analysis of the contribution from scavenger receptors to the uptake of free and IgG-complexed DNP-BSA, LEC were incubated on ice for 60 minutes in the presence or absence of f-BSA (200 nM) before ^{125}I -TC-DNP-BSA or ^{125}I -TC-DNP-BSA-IgG was added and the cells incubated at 37°C for 60 minutes. To remove surface bound ligand the cells were treated on ice for 60 minutes with 0.3% pronase in washing buffer (Seglen, 1976) followed by washing in PBS. After treatment with pronase the cells were scraped in 0.1% sodium dodecyl sulfate and 0.1 M sodium hydroxide, and radioactivity was measured, adjusted according to the protein content and presented in Fig. 1.

The presence of f-BSA completely inhibited the uptake of ^{125}I -TC-DNP-BSA, but did not affect the uptake of ^{125}I -TC-DNP-BSA-IgG in LEC. Similar results were obtained for the KC (data not shown). Thus, scavenger receptors are involved in the uptake of DNP-BSA, but they do not mediate uptake of IgG-ICs in non-parenchymal rat liver cells.

Density distribution of endocytosed ^{125}I -TC-DNP-BSA or ^{125}I -TC-DNP-BSA-IgG following isopycnic centrifugation

To follow the uptake and intracellular fate of IgG-ICs in non-parenchymal liver cells the density distribution of IgG-ICs following isopycnic centrifugation was studied and compared to that of DNP-BSA. LEC or KC were allowed to bind either ^{125}I -TC-DNP-BSA or ^{125}I -TC-DNP-BSA-IgG for 60 minutes on ice followed by incubation at 37°C for the indicated times. After treatment with pronase the cells were homogenized by sonication for 4 seconds to selectively disrupt the plasma membrane. The results obtained after isopycnic centrifugation of postnuclear fractions from LEC or KC in linear sucrose gradients are presented in Fig. 2.

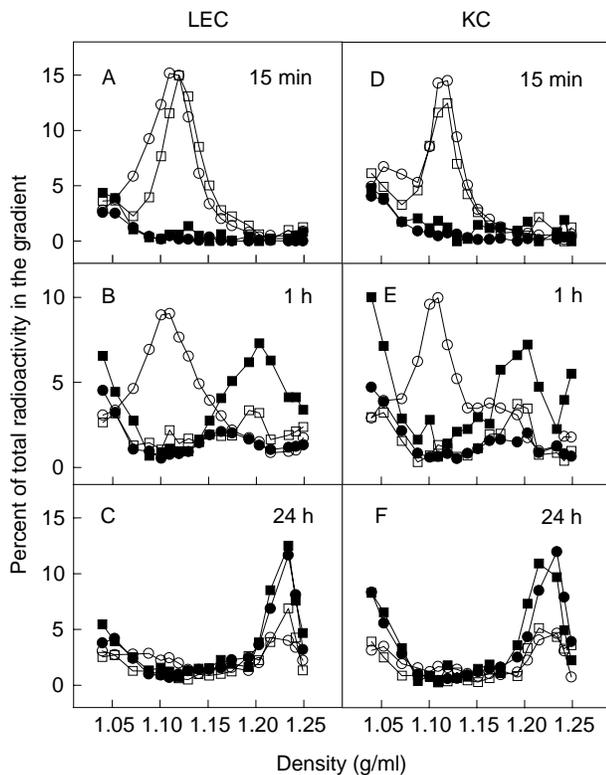


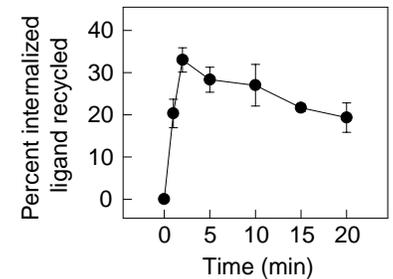
Fig. 2. Density distribution of ^{125}I -TC-DNP-BSA or ^{125}I -TC-DNP-BSA-IgG following isopycnic centrifugation. Monolayer cultures of LEC (left panels) or KC (right panels) were incubated on ice for 60 minutes in the presence of ^{125}I -TC-DNP-BSA (squares) or ^{125}I -TC-DNP-BSA-IgG (circles), followed by incubation at 37°C for 15 (A,D), 60 (B,E) minutes or 24 hours (C,F). The cells were treated with pronase on ice for 60 minutes to remove surface bound ligand, homogenized by sonication and fractionated by centrifugation in linear sucrose gradients. Acid precipitable (open symbols) and acid soluble (filled symbols) radioactivity in trichloroacetic acid in each fraction was measured, expressed as a percentage of the total radioactivity in the gradient and presented as a function of density. The mean of the radioactivity layered on top of the gradients was about 3400 cpm and 12100 cpm for ^{125}I -TC-DNP-BSA and ^{125}I -TC-DNP-BSA-IgG, respectively. The data are representative of four independent experiments.

Initially both ligands banded at low density (1.12 g/ml). After 1 hour of incubation ^{125}I -TC-DNP-BSA was partly degraded and appeared in denser fractions around 1.20 g/ml, whereas ^{125}I -TC-DNP-BSA-IgG was undegraded and still peaked at 1.12 g/ml. Finally, after 24 hours the peak of ^{125}I -TC-DNP-BSA-IgG was again seen coinciding with the peak of ^{125}I -TC-DNP-BSA at 1.23 g/ml and both ligands were degraded. These results clearly demonstrate that both in LEC and KC the initiation of degradation of IgG-ICs were delayed as compared to that of the scavenger receptor ligand DNP-BSA, because the IgG-ICs were transported at a slower rate than DNP-BSA to the lysosomes.

Analysis of retroendocytosis of DNP-BSA-IgG

One possible explanation of the difference in transport kinetics of IgG-ICs and the scavenger receptor ligand DNP-BSA could be that the IgG-ICs remain bound to a recycling receptor and

Fig. 3. Retroendocytosis of DNP-BSA-IgG. Monolayer cultures of LEC were incubated at 37°C for 15 minutes in the presence of DNP-BSA-IgG, followed by binding of goat anti-rabbit IgG on ice for 60 minutes. The cells were then incubated at 37°C for 0, 1, 2, 5, 10, 15 or 20 minutes before



they were allowed to bind goat anti-rabbit ^{125}I -IgG on ice for 60 minutes. Radioactivity was measured and adjusted according to the protein content. Surface bound radioactivity expressed as a percentage of total cell-associated DNP-BSA-IgG is presented as a function of time. Total cell associated goat anti-rabbit ^{125}I -IgG was measured in cells following binding of goat anti-rabbit IgG and treatment on ice with 0.055% digitonin in absolute ethanol. Each point represents the \pm s.e.m. of three independent experiments.

therefore do not make progress along the endocytic pathway. To address this question the LEC were allowed to endocytose unlabeled DNP-BSA-IgG at 37°C for 15 minutes. The cells were then allowed to bind unlabeled goat anti-rabbit IgG antibody (50 μM) on ice for 60 minutes in order to block binding of radiolabeled antibodies to non-internalized DNP-BSA-IgG. Retroendocytosis was subsequently allowed to take place at 37°C for the indicated times and detected by measuring binding of goat anti-rabbit ^{125}I -IgG antibodies (1 nM) to the cells on ice for 60 minutes. The amount of internalized DNP-BSA-IgG was determined in cells that following binding of goat anti-rabbit IgG antibodies were treated with 0.055% digitonin in absolute ethanol on ice for 60 minutes before binding of goat anti-rabbit ^{125}I -IgG antibodies. Treatment with digitonin at low concentrations permeabilize cells by binding to cholesterol (Windaus, 1909), but does not solubilize cells so that exogenously added molecules, here antibodies, gain access to intracellular receptors (Weigel et al., 1983). Radioactivity was measured after lysis of the cells with 0.1% sodium dodecyl sulfate and 0.1 M sodium hydroxide and adjusted according to the protein content. About half of the cell protein content, probably corresponding to the soluble cytoplasmic constituents, was lost following treatment with digitonin. Percentage recycled of total cell-associated radioactivity is presented as a function of time in Fig. 3.

The results show that recycling after 2-5 minutes reaches a maximum of about 35% of the internalized DNP-BSA-IgG, which indicates that the delay of IgG-ICs in the low-density fractions is at least partly due to recycling.

Density distribution of endocytosed ^{125}I -TC-f-BSA in the presence or absence of DNP-BSA-IgG following isopycnic centrifugation

To examine whether transport of f-BSA is affected by simultaneous uptake of IgG-ICs, LEC were allowed to endocytose ^{125}I -TC-f-BSA at 37°C for 15 minutes in the presence or absence of unlabeled DNP-BSA-IgG. Prior to addition of ^{125}I -TC-f-BSA, LEC were allowed to bind DNP-BSA-IgG on ice for 60 minutes. The pulse was either followed by no chase, or by a chase period of 45 minutes at 37°C . After treatment with pronase the cells were homogenized by sonication for 4 seconds.

Fig. 4. Density distribution of ^{125}I -TC-f-BSA in the presence or absence of DNP-BSA-IgG following isopycnic centrifugation. Monolayer cultures of LEC were incubated on ice for 60 minutes with (triangles) or without (squares) DNP-BSA-IgG present, and pulsed at 37°C for 15 minutes in the presence of ^{125}I -TC-f-BSA. The pulse was either followed by no chase (A), or by a chase period of 45 minutes (B) at 37°C . The cells were treated with pronase on ice for 60 minutes to remove surface bound ligand, homogenized by sonication and fractionated by centrifugation in linear sucrose gradients. Acid precipitable (open symbol) and acid soluble (filled symbol) radioactivity in each fraction was measured, expressed as a percentage of total radioactivity in the gradient and presented as a function of density. The data are representative of three independent experiments.

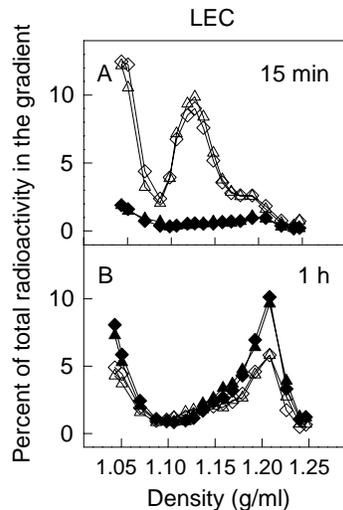


Fig. 4 shows that following isopycnic centrifugation in linear sucrose gradients, ^{125}I -TC-f-BSA was found in sequentially denser fractions both in the presence and in the absence of DNP-BSA-IgG. Initially ^{125}I -TC-f-BSA banded at 1.12 g/ml, but after 1 hour the ligand was mainly found as acid soluble material at 1.20 g/ml. Thus, uptake of IgG-ICs did not affect the intracellular transport and degradation of f-BSA.

Colocalization of Alexa 488-DNP-BSA-IgG and Texas Red-f-BSA studied by confocal laser-scanning microscopy

To determine whether IgG-ICs follow the same itinerary as scavenger receptor ligands, confocal laser-scanning microscopy studies were done. LEC were allowed to bind IgG-ICs in which the DNP-BSA moiety was labeled with Alexa 488. After 60 minutes on ice the cells were pulsed at 37°C for 15 minutes in the presence of Texas Red labeled f-BSA. Alternatively, LEC were pulsed for 15 minutes with the two scavenger receptor ligands Alexa 488-DNP-BSA and Texas Red-f-BSA. The pulses were either followed by no chase, or by a chase period of 45 minutes at 37°C . To remove surface bound ligand the cells were treated with pronase. An overlay of the red and the green labeling is shown in Fig. 5.

Only limited colocalization of Texas Red-f-BSA and Alexa 488-DNP-BSA-IgG detected as yellow labeling was observed, whereas the distribution of Texas Red-f-BSA and Alexa 488-DNP-BSA coincided almost completely both after 15 minutes and 1 hour of incubation. These results suggest that IgG-ICs follow an endocytic pathway that is different from that followed by scavenger receptor ligands.

DAB cross-linking in whole cells and solubilization

To further examine whether the IgG-ICs follow an endocytic pathway that is different from that followed by other endocytic ligands, LEC were allowed to bind ^{125}I -TC-DNP-BSA-IgG on ice for 60 minutes, followed by a 15 minutes pulse at 37°C in the presence of HRP-labeled ovalbumin.

Alternatively, LEC were pulsed for 15 minutes with HRP-ovalbumin and ^{125}I -TC-DNP-BSA. The pulses were either followed by no chase, or by a chase period of 45 minutes at 37°C . To remove surface bound ligand the cells were treated with pronase. In the presence of hydrogen peroxide HRP oxidizes DAB monomers, which polymerize and chemically cross-link all proteins in the organelle to the DAB polymer. One of two parallels was treated with DAB and hydrogen peroxide before all samples were solubilized in Triton X-100, and DAB aggregates pelleted by centrifugation as described in Materials and Methods. Radioactivity in the supernatants and pellets was measured and radioactivity in the supernatant is presented as a percentage of total radioactivity in the sample in Fig. 6.

Almost all the radioactive material from control cells appears in the supernatant. In contrast 10% and 20% of ^{125}I -TC-DNP-BSA, as compared to 30% and 50% of the ^{125}I -TC-DNP-BSA-IgG were found in the supernatants in the presence of DAB and hydrogen peroxide after 15 minutes and 1 hour of incubation, respectively. These results indicate that the scavenger receptor ligand DNP-BSA follows the same intracellular pathway and with the same kinetics as ovalbumin taken up by the mannose receptor. This pathway is to some extent also followed by the IgG-ICs. However, IgG-ICs in addition seem to follow a separate pathway, or, conceivably, the same pathway but with a different kinetics.

Colocalization of DNP-BSA-IgG and Au₅-f-BSA studied by electron microscopy

To further examine whether IgG-ICs and scavenger receptor ligands appear in separate endosomes or not, and to morphologically characterize the organelle in which IgG-ICs are delayed, the intracellular localization of IgG-ICs and f-BSA was studied by electron microscopy. The cells were allowed to bind DNP-BSA-IgG on ice for 60 minutes, followed by a 15 minutes pulse at 37°C with DNP-BSA-IgG and Au₅-f-BSA. The pulse was either followed by no chase, or by a chase period of 45 minutes at 37°C . For chasing overnight LEC were pulsed at 37°C for 30 minutes in the presence of DNP-BSA-IgG and Au₅-f-BSA. To identify the early endosomes Au₂₀-ovalbumin was added 5 minutes prior to fixation.

Electron micrographs show that DNP-BSA-IgG and Au₅-f-BSA after 15 minutes of incubation at 37°C colocalized to some extent in doughnut or horseshoe shaped electron lucent early endosomes (Fig. 7A), but DNP-BSA-IgG was also found in separate early endosomes (data not shown). After 1 hour of incubation Au₅-f-BSA was transferred to spherical electron dense organelles devoid of Au₂₀-ovalbumin (Fig. 7B). The DNP-BSA-IgG, on the other hand, was still found in the early endosomes (Fig. 7B), but was also seen in multivesicular compartments (Fig. 8A). DNP-BSA-IgG seemed to localize to the internal membranes of the multivesicular compartments. They appear either as electron dense or electron lucent structures and the number of internal vesicles varies. Moreover, these multivesicular compartments were apparently depleted of Au₅-f-BSA and Au₂₀-ovalbumin. Finally, DNP-BSA-IgG and Au₅-f-BSA end up in common electron dense spherical lysosomes (Fig. 8B). These results imply that the sorting of IgG-ICs from the early endosomes is different from that of scavenger receptor ligands.

DNP-BSA-Alexa 488
and f-BSA-Texas Red

IgG-IC-Alexa 488
and f-BSA-Texas Red

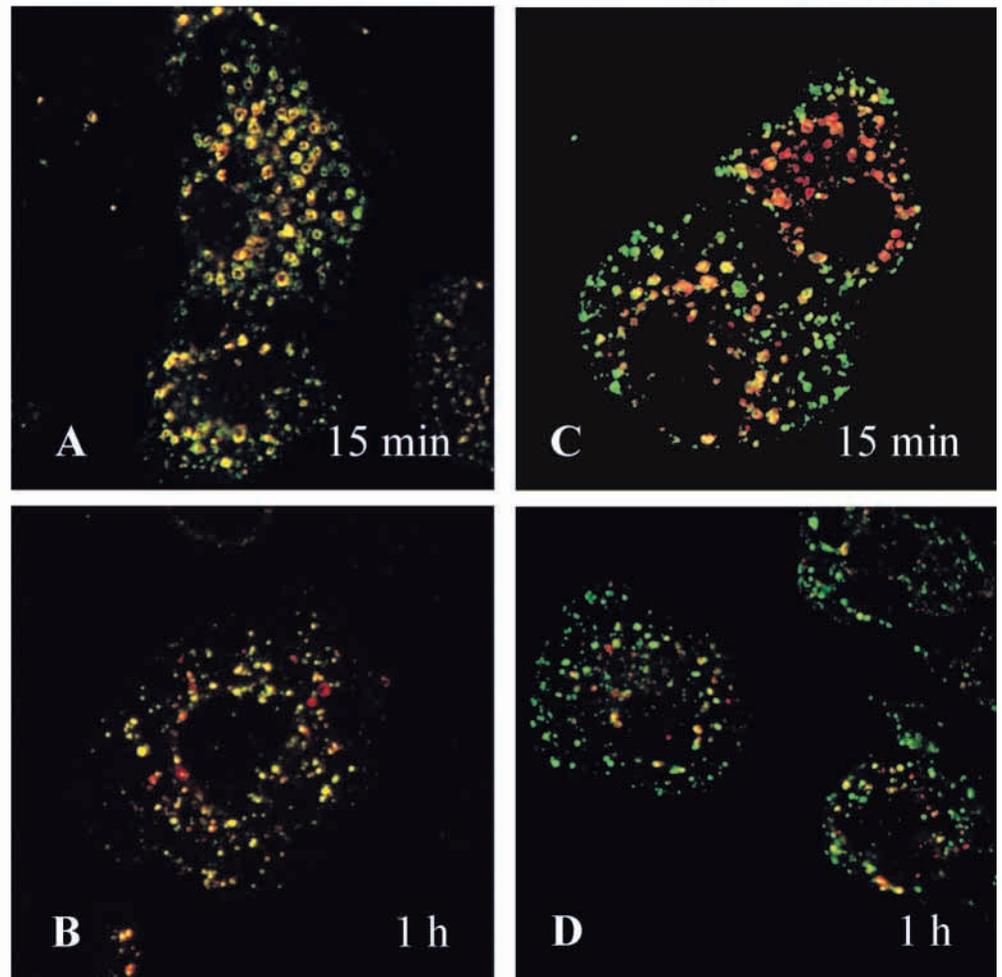


Fig. 5. Colocalization of Alexa 488-DNP-BSA-IgG and Texas Red-f-BSA studied by confocal laser-scanning microscopy. LEC were incubated on ice for 60 minutes with Alexa 488-DNP-BSA-IgG present, followed by a pulse at 37°C for 15 minutes in the presence of Texas Red-f-BSA (C,D). Alternatively, LEC were allowed to endocytose Alexa 488-DNP-BSA and Texas Red-f-BSA (A,B) at 37°C for 15 minutes. The pulses were either followed by no chase (A,C) or by a chase period of 45 minutes (B,D) at 37°C. Surface bound ligand was removed by treatment with pronase on ice for 60 minutes. The cells were fixed in 2% paraformaldehyde for 10-15 minutes at room temperature, washed and mounted upside down in a droplet of FluoroSave. Merged pictures of the green and the red labeling are shown. The cells are representative of two independent experiments.

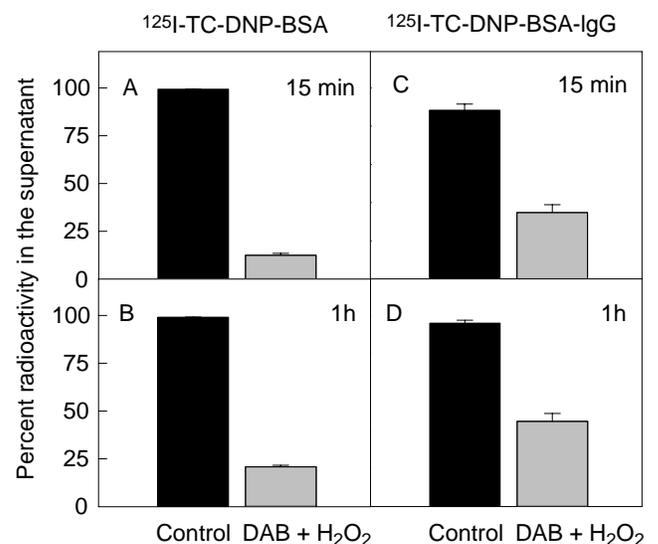
Immunolabeling of MHC class II studied by electron microscopy

Multivesicular compartments enriched in MHC class II have been implicated in antigen presentation. To determine whether the multivesicular compartments observed in LEC could be involved in antigen presentation, we examined whether LEC were enriched in MHC class II. Ultrathin cryosections of LEC were labeled with the monoclonal antibody RT1B OX-6 that

recognizes rat MHC class II. As a positive control cryosections of KC were labeled. Both cell types were treated with pronase before labeling. The results obtained are presented in Fig. 9.

Fig. 6. DAB cross-linking in whole cells and solubilization.

Monolayer cultures of LEC were allowed to bind ^{125}I -TC-DNP-BSA-IgG on ice for 60 minutes, followed by a 15 minutes pulse at 37°C in the presence of HRP-labeled ovalbumin (C,D). Alternatively, LEC were pulsed at 37°C for 15 minutes with HRP-ovalbumin and ^{125}I -TC-DNP-BSA (A,B). The pulses were either followed by no chase (A,C) or by a chase period of 45 minutes (B,D) at 37°C. Surface bound ligand was removed by treatment with pronase on ice for 60 minutes. One of two parallels was treated with DAB and H_2O_2 (gray columns), whereas control cells were not (black columns). All samples were solubilized in Triton X-100 and centrifuged, and radioactivity in the supernatants and pellets was determined. Radioactivity in the supernatants as a percentage of the total radioactivity in the sample is presented in Fig. 6. Each column represents the \pm s.e.m. of three independent experiments.



There is a strong surface labeling of MHC class II in KC, whereas hardly any labeling of MHC class II is detected at the surface of LEC. Thus, it seems unlikely that the multivesicular compartments observed in LEC are involved in antigen presentation.

DISCUSSION

Earlier studies have shown that hepatic uptake and turnover of soluble IgG-ICs takes place in KC and LEC, but not in parenchymal cells (Skogh et al., 1985; Bogers et al., 1991; Johansson et al., 1996). The purpose of the present study was to clarify the intracellular pathway followed by soluble IgG-ICs following FcR mediated uptake in rat LEC and KC. Both KC and LEC have been shown to possess the low affinity Fc γ RII and/or Fc γ RIII (Muro et al., 1993; Ahmed et al., 1995).

DNP-BSA was chosen as model antigen. Conjugation of DNP groups to lysine residues of BSA (Eisen, 1964) prevents ionization of the amino groups and consequently increases the net negative charge (Rhodes and Aasted, 1973; Skogh et al., 1983), which turns the molecule into a ligand for the scavenger receptor (Goldstein et al., 1979; Brown et al., 1980). In both LEC and KC scavenger receptors, probably

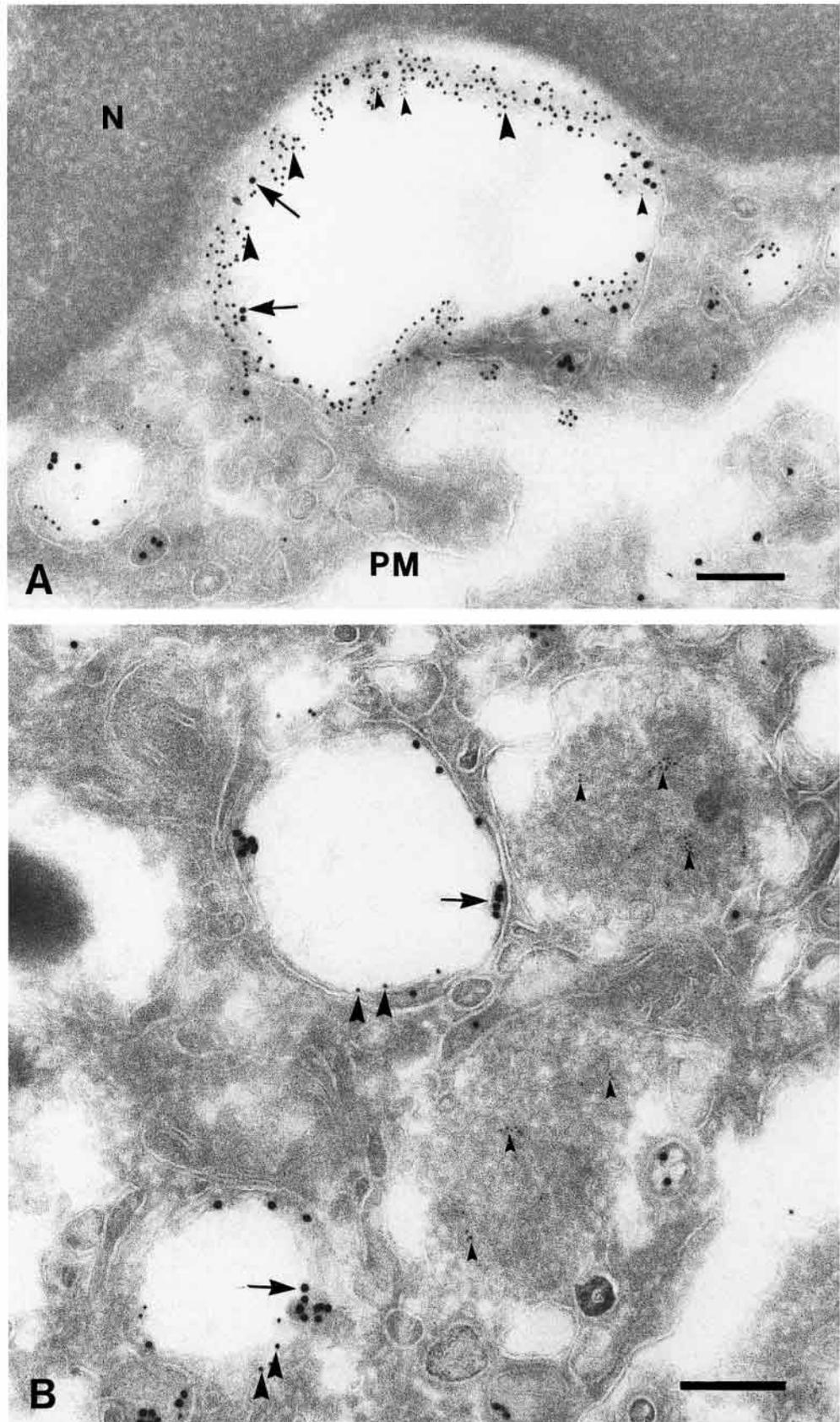


Fig. 7. Colocalization of DNP-BSA-IgG and Au₅-f-BSA studied by electron microscopy. Monolayer cultures of LEC were incubated on ice for 60 minutes in the presence of DNP-BSA-IgG, and pulsed at 37°C for 15 minutes in the presence of DNP-BSA-IgG and Au₅-f-BSA (small arrowheads). The pulses were either followed by no (A), or by a chase period of 45 minutes (B) at 37°C. To label the early endosomes Au₂₀-ovalbumin (arrows) was added for the last 5 minutes of the incubation. After removal of surface bound ligand by treatment with pronase on ice for 60 minutes, the DNP-BSA-IgG in the ultrathin cryosections was labeled with biotinylated goat anti-rabbit IgG antibodies, followed by Streptavidin conjugated to 10 nm colloidal gold particles (large arrowheads). Nucleus (N) and plasma membrane (PM). Bars, 200 nm.

DNP-BSA-IgG in the ultrathin cryosections was labeled with biotinylated goat anti-rabbit IgG antibodies, followed by Streptavidin conjugated to 10 nm colloidal gold particles (large arrowheads). Nucleus (N) and plasma membrane (PM). Bars, 200 nm.

class A scavenger receptors, were found to mediate uptake of DNP-BSA but not uptake of IgG-ICs. Recognition of carbohydrate moieties via hepatic lectins is not responsible for internalization of the antigen (Skogh, 1982). To exclude contribution from complement the IgG-ICs were made in serum free buffer and the experiments performed either without serum present, or in the presence of heat inactivated serum.

A striking feature of the turnover of IgG-ICs in KC and in LEC is that the degradation of IgG-ICs is delayed as compared to that of DNP-BSA taken up by a scavenger receptor. Scavenger receptor ligands on the other hand, showed similar degradation kinetics as mannose receptor ligands (Kjeken et al., 1995). The degradation of the DNP-BSA moiety of the ICs, was not inhibited because of its binding to IgG, since the free and bound antigen were degraded at the same rate by a crude fraction of lysosomal enzymes (Andersen et al., 1997). Subcellular fractionation experiments revealed that the delayed degradation was instead due to a late arrival of the IgG-ICs at the lysosomes. The DAB cross-linking experiments also showed that less IgG-ICs than scavenger receptor ligands had reached lysosomes 1 hour after initiation of uptake, although more IgG-ICs seemed to be associated with lysosomes with this method than by subcellular fractionation. The discrepancy is probably due to variation in the amount of free DNP-BSA in the IgG-IC preparations used. It should also be noted that endocytosis of IgG-ICs does not change the kinetics of the endocytic process as such, since endocytosis of scavenger receptor ligands was unaffected by simultaneous uptake of IgG-ICs.

The intracellular transport of the IgG-ICs is probably delayed between the early endosomes and the lysosomes. Experiments using both biochemical and morphological techniques indicated that the IgG-ICs follow the same pathway and with the same kinetics as ligands of

scavenger and mannose receptors to the early endosomes. The presence of IgG-ICs in separate early endosomes is probably a result of a higher number of Fc γ R than scavenger and mannose receptors at the surface of LEC. In addition, the limited colocalization of scavenger receptor ligand and IgG-ICs in

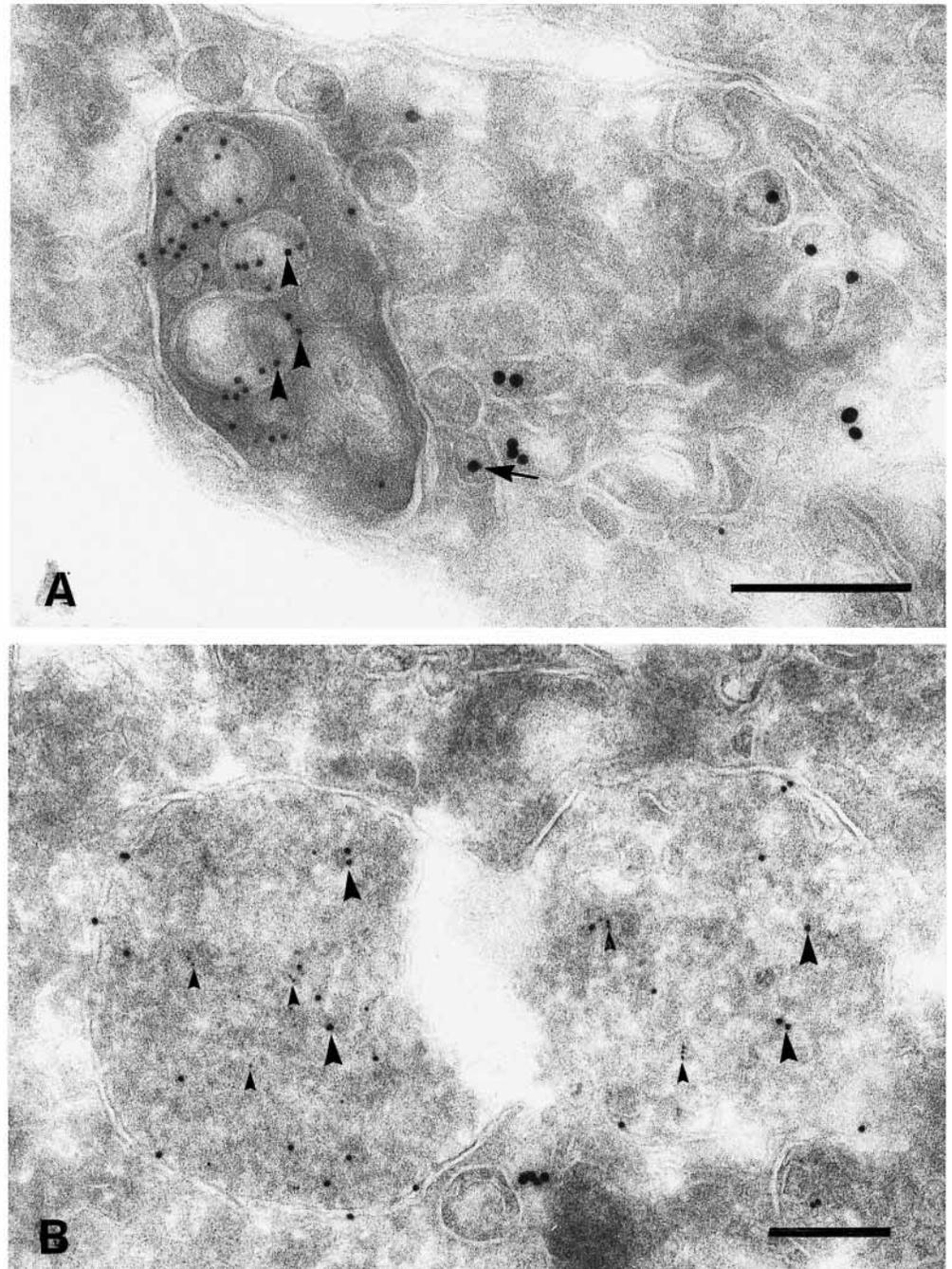


Fig. 8. Colocalization of DNP-BSA-IgG and Au₅-f-BSA studied by electron microscopy. Monolayer cultures of LEC were incubated on ice for 60 minutes in the presence of DNP-BSA-IgG, and pulsed at 37°C in the presence of DNP-BSA-IgG and Au₅-f-BSA (small arrowheads). The cells were either pulsed for 15 minutes followed by a chase period of 45 minutes (A), or for 30 minutes (B) followed by chasing overnight at 37°C. To label the early endosomes Au₂₀-ovalbumin (arrows) was added for the last 5 minutes of the incubation. After removal of surface bound ligand by treatment with pronase on ice for 60 minutes, the DNP-BSA-IgG in the ultrathin cryosections was labeled with biotinylated goat anti-rabbit IgG antibodies, followed by Streptavidin conjugated to 10 nm colloidal gold particles (large arrowheads). Nucleus (N) and plasma membrane (PM). Bars, 200 nm.

endosomes observed by confocal laser-scanning microscopy may be explained by the two ligands being localized to different parts of the same organelle.

The delayed entrance in lysosomes of IgG-ICs as compared to scavenger and mannose receptor ligands may conceivably have two different explanations: Firstly, the IgG-ICs may follow the same pathway as the two other types of ligands, but with slower kinetics; secondly, the IgG-ICs may partly follow its own path from the early endosomes to the lysosomes. The first alternative raises a question as to how the IgG-ICs are selectively delayed in the common itinerary. An answer that at least partly explains the delay is that the IgG-ICs are prevented from leaving the early endosomes because they remain bound to the recycling Fc γ R (Fig. 3). Ligand binding to Fc γ Rs is insensitive to the pH of the early endosomes (Mellman and Plutner, 1984; Mellman et al., 1984; Finbloom, 1986; Ukkonen et al., 1986), whereas scavenger and mannose receptors loose their affinity for ligands in the early endosomes due to low pH (Wileman et al., 1985; Naito et al., 1991; Doi et al., 1994). Scavenger and mannose receptor ligands are therefore more readily available for making progress to the lysosomes than the membrane bound IgG-ICs. The recycling of the Fc γ Rs is, however, rather limited and may not alone explain the relative delay of the IgG-ICs in early endosomes. This notion is supported by the finding that IgG-ICs never were observed in tubular structures similar to those in which ricin is localized (Brech et al., 1993). Moreover, 70-80% of ricin (Magnusson et al., 1991) and only 35% of the IgG-IC are recycled after a 15 minute pulse (Fig. 3). The itinerary of the IgG-ICs may be compared to that of ricin as ricin remains bound to recycling galactose receptors in liver endothelial cells (Magnusson et al., 1991, 1993). Further experiments are needed to decide whether the Fc γ R-IgG-ICs are merely delayed in early endosomes or whether they are, in addition, directed to the lysosomes via a pathway different from that followed by the ligands of the scavenger and mannose receptor. We found by electron microscopy that the IgG-ICs were located in a multivesicular compartment apparently devoid of the other endocytic markers (e.g. Au₅-f-BSA). Whether IgG-ICs are selectively sorted to this compartment, or whether the early endosomes matures into a multivesicular compartment that no longer are accessible to newly endocytosed ligands, remains to be solved. Other studies have shown that even the mannose-receptor ligand ovalbumin is found in multivesicular bodies (Kjeken et al., 1995), and multivesicular endosomes have been shown in numerous studies to constitute a 'compulsory' step in the endocytic pathway. Our data may therefore mean that the Fc γ R-IgG-ICs follow the same

endocytic pathway as the ligands of the scavenger and mannose receptor, but with a different kinetics.

Aggregation and oligomerization of proteins have been reported to alter receptor trafficking in the endocytic system. Depending on the degree of cross-linking by oligomerized transferrin the transferrin receptor is either retained in an endocytic recycling compartment, or directed to the lysosomes (Marsh et al., 1995). Cross-linking of Fc γ Rs directs the FcR-ligand complex to degradation in lysosomes (Mellman and Plutner, 1984), whereas monovalent antibodies bound to Fc γ Rs are recycled with the receptor to the cell surface (Mellman et al., 1984; Harrison et al., 1994). Lysosomal trafficking of the membrane bound IgG-ICs requires active sorting from the recycling membrane components. This is probably a time-consuming process that may lead to a further delay of the IgG-ICs in prelysosomal compartments. Similar to the EGF-EGF-R complex, sorting of IgG-ICs to the inner vesicles of the multivesicular compartments may lead to degradation of both the Fc γ R and IgG-ICs in the lysosomes. It has been demonstrated that both the EGF-R (Haigler et al., 1979; McKanna et al., 1979; Felder et al., 1990; Hopkins et al., 1990) and the Fc γ R (Hedin et al., 1984; Ukkonen et al., 1986) is associated with the luminal

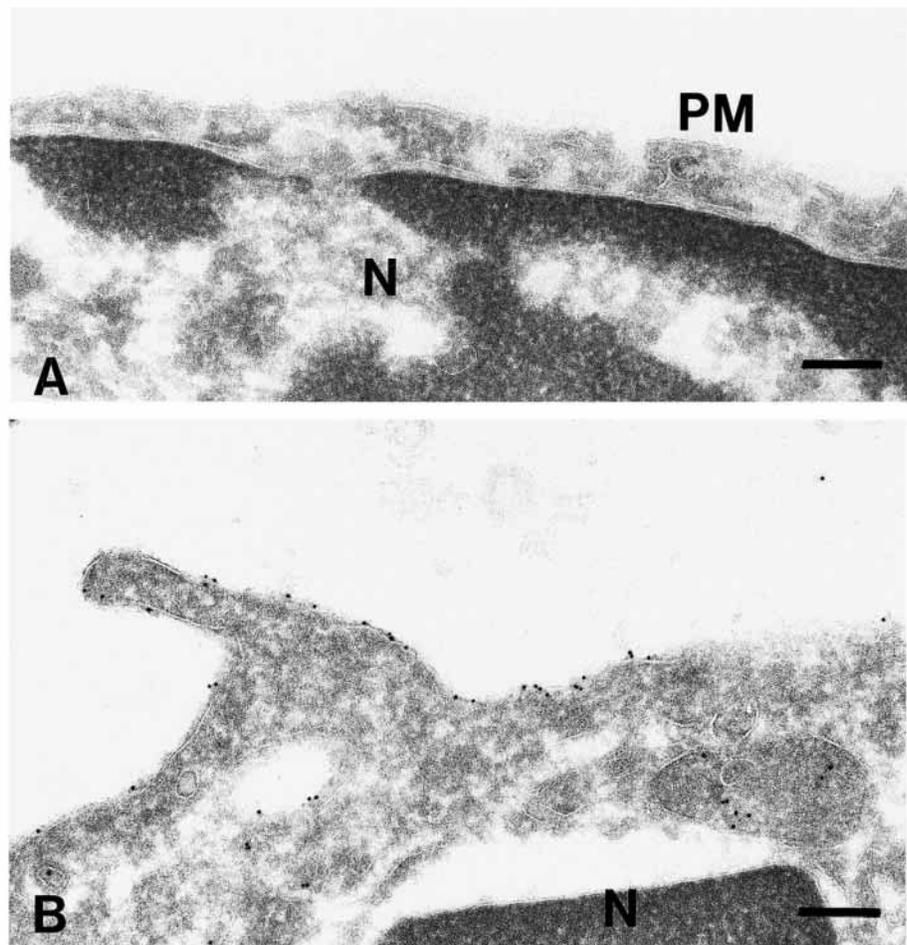


Fig. 9. Immunolabeling of MHC class II studied by electron microscopy. Ultrathin cryosections of cultured LEC (A) or KC (B) treated with pronase on ice for 60 minutes, were labeled with the monoclonal antibody anti-RT1B OX-6 that recognizes rat MHC class II, and detected by goat anti-mouse IgG conjugated to 10 nm colloidal gold particles. Nucleus (N) and plasma membrane (PM). Bars, 200 nm.

vesicles of multivesicular compartments. These vesicles will be delivered to the lysosomes, and the receptors associated with the vesicles will be completely degraded (i.e. – transmembrane, intravesicular and luminal domains) (Ukkonen et al., 1986; Renfrew and Hubbard, 1991).

FcR mediated uptake of IgG-ICs has been shown to enhance presentation of antigens by MHC class II (Manca et al., 1991; Amigorena et al., 1992a,b; Gosselin et al., 1992; Liu et al., 1996). This process may take place in a compartment reminiscent of the multivesicular compartments observed in the LEC. It is therefore conceivable that these organelles are involved in processing and presentation of antigenic peptides. The earlier described multivesicular compartments for peptide loading are enriched in MHC class II (for review see Nordeng, 1998). In accordance with Pulford and Souhami (1981) and Smedsrød et al. (1985), we found MHC class II in KC but not in LEC. The lack of MHC class II in LEC does not support a role of these cells in antigen presentation. However, this result is at variance with data indicating that both KC and LEC in mice contain MHC class II, and that these cells are involved in antigen presentation (Lohse et al., 1996; Knolle et al., 1999a,b). It is noticeable, however, that LEC failed to induce differentiation toward inflammatory T cells (T_H1) (Knolle et al., 1999b). The discrepant data may be due to differences in the experimental design. We used LEC that had been cultured for only 3-4 hours whereas the cells used by Knolle and co-workers were cultured for 3 days. It has been reported that endotoxin in portal blood down regulates MHC class II on LEC (Knolle et al., 1999a), and such an effect of endotoxin would be seen in newly prepared LEC but maybe not in cells cultured for 3 days.

We conclude that following FcR mediated endocytosis IgG-ICs mainly follow the same pathway and kinetics as scavenger and mannose receptor ligands to the early endosomes. However, there is a clear delay in the intracellular transport of IgG-ICs from the early endosomes as compared to the scavenger and mannose receptor ligands, which partly is explained by recycling of IgG-ICs to the plasma membrane. In addition to being retained in early endosomes, IgG-ICs were seen in multivesicular compartments mostly devoid of other endocytic markers. Finally, degradation products from all markers end up in common lysosomes. Thus, IgG-ICs seem to follow an endocytic pathway that is kinetically or maybe morphologically different from that followed by scavenger and mannose receptor ligands.

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