Ultrastructural study of the clearance of intracerebrally infused native and modified albumin-gold complexes

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Summary. The main objective of this ultrastructural study was to gain a better understanding of the involvement of brain vasculature in clearance of proteins from edematous fluid. For this purpose, both native and modified (cationized, glucosylated, and mannosylated) bovine serum albumin-gold complexes (BSA-G, catBSA-G, glucBSA-G and manBSA-G respectively) dissolved in phosphate-buffered saline (PBS) were infused (10 µl) into mouse cerebral cortex. Samples of brain were taken at 30 min, 1 h, and 24 h post-infusion for electron microscopical examination. All BSA-G complexes were rapidly taken up and deposited inside the cytoplasm of pericytes and of various glial cells (microglia and eventually astrocytes) located in the area adjacent to the infusion site. Only glucBSA-G particles also appeared inside the nuclei of some cells. In the applied experimental conditions and at the examined time intervals, neither BSA-G nor catBSA-G and glucBSA-G complexes were transported back to the bloodstream, although they entered vascular basement membrane and were eventually internalized in the endosomes or multivesicular bodies of the endothelial cells. Only a few gold particles representing the manBSA-G complex were found inside the vascular lumen, suggesting their reverse transport to a rather small degree. The mechanism of this transport, however, remains unclear. Complexes of catBSA-G were apparently trapped by the negatively charged vascular basement membrane and remained in this structure without any further significant uptake by the endothelial cells. These observations suggest that large size and multimeric nature of albumin-gold complexes are limiting factors making it difficult to interpret the results and hampering their relevance to the clearance in vivo of native albumin from brain edematous fluid.

Key words: Intracerebral infusion, Clearance of albumin, Albumin-gold complex, Reverse transport, Blood-brain barrier

Introduction

The pathways and mechanisms of removal of extravasated serum proteins from brain are still not fully understood. This problem has not only theoretical but also practical aspects because it relates to the formation and resolution of brain edema.

It was postulated by Klatzo et al. (1980) that the intracellular uptake of extravasated serum proteins, predominantly by various glial cells such as astrocytes and microglia, constitutes the principal mechanism for vasogenic brain edema (VBE) resolution. Our further light microscopical (LM) and electron microscopical (EM) observations suggested that reverse transport of edematous fluid labeled with horseradish peroxidase (HRP) across some segments of blood vasculature (presumably post-capillary venules) can also represent an additional mechanism responsible for removal of plasma proteins extravasated during formation of VBE (Vorbrodt et al., 1985).

These observations prompted us to perform additional experiments, the main objective of which is to gain insight into the cellular mechanisms that govern the clearance of intracerebrally infused native and modified albumin-gold complexes, with special emphasis on brain blood vasculature. The use of albumin is justified because, contrary to HRP used in our previous study, this protein is one of the normal components of circulating blood which secures the oncotic pressure of plasma and interstitial fluid, and serves as a carrier for many metabolically important substances (Ghitescu et al., 1986; Milici et al., 1987; Raicu et al., 1991). In addition, albumin can be easily modified and complexed with colloidal gold of a wide range of sizes, providing excellent visibility for EM. After complexing with colloidal gold the albumin does not change its biological
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and immunological properties (Handley and Chien, 1987; Millici et al., 1987; Villaschi, 1989). These complexes, however, have relatively large dimensions and probably behave like polymerized albumin. According to data presented by Millici et al. (1987), the diameter of the complex of albumin with colloidal gold of 7-nm diameter is approximately 14.4 nm.

In spite of this disadvantage, albumin-gold complexes appeared to be an excellent and useful tracers in studying the cellular mechanisms of transendothelial transport from blood plasma to tissue in non-barrier vasculature of lungs, myocardium and skeletal muscle (Ghitescu et al., 1986; Millici et al., 1987). They were also successfully used in studies on the transendothelial reverse transport (retrotransport from the interstitium to the blood vessel lumen) by Raicu et al. (1991). These authors, after interstitial injection of albumin-gold complexes into the adipose tissue, demonstrated that albumin-binding sites are expressed on the abluminal plasmalemma of the vascular ECs. It seems interesting, that experiments performed in vitro on EC cultures showed ten time more active retrotransport of albumin than in the opposite direction (Shasby and Shasby, 1985).

The mechanisms of reverse transport are of special interest because if brain vasculature is at all engaged in clearance of proteins from the brain edematous fluid, one can expect that this type of transport is involved. This problem, however, still remains unsolved and controversial. It relates to the existence not only of the blood-brain barrier (BBB) which controls the movement of various solutes from blood to brain parenchyma (luminal transport) but also the brain-blood barrier controlling the movement in the opposite direction (Broadwell et al., 1983).

In our present study we decided to use both native and modified albumin because it was observed that the interaction of protein tracers such as HRP and albumin with the vascular endothelium changes after their molecules become modified by cationization (Houthoff et al., 1984; Vorbrot et al., 1996), glucosylation (Villashi et al., 1986; Predescu et al., 1988; Esposito et al., 1989; Poduslo and Curran, 1994) or conjugation with fatty acids (Raicu et al., 1991; Antohe et al., 1993). It should be emphasized that these modifications enhance binding, endocytosis and transcytosis of albumin by ECs in vitro (Esposito et al., 1989; Smith and Borchardt, 1989; Raicu et al., 1991) and in vivo (Villaschi et al., 1986; Predescu et al., 1988; Antohe et al., 1993) including the BBB (Poduslo and Curran, 1994). It was also shown that mannosylation of liposomes facilitates their transport to the brain across the BBB and concomitantly enhances their uptake by glial cells (Umezawa and Eto, 1988).

Taking all these data into consideration, we decided to use both native and modified (cationized, glucosylated and mannosylated) albumin-gold complexes in our experiments to evaluate whether these modifications have any influence on their clearance after intracerebral infusion. More specifically, using the infusion brain edema (IBE) model of Marrasorou et al. (1980), we targeted this study to observe the involvement of cellular and especially vascular elements in clearance of infused proteins.

Because the results of previously performed experiments (Blasberg, 1976; Houthoff et al., 1984; Vorbrot et al., 1985) indicate that the most dramatic events related to clearance of edema proteins occur during the first day, we decided to limit our observations to three early time intervals (up to 24 h) after intracerebral infusion of albumin-gold complexes.

Materials and methods

Preparation of albumin-gold complexes

Monodisperse suspensions of colloidal gold with two different particle sizes were prepared as follows:

(a) To obtain 5-nm particles, the method of Mühlpfordt (1982) was used with tannic acid as an additional reducing agent.

(b) To prepare 13-nm particles, the procedure of Frens (1973) was used with sodium citrate as a reducing agent.

Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO, USA), glucosylated BSA (glcBSA) and mannosylated BSA (manBSA), also called neoglycoproteins, from EY Laboratories (San Mateo, CA, USA), and cationized BSA (catBSA) from Chemicon (Temecula, CA, USA).

The method for preparing albumin-gold complexes was based essentially on the procedure of Ghitescu et al. (1986). The minimal amounts of albumin required to stabilize the gold colloids were determined by using the standard flocculation assay described by Horrisberger and Rosset (1977). The pH of the colloidal gold solution for preparation of the complex with BSA was 5.8 (pH of BSA ~5.2); with glcBSA and manBSA, was 5.2 (pH of these compounds was ~4.8); and with catBSA, was 8.5 (pH of catBSA was ~9.5). These complexes were sedimented by centrifugation, and red sediment was dissolved in PBS containing 0.1% polyethylene glycol (M.W. 20,000). In the further descriptions that follow, the size of gold particles in a given albumin complex is indicated by G5 for 5-nm gold particles and G13 for 13-nm gold particles.

Experimental procedure

Twenty-four adult (4- to 6-month-old) BALB/c mice of both sexes, weighing approximately 25 g, were used. The procedure of intracerebral infusion was essentially similar to that used previously in our Institute (Houthoff et al., 1984). Under Nembutal (sodium pentobarbitol) anesthesia, the skull was exposed, and a hole was drilled through the right side of the skull approximately at a point 1 mm caudal to bregma and 2 mm lateral to the sagittal suture. A 30-gauge needle was
inserted into the parietal cortex to a depth of approximately 2 mm below the dura. This needle was connected to the Hamilton RN precision syringe (10 μl capacity) used for infusion of 10 μl of native or modified BSA-gold complex solutions in PBS (approx. 2.5 mg of protein/ml) into cerebral cortex (2 μl/min). The needle was left in place for an additional 2 min to reduce backflow and then removed. After 30 min, 1 h, and 24 h, the mice were decapitated (two at each time interval). To minimize washing out of the infusionate and blood plasma, where eventually tracer particles could appear, fixation was applied immediately during rapid excision of the brain (in situ fixation). This step was followed by immersion fixation in our standard fixative for 3 h (2% formaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, supplemented with 0.2M sucrose). After fixation, small samples of the cerebral cortex, under control with a low-power dissection microscope, were taken from the area of infusion, and after washing, they were additionally fixed for 2 h in buffered 2% osmium tetroxide. They were washed again and stained en bloc with 0.5% uranyl acetate (pH 5.0) overnight in the refrigerator, dehydrated in ethanol and embedded in Spurr low-viscosity resin. Ultrathin sections were cut on a Sorvall (DuPont) MT-5000 ultramicrotome, stained with lead citrate for 6 min at room temperature (22 °C), and analyzed in a Hitachi 7000 electron microscope.

Before ultrathin sectioning, all embedded blocks were carefully examined with light microscopy, using 1 μm-thick sections stained with toluidine blue for precise identification of the sites of the tracer infusion. In addition, such screening helps eliminate from further examination the injuries of brain tissue in the area of the needle track. Consequently, all areas showing mechanical injury of the vascular wall segments marked by the extravasated red blood cells, were eliminated from our observations and were not included in the descriptions that follow.

Results

Infusion of native BSA-gold complexes

At 30 min post-infusion, solitary and clustered gold particles were found in the cerebral cortex, mainly located in the extracellular space (ECS) of the neuropil (Fig. 1). In these areas, however, only a few sectioned blood microvessels, mostly capillaries or post-capillary venules, could be found. The precapillary arterioles were less numerous, and only seldom were we able to observe this type of vessel located in close proximity to the infused BSA-G complexes. Nevertheless, at the indicated time interval (30 min post-infusion), only in a few vascular profiles did solitary gold particles appear inside the cytoplasm of pericytes (Fig. 2) or in the vascular BM (Fig. 3). Occasionally, a few gold particles were also observed to be endocytosed and internalized in small cytoplasmic vesicles or endosomes of perivascular microglial cells or presumably of astrocytic cell processes. No differences were noted in the fate of infused BSA-G solutions related to the size of the colloidal gold particles (G5 and G13) used for complexing with BSA.

At 60 min after infusion, the general pattern of distribution of the tracer particles was essentially similar to that already described, except that more gold particles were endocytosed by pericytes and by perivascular microglial and presumably other glial cells (Fig. 4). Several gold particles, however, remained in the ECS or were taken up by structural elements of the neuropil, presumably into the processes of astrocytes.

At 24 h after infusion, numerous gold particles were still located in some areas of neuropil, mostly in the apparently swollen perivascular processes of astrocytes. One could note some tendency to the accumulation of gold particles on the borderline of the microvascular wall (mainly capillaries or postcapillary venules), in close vicinity of the BM (Figs. 5-7). Some tracer particles appeared also in the subendothelial BM (Figs. 6, 7), whereas only solitary or a few gold particles entered the EC cytoplasm (Fig. 5) or were internalized inside the multivesicular body located in the EC cytoplasm (Fig. 6). No tracer particles, irrespective of their size, were found in the interendothelial junctional clefts or in the vascular lumen.

Numerous gold particles were endocytosed and apparently internalized in the dense cytoplasmic vacuoles, presumably phagolysosomes (Fig. 7), located in the perivascular neuropil, presumably inside the astrocytic processes. Small or large conglomerates of the infused tracer particles were also endocytosed and internalized in various cytoplasmic vacuoles of microglial and other, presumably glial, cells (Fig. 8).

Infusion of cationized BSA-G complexes (catBSA-G)

The fate of infused catBSA-G complexes was essentially similar to that described above for native BSA-G.

The main difference was restricted to relatively faster passage of the infused catBSA-G into the area of the vascular wall followed by fast penetration into the BM. At 30 min post-infusion, this phenomenon was hardly noticeable, whereas at 60 min, almost all vascular profiles located in the area of infusion were extensively labeled with numerous gold particles, accumulating irregularly in the subendothelial BM (Fig. 9). Concomitantly, many gold particles were still present in the ECS of neuropil (Fig. 9) or were taken up mainly by perivascular microglial cells.

The accumulation of catBSA-G particles in the BM of blood microvessels was even more pronounced at 24 h post-infusion (Fig. 10), irrespective of the size of the catBSA-G complex (G5 or G13). It is noteworthy that the vast majority of the tracer particles remained in the subendothelial BM and did not enter the EC cytoplasm. Concomitantly, solitary or clustered gold particles were
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endocytosed by perivascular cells (presumably microglia) or by perivascular astrocytic processes (Fig. 10).

Generally, the pattern of distribution of gold particles suggests that the catBSA-G complex is "trapped" by the components of the BM more efficiently than it is adsorbed and endocytosed by the cellular elements of the neuropil such as microglia or astrocytes.

**Infusion of glucosylated BSA-G complexes (glucBSA-G)**

As soon as 30 min after infusion of glucBSA-G complexes, the infusate spread rapidly throughout the ECS of the neuropil. Although some capillaries remained free of the tracer (Fig. 11), many vascular profiles, including precapillary arterioles, were labeled with numerous gold particles, which penetrated both peripheral and subendothelial BM and were taken up by some perivascular cells. In contrast, smooth muscle cells present in the wall of arterioles remained unlabeled (Fig. 12). At this early post-infusion time, many microglial cells and presumably astrocytes rapidly endocytosed the infused tracer.

After 60 min, the endocytosed gold particles were frequently found in the perinuclear areas of the cytoplasm of these cells and also entered their nuclei (Figs. 13, 14). Numerous gold particles also penetrated the astrocytic processes and synapses, where they were associated with synaptosomes and also with the postsynaptic densities (Fig. 15).

At 24 h after infusion of glucBSA-G complexes, the pattern of distribution of gold particles was essentially similar. Numerous gold particles appeared in the BM of all types of blood microvessels, but no evidence of their reverse transport from the neuropil to circulating blood was found.

Although both complexes of glucBSA-G were endocytosed by glial cells, our impression was that more smaller (G5) than larger (G13) particles penetrated the cell nuclei.

**Infusion of mannosylated BSA-G complexes (manBSA-G)**

At 30 min after intracerebral infusion of manBSA-G complexes, the deposits of gold particles were found mainly in the ECS of neuropil. At this time interval, a few gold particles also penetrated the wall of neighboring blood microvessels and occasionally appeared in their BM (Fig. 16). Single gold particles were also endocytosed by perivascular microglial cells and pericytes.

At 60 min after infusion, in some microvessels, especially in postcapillary venules, several gold particles appeared not only in the subendothelial BM but also inside the EC cytoplasm (Fig. 17). Concomitantly, numerous gold particles were dispersed irregularly throughout the ECS of the neuropil and also were internalized into the cytoplasmic vesicles or endosomes of pericytes, microglial cells, and eventually astrocytes.

Occasionally, we noted several gold particles to be associated with blood plasma located inside the lumen of some vascular profiles (Fig. 18). In these microvessels, however, we did not find any gold particles located in the interendothelial junctional clefts or in the transport-related EC organelles, such as transcytotic or clathrin-coated vesicles.

In brain specimens taken 24 h after infusion, the deposits of manBSA-G particles were evidently less dense than those observed at 30- or 60-min post-infusion. Several gold particles, however, were located inside the cytoplasmic vesicles or endosomes, apparently endocytosed by various cells, mainly pericytes,

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**Fig. 1.** A section of the cerebral cortex of mouse 30 min after infusion of native BSA-G13. The area shown is located in close proximity to, but outside the needle track. The infused complex represented by gold particles is located in the extracellular space of perivascular neuropil (arrows). No gold particles penetrate the basal lamina (B) or the endothelium (E) of the capillary. The following symbols are used in this and all further figures: B: basement membrane (basal lamina); E: endothelial cell; L: vessel lumen; M: mitochondria; N: cell nucleus; S: smooth muscle cell. × 27,000

**Fig. 2.** In this cross-sectioned blood microvessel located in the same area as shown in Fig. 1, a single gold particle representing infused BSA-G13 (arrow) appears inside the pericyte cytoplasm. × 30,000

**Fig. 3.** A portion of the capillary wall from the same specimen as shown in Figs. 1 and 2 with a single gold particle (arrow) located at the borderline between the BM (B) and the abluminal front of the EC (E). × 37,000

**Fig. 4.** In this section of the cerebral cortex taken 60 min after infusion of BSA-G13, a perivascular microglial cell is loaded with gold particles apparently representing endocytosed BSA-G13 complex (arrows). A few gold particles are scattered in the adjacent neuropil. × 21,000

**Fig. 5.** Area of cerebral cortex taken 24 h after infusion of BSA-G13. In close proximity to the needle track, numerous gold particles are scattered throughout the perivascular blood microvessel (arrowshead), whereas only a single gold particle enters the EC cytoplasm (arrow). × 46,000

**Fig. 6.** A portion of the wall of another blood microvessel from the same specimen as shown in Fig. 5. Several gold particles appear in the BM area (arrowheads), and two particles are internalized inside the multivesicular body (arrow) of the EC (E). × 46,000

**Fig. 7.** Cerebral cortex of the mouse taken 24 h after infusion of BSA-G5 complex. Numerous gold particles are endocytosed into the phagolysosomes (arrowheads), presumably located in tangentially sectioned perivascular astrocytic processes. Several gold particles are also scattered throughout the subendothelial BM (arrows). × 35,000

**Fig. 8.** In the same section as shown in Fig. 7, the cytoplasm of a glial cell (presumably an astrocyte) is loaded with gold particles (arrows). × 30,000
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microglia, and eventually astrocytes.

**Discussion**

The results obtained clearly indicate that both native and modified BSA-G complexes are rapidly taken up and deposited inside the cytoplasm of perivascular macrophages, pericytes and glial cells (mainly microglia and eventually astrocytes), located in the area adjacent to the site of intracerebral infusion. Thus, these findings are compatible with previous reports of Klatzo et al. (1980) and Wolman et al. (1981) related to the clearance of serum proteins from edematous fluid in the VBE model. In addition, our present observations strongly suggest that the main or at least the most conspicuous mechanism of clearance of intracerebrally infused albumin-gold complexes, consists in their rapid cellular uptake.

The results of the present study do not support our previous observations suggesting that edematous fluid which was labeled with HRP is, at least to some degree, transported across selected segments of blood microvessels back to the bloodstream (Vorbrodt et al., 1985). In contrast, we observed that although the infused BSA-G complexes penetrated the vascular BM and even entered the EC cytoplasm, they were not transported and exocytosed into the blood plasma located in the vessel lumen - with the exception of the manBSA-G complex - which was found occasionally inside some vascular segments.

These differences can result from the utilization of different tracers and from the application of different experimental models. One cannot exclude the possibility that in our previous study, intravenously injected HRP induces, by itself or by histaminic reaction, the reverse vesicular transport of edematous fluid across the endothelial lining of some segments of blood microvasculature, noted previously by van Deurs (1977). Such a possibility should be taken into consideration also on the basis of observations and findings of Cotran and Karnovsky (1967), Straus (1979), and Dux et al. (1988).

In addition, histaminic reaction can be triggered by cold lesion injury of brain tissue, ultimately leading to induction of reverse transport of edematous fluid in the VBE model, as suggested by Ohata et al. (1990).

The application in our experiments of modified albumin-gold complexes enabled us to make some interesting observations, which deserve a few comments.

The fate of albumin after infusion was evidenced by cationization, as evidenced by rapid and intense labeling of the vascular BM. A similar phenomenon was observed by Houthoff et al. (1984) after intracerebral infusion of three different derivatives of HRP: native, anionic, and cationic. These authors also observed that the clearance of cationic HRP was much slower than that of native and anionic derivatives. It should be emphasized that despite relatively high accumulation of catBSA-G complexes in subendothelial BM, no signs of reverse transendothelial transport of the tracer across the endothelium were noted. Our impression is that catBSA-G complexes are trapped in the BM and that this phenomenon consists in electrostatic attraction of these cationic molecules by anionic components of the BM. This assumption is based on reports indicating that the BM of non-barrier vessels (Simionescu et al., 1982) as well as of BBB-type vessels (Vorbrodt, 1989) is strongly anionic and presumably behaves as a semipermeable screen or barrier restricting the movement of various molecules, according to both their size and charge. Consequently, cationized molecules easily penetrate the BM and remain in this structure without possibility of further passage to or across the endothelium. Thus, their eventual reverse transport appears to be impaired, in addition to structural obstacles, also by electrostatic forces.

The modification of albumin molecules by glycosylation also affects the fate of intracerebrally infused tracer. One easily noticeable effect is the relatively rapid uptake of glucBSA-G particles by various cells located in the area of infusion, with concomitant fast passage through the cell cytoplasm followed by penetration into the cell nucleus. This finding suggests that the addition of glucose molecules affects the interaction of BSA with cell organelles such as...
as the plasma membrane, intracytoplasmic vesicles and vacuoles, nuclear membrane, and cell nucleus. We do not know, however, what mechanism is responsible for rapid entrance of these modified molecules into the cell nucleus. This process is probably controlled by some mechanisms restricting the passage also according to the size of the tracer, because considerably more particles of glucBSA complexed with G5 than with G13 appeared inside the cell nuclei. Perhaps, the penetration of glucBSA-G complexes into the cell nuclei can be associated with or is related to the presence of specific glucose-binding sites, which were recently demonstrated at the ultrastructural level in the cell nucleus by Gugliucci and Bendayan (1995).

Our observations indicate that neither native nor modified by cationization or glucosylation albumin-gold complexes are crossing the vascular wall via reverse transport, back to the vessel lumen. We are aware that already mentioned large dimensions of these complexes may influence their eventual transport and in this respect they can behave differently than native albumin. Nevertheless, among the four BSA derivatives used in our present study, only mannosylated derivatives, i.e., manBSA-G complexes, were observed to find their way

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**Fig. 15.** In the same specimen as shown in Fig. 14, several glucBSA-G5 particles appear to be associated with synapses, especially with postsynaptic densities (arrowheads). × 34,000

**Fig. 16.** In the cerebral cortex, taken 30 min after infusion of manBSA-G5, a large conglomerate of gold particles (arrows) is deposited in the neuropil between a perivascular microglial cell and capillary profile. A few gold particles apparently penetrate the vascular BM (arrowhead). × 20,000

**Fig. 17.** A portion of the capillary wall from the cerebral cortex taken 60 min after infusion of manBSA-G5. A few gold particles are located in the perivascular neuropil (arrowheads) and also appear inside the capillary endothelium (arrows). × 37,000

**Fig. 18.** Another blood microvessel from the section shown in Fig. 17. Few manBSA-G5 particles are present in the perivascular neuropil (arrowheads) and also appear inside the vessel lumen (arrows). × 30,000
to the bloodstream during the first 24 h post-infusion. Their exact pathway across the vascular segments (presumably postcapillary venules), however, was not defined and remained obscure because we do not know whether it was an intra- or extra-endothelial (through interendothelial junctional clefts) passage. Umezawa and Eto (1988) observed that mannosylation of liposomes renders possible their passage into the brain across the BBB and concomitantly enhances their uptake by glial cells, suggesting that these cells as well as BBB-associated endothelia recognize mannosine molecules and possess specific receptors. Perhaps these receptors are identical with endogenous lectins showing specific affinity to mannosyl residues, which were found histochemically to be highly concentrated in the brain vasculature (Debbage et al., 1988, Plendl et al., 1995).

In accordance with the data presented by Gabius et al. (1993), one can assume that the different endocytic and eventually transcytotic pathways of carbohydrate-modified BSA complexes used in our present experiments can be explained by their interaction with various endogenous lectins present in the brain tissue. These authors demonstrated previously that neoglycoproteins can be used for visualization of the lectin-dependent endocytic process. The role of specific receptors or endogenous lectins, however, in binding, and facilitation of luminal or reverse transendothelial transport of neoglycoproteins such as glycosylated or mannosylated BSA-G is not yet well known and requires further detailed studies.

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