Vesicular Transport of Cationized Ferritin by the Epithelium of the Rat Choroid Plexus

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ABSTRACT We have studied the transport of ferritin that was internalized by coated micropinocytic vesicles at the apical surface of the choroid plexus epithelium in situ. After ventriculocisternal perfusion of native ferritin (NF) or cationized ferritin (CF), three routes followed by the tracers are revealed: (a) to lysosomes, (b) to cisternal compartments, and (c) to the basolateral cell surface.

(a) NF is micropinocytosed to a very limited degree and appears in a few lysosomal elements, whereas CF is taken up in large amounts and can be followed, via endocytic vacuoles and light multivesicular bodies, to dark multivesicular bodies and dense bodies. (b) Occasionally, CF particles are found in cisterns that may represent GERL or trans-Golgi elements, whereas stacked Golgi cisterns never contain CF. (c) Transepithelial vesicular transport of CF is distinctly revealed. The intercellular spaces of the epithelium, below the apical tight junctions, contain numerous clusters of CF particles, often associated with surface-connected, coated vesicles. Vesicles in the process of exocytosis of CF are also present at the basal epithelial surface, whereas connective tissue elements below the epithelium are unlabeled.

Our conclusion is that fluid and solutes removed from the cerebrospinal fluid by endocytosis either become sequestered in the lysosomal apparatus of the choroidal epithelium or are transported to the basolateral surface. However, our results do not indicate any significant recycling via Golgi complexes of internalized apical cell membrane.

The choroid plexus secretes a major fraction of the cerebrospinal fluid (CSF) (12, 18, 34). In addition, the choroidal epithelium has an absorptive function, since it removes various substances from the CSF. Isolated chick choroid plexus absorbs fluorescein-labeled serum proteins (22), and a decreased rate of absorption from the CSF of labeled albumin has been shown in pleurectomized hydrocephalic patients (14). Several smaller molecules normally absent in the CSF (penicillin, diodrast, inulin, and phenolsulfonphthalein) are transported from the CSF to the blood, presumably through the choroidal epithelium (17, 24, 33). Also, the CSF contains small amounts of several proteins synthesized in the brain that are of importance for brain function. An example is the D2 protein (9, 10), which is released into the CSF in increased amounts in several psychiatric disorders (11). Immunohistochemical studies have shown the presence of the D2 protein as well as of other brain-specific proteins in the choroid plexus, indicating that the plexus plays a part in the homeostatic regulation of the CSF.

A likely mechanism for the clearance of solutes from the CSF is endocytic uptake and transport by the choroidal epithelium (1, 23). Recent studies from our laboratory involving ventriculocisternal perfusion of horseradish peroxidase (HRP; 44,000 mol wt) and microperoxidase (MP; 1,900 mol wt) in physiologically well-controlled rats showed a pronounced uptake of these molecules by micropinocytosis at the apical (ventricular) epithelial surface, followed by lysosomal sequestration (25, 26, 28, 32). Moreover, a vesicular transepithelial pathway, circumventing the epithelial tight junctions, was indicated. This was based mainly on the observation that, after the ventriculocisternal perfusion of HRP, several heavily HRP-loaded, coated vesicles opened into the basolateral intercellular spaces. The spaces contained no HRP, or HRP was present only immediately outside the vesicle opening, a situation suggesting exocytosis (32). However, the interpretation of such observations with a diffusible, low molecular weight enzymatic tracer is not beyond question. For instance, retrograde tracer labeling of the intercellular spaces from the connective tissue interstitium of the plexus, the influence of variable fixation on
enzymatic activity, and the diffusion of reaction product must be considered. The pertinent question, therefore, is to establish, by other approaches, that the coated vesicles are actually discharging their contents into the intercellular spaces, rather than endocytosing them.

In the present study, native (anionic) ferritin (NF) and cationized ferritin (CF), which are high molecular weight (500,000 mol wt), nonenzymatic tracers, have been administered by ventriculocisternal perfusion in rats. The micropinocytic absorption into the choroidal epithelium is clearly cation-selective. We demonstrate that a vesicular transepithelial pathway exists in the choroidal epithelium in situ, and also, that considerable amounts of endocytosed CF become sequestered in elements of the lysosomal apparatus, especially multivesicular bodies, whereas presumptive trans-Golgi or GERL elements only on occasion exhibit CF labeling, and stacked Golgi cisterns remain unlabeled.

Part of this work has been communicated in abstract form (30, 31).

MATERIALS AND METHODS

Ventriculocisternal Perfusion

Male Wistar rats (325-375 g) were anesthetized with 1 ml/100 g i.p. of a 10% pentobarbital solution (Mebumal; DAK, Denmark) diluted 1:20 with physiological saline. The animals were tracheostomized, and, when necessary, respiration was artificially assisted with a rodent respirator (Harvard Apparatus Co., Inc. Millis, Mass.). Rectal temperature was monitored thermoelectrically (Thermometer type DU3-S; Eilab A/S, Copenhagen, Denmark) and maintained at 37.5°C with a 250-W heating lamp. Blood pressure was continuously monitored in the left femoral artery with a Statham pressure transducer (Gould Statham Instruments, Inc., Hato Rey, P. R.).

The animals were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, Calif.). A cannula with an o.d. of 1.0 mm was placed in the anterior horn of the right lateral ventricle (coordinates according to reference 13: anterior, 7.02 mm; lateral, 1.0 mm; superior-inferior, 3.0 mm measured from the pial surface of the brain). The neck muscles were removed from the occipital bone and a cannula was inserted through the atlanto-occipital membrane into the cisterna magna by the use of a micromanipulator (Leitz; NOCO A/S, Copenhagen, Denmark). The correct position of the cannula was verified by the presence of a free fluid flow through the ventricles at a hydrostatic pressure of 20 cm of H₂O.

Material from 10 successful experiments were used for the electron microscopic investigation. In these experiments, 5 mg CF or NF (Miles Yeda; Miles, Millis, Mass.) was placed in the anterior horn of the right lateral ventricle. The correct position of the cannula was verified by the presence of a free fluid flow through the ventricles at a hydrostatic pressure of 20 cm of H₂O.

The incubation medium was bubbled with 95% O₂ and 5% CO₂ l h before, and during, the incubation. After 1 or 5 min of incubation, 2 ml of 5% glutaraldehyde and 4% formaldehyde in 0.2 M Na-cacodylate buffer, pH 7.4, was added to the incubation medium. After 2 h of fixation at room temperature, the material was further processed for electron microscopy.

Processing for Electron Microscopy

After aldehyde fixation, tissue pieces were postfixed for 2 h in 2% OsO₄, 0.1 M cacodylate buffer, pH 7.4, dehydrated in ethanol, and embedded in Epon. Survey sections 1 μm thick were cut (in the case of the third ventricle, in the frontal plane) and stained with toluidine blue. Thin sections were cut from several preselected areas of different parts of the plexus, contrasted with 5% uranyl acetate in 50% methanol at 40°C for 10-20 min, and examined in a Philips 100 electron microscope.

RESULTS

The ultrastructural preservation of the choroid plexus after ventriculocisternal perfusion of tracer was good, and corresponds to that obtained in control material. No abnormal changes in cell structure were seen, even after the longer periods of tracer perfusion.

Surface Labeling

When CF was perfused through the ventricular system, after the choroid plexus had been fixed by ventriculocisternal perfusion, labeling of the apical epithelial surface was distinct (Fig. 1). Although the degree of CF labeling varied from region to region of the plexus, it was consistently uniform from the tip to the base of the microvilli. This distribution of CF suggests that anionic groups are uniformly distributed on the apical cell surface, although it may reflect, in part, reaction of tracer with unblockaded aldehyde groups. Coated pits between the bases of the microvilli were also labeled. Occasionally, vesicular profiles with CF were seen in the apical cytoplasm very close to the surface (Fig. 1), indicating that such vesicles must be connected to the surface at another plane of sectioning.

When CF was perfused through the ventricular system before fixation (Figs. 2-4), a very uneven surface labeling of the plexus was seen. Many choroidal villi completely lacked a labeling with CF, even after the longest periods of CF administration. This emphasizes the need for thin sections of tissue blocks from several parts of the plexus in each experiment to obtain a reliable picture of the events following CF perfusion. When the CF particles were present in large amounts, they were uniformly distributed and bound to the epithelial surface and associated pits. In regions with only a little CF, the particle distribution on the surface was less uniform. Sometimes the surface labeling by CF could be distinctly followed into coated pits (Fig. 2). Other times, the coated pits between the microvilli appeared loaded with CF (Fig. 3). This may be due to their small curvature, or to binding to a glycolycalyx at a distance from the membrane bilayer, or the pits might contain CF in excess of that bound to the membrane or glycolycalyx (fluid phase uptake).

Adjacent microvilli often appeared to be bound together by the CF particles (Fig. 4), in contrast to the situation with CF perfused after fixation (Fig. 1).

In Vitro Incubations

Choroid plexus from the lateral, the third, and the fourth ventricles of two male Wistar rats (325 g) anesthetized with ether was rapidly removed, divided into smaller pieces with razor blades in drops of mock CSF at 37°C, and incubated at 37°C in 5-ml vials with 2 ml of mock CSF containing 0.1 mg CF/ml under constant agitation (Thermomix; Braun Melsungen AG, W. Germany). The incubation medium was bubbled with 95% O₂ and 5% CO₂ for 1 h before, and during, the incubation.

After 1 or 5 min of incubation, 2 ml of 5% glutaraldehyde and 4% formaldehyde in 0.2 M Na-cacodylate buffer, pH 7.4, was added to the incubation medium. After 2 h of fixation at room temperature, the material was further processed for electron microscopy.
FIGURES 1–6  Labeling of the apical surface of the choroidal epithelium. In Fig. 1 the plexus was fixed before the perfusion of the ventricles with CF. Note the binding of the CF to the cell surface. Arrow indicates an apparent free vesicle with CF. In Figs. 2–4, CF has been perfused ventriculocisternally for 35 min, followed by 30 min of perfusion with mock CSF without CF before perfusion with fixative. CF labeling of the apical cell surface is seen and, in Fig. 4, CF particles bind adjacent microvilli together (arrowheads). Coated pits and vesicles (arrows) with CF are seen. The CF particles in one of the coated vesicles in Fig. 4 (arrow) are apparently detached from the membrane. Fig. 5 shows CF labeling of the apical epithelial surface and of two coated pits (arrows), after 5 min of in vitro incubation. In Fig. 6, NF has been perfused ventriculocisternally for 60 min before perfusion with fixative. NF particles are seen between the microvilli and in a coated pit (arrow), but are not bound to the cell surface. Fig. 1, × 52,200; Figs. 2 and 3, × 76,600; Fig. 4, × 72,000; Fig. 5, × 70,000; and Fig. 6, × 55,000.
distinct and uniform labeling from the tip to the base of the microvilli, as well as of the coated pits. Also, the microvilli were often bound together, as observed after ventriculocisternal perfusion (Fig. 5). The distribution of CF after only 1 min of in vitro incubation before fixation supports the notion that the anionic sites are uniformly distributed on the apical cell surface.

After ventriculocisternal perfusion of NF before fixation, the ferritin particles were often aggregated between the choroidal villi, and were present in varying concentrations in the spaces between the microvilli; but they were not bound to the apical cell surface (Fig. 6).

**Micropinocytosis**

In all experiments with CF administration before fixation, coated and uncoated vesicles containing CF were present within the epithelial cells. As described for the surface-connected pits, some of the vesicles exhibited only CF particles bound to the inner aspect of the membrane, whereas others apparently were filled with CF. In some coated vesicles, the CF particles appeared detached from the membrane (Fig. 4).

Even in the experiments with perfusion of CF for 35 min, followed by perfusion of mock CSF without CF for another 30-50 min before fixation, labeling of the apical surface was pronounced regionally, and micropinocytosis of CF occurred. Micropinocytic vesicles containing NF were rarely seen in the epithelial cells.

**Labeling of Lysosomes**

The various elements of the lysosomal apparatus of the choroidal epithelium are described in detail elsewhere (25, 28). The elements are clearly revealed both in tracer experiments and in controls. They comprise endocytic vacuoles, which have an electron-lucent content; light multivesicular bodies (MVB), which are endocytic vacuoles with a few internalized vesicular profiles; dark MVB, which are vacuoles with numerous small vesicular profiles in an electron-dense matrix; and dense bodies, which resemble dark MVB because they sometimes contain vesicular profiles, in addition to lipidlike droplets and myelin figures in the dense matrix. The dark MVB and dense bodies are reactive for acid phosphatase activity and represent secondary lysosomes (25).

After 5 min of in vitro incubation and, in particular, after 15 min of CF perfusion, endocytic vacuoles contained CF. The ferritin particles usually covered the inner aspect of the membrane (Figs. 7–9), but some endocytic vacuoles also exhibited clustered CF particles freely in the matrix or associated with some nonmembranous material (Fig. 10).

Apparent fusions of small, CF-labeled vesicles with larger vacuoles were seen, suggesting discharge of micropinocytosed material into endocytic vacuoles and addition of membrane to the vacuole (Fig. 7 and 8). Tracer-labeled vesicles transporting material to lysosomal elements appeared uncoated, suggesting that the cytoplasmic coat of the micropinocytosed vesicles is shed before fusion takes place.

After 15 min of CF perfusion, some of the light MVB also contained CF. The ferritin particles were usually bound to the inner aspect of the limiting membrane as well as to the outer surface of the small vesicles (Fig. 11). In addition, CF particle clusters might be localized freely in the light MVB or bound to nonmembranous material (Fig. 11).

After 30 min of CF perfusion, a few dense bodies and dark MVB as well were labeled with CF. The matrix of the dark MVB contained numerous CF particles apparently not bound to membranes (Fig. 12), and the dense bodies contained various amounts of CF aggregated within the matrix (Fig. 13).

After 35 min of CF perfusion, followed by 30–50 min of perfusion with mock CSF without CF, the predominant CF-labeled compartments were dark MVB and dense bodies (Fig. 14). However, in accordance with the observation that micropinocytic activity was still appreciable in these experiments, light MVB and endocytic vacuoles also showed CF labeling. NF was rarely seen in lysosomal elements of the epithelium, even in situations where Kolmer cells (ventricular macrophages) on the epithelial surface exhibited heavily NF-loaded lysosomes, demonstrating that the tracer had been present long enough, and in sufficient amount, for uptake to occur. NF particles in lysosomal elements of the epithelium were always located in the matrix.

**Labeling of Cisternal Compartments**

In all experiments with CF administration before fixation (except for the 1-min in vitro incubation), a few cisterns in the epithelial cells contained CF. In general, CF did not appear to be bound to the cisternal membrane (Fig. 15). The CF-labeled cisterns were often located in Golgi regions, sometimes close to stacked Golgi elements (Fig. 15). However, stacked Golgi cisterns never contained CF (Figs. 10–12). NF was not seen in cisternal compartments.

**Labeling of the Basolateral Epithelial Spaces**

In all experiments with ventriculocisternal perfusion of CF before fixation, clusters of CF particles were present in the intercellular spaces of the epithelium. This was particularly distinct in experiments with CF administration for 30 min or more, where CF particles in the intercellular spaces, predominantly in the regions of basolateral foldings, were numerous and consistently observed (Fig. 16). The CF particle clusters were often associated with coated pits or vesicles opening into the intercellular space (Fig. 17). CF particle clusters were also frequently encountered between the basal epithelial cell membrane and the basement membrane (Figs. 18 and 19). However, CF particles were not seen within the basement membrane.
Figures 9-13  CF labeling of the lysosomal apparatus of the choroidal epithelium. Fig. 9 (15 min of CF perfusion) shows a large endocytic vacuole with membrane-bound CF. Fig. 10 (30 min of perfusion) shows an endocytic vacuole with CF particles freely in the matrix, or bound to nonmembranous material, in addition to the membrane bound CF. No CF is present in the Golgi cisterns (Go). Fig. 11 (30 min of perfusion), shows a light MVB with numerous CF particles, an unlabeled lysosome (Ly), and an unlabeled Golgi complex (Go). Fig. 12 (30 min of CF perfusion) and Fig. 13 (35 min of CF perfusion followed by 30 min of perfusion with mock CSF without CF before fixation) show a dark MVB filled with CF and a dense body with CF in the matrix. Note that no CF is present in the Golgi cisterns (Go) in Fig. 12. 9-13, ×75,600.
bound to elements of the connective tissue interstitium between the epithelium and the choroidal blood vessels, or in the blood vessels (Figs. 18 and 19).

It was characteristic that CF particles or particle clusters in the intercellular spaces, and in the coated pits and vesicles at the basolateral epithelial surface, did not show any distinct binding to the membranes (Figs. 16–19).

The apical tight junctions of the epithelium never contained CF, and CF particles were not present in the most apical parts of the intercellular space, i.e., just beneath the junctions.

The in vitro incubation experiments with CF were not considered appropriate to evaluate whether a possible vesicular transport of CF takes place across the epithelium or not, because CF labeling of connective tissue elements could be seen in these experiments to be a result of diffusion from the incubation medium.

NF was never found in the intercellular spaces, nor in the connective tissue below the epithelium.

DISCUSSION

We have used CF perfused ventriculocisternally in rats to analyze the transport of molecules internalized by coated micropinocytic vesicles at the apical surface of the choroid plexus epithelium in situ. Three different routes are followed by the tracer: to elements of the lysosomal apparatus, to cisternal compartments; and to the basolateral epithelial surface (Fig. 20). The latter route indicates a transepithelial vesicular transport mechanism. So far, such a mechanism has not been documented in studies with CF, although it has been mentioned as a possibility for CF transport into closed thyroid follicles (4).

Transport to Lysosomes

CF was introduced as an ultrastructural marker of anionic sites on the cell surface (see references 3, 7, 20, 21), and is used for the localization of anionic groups of extracellular tissue components, as for example, in the glomerular basement membrane (19).

Recently, CF has also been used to trace the fate of internalized membrane in isolated pituitary cells (5, 6) and isolated thyroid follicle epithelium (4, 8). It was demonstrated that whereas NF (pI = 4.6; net negative charge at physiological pH) is absorbed only in small amounts and becomes sequestered exclusively in lysosomes, CF (pI = 8.5; net positive charge at physiological pH) is taken up in large amounts and can be followed also to Golgi elements and, in the pituitary cells, to secretory granules (6, 8).

However, although CF labels surface membranes distinctly, we found that, to some extent, CF becomes detached from the membranes when internalized (see also references 6 and 8). In the present study, apparent detachment of CF particles was seen even in coated vesicles close to the apical surface (and, therefore, presumably newly formed) as well as in coated vesicles close to the basolateral surface. The reason for this detachment from membranes is not clear, but the reliability of CF labeling in the evaluation of membrane flow within the cytoplasm becomes questionable, since CF may only be regarded as a content marker when considering cytoplasmic membrane compartments. On the other hand, because CF is a particulate tracer, it is in certain respects a more accurate probe than the previously used enzyme tracers (25, 26, 32) (for example, when analyzing progressive loading of lysosomal

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FIGURES 16-19  CF particles present at the basolateral epithelial surface after 35 min of CF perfusion, followed by 30 min of perfusion with mock CSF without CF before fixation. Fig. 16 is a section through the basolateral foldings. CF particle clusters are present at several locations in the intercellular space (IS), often in association with coated pits (arrows). Fig. 17 shows CF particles in the intercellular space in association with coated vesicles. In Fig. 18, several CF particle clusters (arrowheads) are present between the basal epithelial cell membrane and the basement membrane (BM), as well as in the most basal part of an intercellular space (IS). Below the basement membrane, and in the connective tissue which here contains much collagen (Col), no CF is visible. In the portion of the plexus shown in Fig. 19, the epithelium and the endothelium (En) are close together, separated only by the basement membranes (BM) and a narrow connective tissue space (arrows). A CF-labeled, coated pit at the basal epithelial surface is present, but the basement membranes, the narrow space between them, and the blood vessel are not CF labeled. Er, erythrocyte. Fig. 16, X 54,000; Fig. 17, X 75,600; Fig. 18, X 56,000; Fig. 19, X 75,600.
Thus, the velocity and direction of the flow of exogenous molecules through the various cytoplasmic compartments elements or transepithelial transport), provided it can be administered in the presence of only little, or no, protein. This is possible with in vitro incubations (4, 6, 8) or ventriculocisternal perfusion.

The present results show that, compared with CF, NF is taken up only in very small amounts by the choroidal epithelial cells. The sequence of events established in this study agrees with our previous results obtained with HRP and MP (25, 26, 28, 32). Thus, the velocity and direction of the flow of exogenous molecules through the various cytoplasmic compartments to secondary lysosomes appear independent of the nature of the molecules, whereas the amount of uptake is determined by their net surface charge. In the choroidal epithelium the numerous MVB are a characteristic feature, and possibly play a central role in the handling of internalized membrane, since they, with increasing time, contain increasing amounts of CF as well as vesicular profiles binding CF. A large amount of internalized apical membrane presumably becomes degraded in the dark MVB as these develop into dense bodies.

Transport to Cisternal Compartments

In agreement with previous experiments with HRP and MP (25, 26), CF particles were seen in a few cisternal compartments of the choroidal epithelial cells. The CF-labeled cisterns were often located in Golgi regions, and may represent trans-Golgi elements or correspond to GERL (16), but stacked Golgi cisterns never contained CF.

A consistent CF labeling of Golgi elements, including stacked Golgi cisterns, in isolated pituitary and thyroid follicle cells has been interpreted as indicating recycling of membrane internalized by endocytosis (5, 6, 8). In the plexus, internalized membrane does not appear to reach the stacked Golgi cisterns directly from the apical surface, but, because CF is a doubtful membrane marker, a lack of CF labeling of stacked Golgi elements does not necessarily indicate that internalized membrane has not reached the Golgi complex from the lysosomal compartment. It is therefore difficult to evaluate whether or not recycling of internalized membrane takes place in the choroidal epithelium. But, in view of the considerable amount of internalized membrane trapped in the numerous MVB, recycling to any significant degree seems unlikely. Dark MVB and dense bodies appear to be the final station for most intact internalized membrane in the choroidal epithelium.

Transepithelial Vesicular Transport

CF particle clusters were consistently present in the intercellular spaces at the basolateral epithelial surface after 30 min or more of CF administration. The CF particle clusters were frequently seen in association with coated vesicles or pits opening into the intercellular space. Narrow pores are present in the tight-junctional strands of the rat choroidal epithelium, as revealed by complementary freeze-fracture replicas, and they may be of importance in the CF particle clusters in the intercellular spaces and at the basolateral epithelial surface, because CF was not bound in the epithelial basement membrane or in the connective tissue interstitium below the epithelium. Therefore, the CF particle clusters at the basolateral epithelial surface must be discharged by the coated vesicles.

An important question is whether the coated vesicles that ferry CF to the basolateral surface come directly from the apical epithelial surface (which is indeed a prerequisite to transepithelial transport of fluid and solutes) or via the lysosomal compartment, where the vesicles may detach from the lysosomes after discharge of endocytosed material. It should be remarked against the latter view that (a) MVB seem to trap endocytosed membrane progressively, rather than budding off vesicles, (b) coated vesicles were never seen in connection with dark MVB and dense bodies; and (c) CF labeling of the basolateral surface was already observed at a time (15 min) when the dark MVB and dense bodies were labeled only slightly or not at all.

We conclude that a vesicular transport mechanism operates in the choroidal epithelium, transferring material directly from the apical to the basolateral epithelial surface, thus bypassing both the tight junctions and the lysosomal compartment. From the basolateral surface, the blood vessels of the plexus are readily accessible (27).

A likely mechanism to compensate for the flow of membrane from the apical to the basolateral epithelial surface would be vesicular transport in the opposite direction. Such a mechanism has not been documented so far in electron microscopic tracer studies (2). However, coated microinocytic vesicles do internalize intravenously injected tracer and transport it to lyso-
somes in the choroid plexus epithelium (2, 25), although to a lesser extent than at the apical surface (25).

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