

# Behavior of $\alpha$ -, $\beta$ -, and $\gamma$ -Cyclodextrins and Their Derivatives on an in Vitro Model of Blood-Brain Barrier

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## ABSTRACT

Cyclodextrins (CDs) can be envisaged to cure some diseases related to the brain, but the behavior of these compounds toward the blood-brain barrier (BBB) remains largely unexplored to envisage such clinical applications. To fulfill this gap, the toxicity and endothelial permeability for native, methylated, and hydroxypropylated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CDs have been studied on an in vitro model of BBB. As shown by the endothelial permeability for sucrose and immunofluorescence stainings, the native CDs are the most toxic CDs ( $\alpha$ - >  $\beta$ - >  $\gamma$ -CD). Whereas the chemical modification of  $\beta$ -CD did not affect the toxicity of this

CD, differences are observed for the  $\alpha$ - and  $\gamma$ -CD. To determine the origin of toxicity, lipid effluxes on the brain capillary endothelial cells were performed in the presence of native CDs. It was found that  $\alpha$ -CD removed phospholipids and that  $\beta$ -CD extracted phospholipids and cholesterol.  $\gamma$ -CD was less lipid-selective than the other CDs. Finally, the endothelial permeability of each CD has been determined. Surprisingly, no structure/permeability relationship has been observed according to the nature and chemical modifications of CDs.

Cyclodextrins (CDs) are cyclic oligosaccharides composed of 6, 7, or 8 glucose units named  $\alpha$ -,  $\beta$ -, or  $\gamma$ -cyclodextrin, respectively. These compounds are widely used in the pharmaceutical field to improve the dissolution rate, chemical stability, and bioavailability of drugs (Uekama, 1999). Interestingly, CDs can also promote drug absorption across the dermal, nasal, or intestinal barrier by extracting cholesterol, phospholipids, or proteins from membranes (Irie and Uekama, 1997). Thus, a pretreatment of the skin surface during 24 h with randomly methylated  $\beta$ -CD enhanced the flux of piritol 3-fold. Nevertheless, such an effect was not observed with  $\beta$ -CD and hydroxypropyl- $\beta$ -CD (HP- $\beta$ -CD) (Legendre et al., 1995). A beneficial effect of CDs on the nasal absorption of peptide and protein drugs was also described (Merkus et al., 1999). In particular, it was found that dimethyl- $\beta$ -CD increased the nasal absorption of insulin up to 100% (Merkus et al., 1991). The effect of CDs on the transport of polyethylene glycol 4000 across Caco-2 monolayers as a human intestinal epithelial model was also investigated (Hovgaard and Brondsted, 1995). Among the different CDs evaluated, the dimethyl- $\beta$ -CD was the most efficient absorption promoter and caused an increase in the permeability of the

membrane in a concentration-dependent manner. With this methylated CD, the transport can be improved by a factor of 10 without significant toxic effects on the monolayer.

Only a few studies have been reported in the literature concerning the blood-brain barrier (BBB), and the results are rather contradictory. Indeed, a  $\beta$ -CD coupled with a  $\delta$ -opioid receptor peptide exhibited potent antinociceptive properties by intravenous administration, suggesting a passage through the BBB (Hristova-Kazmierski et al., 1993). In contrast, the slight effect of HP- $\beta$ -CD on Niemann-Pick type C disease was attributed to the nonpermeation of this CD across the BBB (Camargo et al., 2001). In line with this result, Pitha et al. (1994) reported that HP- $\beta$ -CD was cleared from the brain via cerebrospinal fluid when an intracerebral injection of testosterone incorporated in HP- $\beta$ -CD was performed on rats. Finally, the use of a chemical delivery system associated to a HP- $\beta$ -CD allowed the brain delivery of testosterone to increase (Anderson et al., 1988). The beneficial effect of the HP- $\beta$ -CD was ascribed to the better solubility of the testosterone/chemical delivery system in the blood, and the effect on this CD on the BBB was mysteriously ignored. Because CDs are potential agents in curing neurodegenerative disorders [Niemann-Pick or Alzheimer diseases (Camilleri et al., 1994)] or can be used to facilitate intracerebral injection of drugs [neurotoxin capsaicin or opioid alkaloids

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**ABBREVIATIONS:** CD, cyclodextrin; HP, hydroxypropyl; BBB, blood-brain barrier; Me, methyl; DS, degree of substitution; DMEM, Dulbecco's modified Eagle's medium; BCEC, brain capillary endothelial cell; CMF-PBS, calcium and magnesium-free phosphate-buffered saline; PC, phosphatidylcholine; SM, sphingomyelin.

(Yaksh et al., 1991)], it is of great interest to clarify the behavior of cyclodextrins toward BBB. In the present work, we have studied the toxicity and endothelial permeability for native, methylated, and hydroxypropylated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CDs on an *in vitro* model of the BBB developed in our laboratory (Dehouck et al., 1992; Cecchelli et al., 1999). The action mechanism of CDs was also discussed from immunofluorescence stainings and lipid efflux experiments.

## Materials and Methods

**Chemicals and Antibodies.** [ $^3\text{H}$ ]cholesterol (38 Ci/mmol), [methyl- $^3\text{H}$ ]choline chloride (80 Ci/mmol), and [ $^{14}\text{C}$ ]sucrose (58 mCi/mmol) were obtained from Amersham Biosciences Inc. (Piscataway, NJ).  $\alpha$ -CD was purchased from Acros Organics (Noisy le Grand, France),  $\beta$ -CD was purchased from Roquette Frères (Lestrem, France), and  $\gamma$ -CD was purchased from Cyclolab (Budapest, Hungary). Randomly methylated  $\alpha$ -CD with an average degree of substitution (DS) of 10.8 was purchased from Cyclolab, randomly methylated  $\beta$ -CD (DS, 12.6) was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), and Me- $\gamma$ -CD (DS, 14.4 in position 2 and 6) was synthesized according to the method described by Takeo (1990). HP- $\alpha$ -CD (DS, 3.6) and HP- $\beta$ -CD (DS, 5.6) were purchased from Sigma-Aldrich, and HP- $\gamma$ -CD (DS, 4.8) was purchased from Wacker Chemie GmbH (Lyon, France). They were used as received without further purification. Primary antibody was detected with the following appropriate combination of fluorescently labeled secondary antibodies (Molecular Probes, Inc., Eugene, OR): rabbit polyclonal anti-human occludin (Zymed Laboratories, South San Francisco, CA) and Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), respectively. Hoescht 33258 (bis-benzimide) was purchased from MP Biomedicals (Irvine, CA).

**Cell Culture.** Primary cultures of mixed glial cells were made from newborn rat cerebral cortex. After the meninges had been removed, the brain tissue was gently forced through a nylon sieve, as described by Booher and Sensenbrenner (1972). Glial cells were plated in six-well dishes at a concentration of  $1.2 \times 10^5$  cells/ml in 2 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), and the medium was changed twice a week.

Three weeks after seeding, glial cells were stabilized, and coated filters (Millicell PC 3  $\mu\text{m}$ , 30-mm diameter; Millipore SAS, Molsheim, France) were set in six-well dishes containing glial cells. Brain capillary endothelial cells (BCECs), isolated and characterized as described by Méresse et al. (1989), were plated at a concentration of  $4 \times 10^5$  cells/ml on the upper side of the coated filters in 1.5 ml of medium (Bornstein, 1958). The coculture medium shared by both cell types was DMEM-supplemented with 10% (v/v) heat-inactivated calf serum and 10% (v/v) horse serum (Invitrogen), 2 mM glutamine, 50  $\mu\text{g}/\text{ml}$  gentamycin, and basic fibroblast growth factor (1 ng/ml, added every other day). The medium was changed every other day. Under these conditions, BCECs formed a confluent monolayer after 7 days. Experiments were performed 5 days after confluence.

**Transport Experiments.** All transport studies were conducted at 37°C in buffered Ringer's solution at pH 7.4. Prior to the transport experiment, cell monolayers were washed with Ringer's solution. At the start of the experiment, 2.5 ml of buffered Ringer's solution was added to wells of a six-well plate. One insert containing a confluent BCEC monolayer was placed in the first well of the six-well plate. Then 1.5 ml of buffered Ringer's solution containing CDs or not (control) at the required concentrations was placed at time 0 in the apical compartment. The plates were then placed on a rocking platform. At selected times (30, 60, and 120 min after the addition of the solution containing CDs), the insert was moved to other wells of the plate to minimize back diffusion of the compound to the upper compartment. Three inserts with the BCEC monolayer and three

without cells were assayed for each solution. Amounts of CD in the lower compartment were analyzed by high-performance liquid chromatography. The conditions were as follows: a Finnigan P4000 pump and LCQ-DUO mass spectrometer detector (Thermo Finnigan, Courtaboeuf, France) and a Polymer Laboratories' PLRPS-S column ( $250 \times 4.65$  mm; Polymer Laboratories Ltd., Marseille, France).

Using the same procedure, the integrity of the BCEC monolayer was checked by adding [ $^{14}\text{C}$ ]sucrose in the upper compartment containing the tested solutions. Amounts of radiotracers in the lower compartment were measured in a liquid scintillation counter (Tri-Carb 2100TR; PerkinElmer Life and Analytical Sciences, Boston, MA).

**Data Analysis and Calculation.** The amount of sucrose crossing the BBB was expressed in endothelial permeability (Pe, centimeters per minute). The cleared volume was calculated as described by Siflinger-Birnboim et al. (1987) by dividing the amount of compound in the receiver compartment by the drug concentration in the donor compartment at each time point. The average cumulative volume cleared was plotted versus time, and the slope was estimated by linear regression analysis to give the mean and standard deviation of the estimate. The slope of the clearance curve with inserts alone and inserts with BCEC monolayer is equal to PSf and PSt, respectively, where PS = the permeability surface area product. The units of PS and S are microliters per minute and square centimeters, respectively. The PS value for endothelial monolayer (PSe) was obtained as follows:  $1/\text{PSe} = 1/\text{PSt} - 1/\text{PSf}$ . To generate the endothelial permeability coefficient Pe (centimeters per minute), the PSe value was divided by the surface area of the insert.

For the CDs, results were expressed as a percentage of transport across the BCEC monolayer alone and were obtained from the transport across the inserts coated with collagen and seeded with BCECs and the transport across the inserts coated only with collagen.

**Fluorescence Microscopy.** To visualize tight junction-associated protein, BCECs were fixed with 1% paraformaldehyde in calcium and magnesium-free phosphate-buffered saline (CMF-PBS) at room temperature. After washing with CMF-PBS, cells were permeabilized with 0.1% (v/v) Triton X-100/CMF-PBS for 10 min and preincubated in 10% (v/v) normal goat serum/CMF-PBS for 30 min. Primary antibody against occludin was added [1:200 dilution in 2% (v/v) normal goat serum/CMF-PBS] for 60 min. Secondary antibody (Alexa 568) was added for 60 min. For the nuclei staining, DNA was stained using Hoescht reagent (4  $\mu\text{l}/10$  ml CMF-PBS) after secondary antibody labeling. The filters and their attached monolayers were mounted on glass microscope slides with a Mowiol mountant (Aventis, Strasbourg, France). The specimen was visualized and photographed with a Leica fluorescence microscope (Leica, Wetzlar, Germany).

**Release of Cholesterol from BCECs.** On the 10th day of coculture, [ $^3\text{H}$ ]cholesterol (0.66  $\mu\text{Ci}/\text{ml}$ ) was added in the cell-conditioned medium in the upper compartment. BCECs were incubated in this radioactive medium for 48 h, and, under these conditions, cells were radiolabeled with cholesterol. BCECs were washed with CMF-PBS and incubated in DMEM supplemented with 5% (v/v) heat-inactivated calf serum and horse serum for 4 h. The cell monolayers were washed with CMF-PBS and incubated in transport buffer containing CDs or not (control) for 2 h. The control was incubated only with the transport buffer for 2 h. The concentration of [ $^3\text{H}$ ]cholesterol in BCECs in the presence and absence (control) of CDs after 2 h of experiment was determined in a liquid scintillation counter (Tri-Carb 2100TR). Results are expressed as a percentage of the total cholesterol released from BCECs in the presence of CD compared with the control.

**Release of Phosphatidylcholine and Sphingomyelin from BCECs.** On the 10th day of coculture, [ $^3\text{H}$ ]choline (1.32  $\mu\text{Ci}/\text{ml}$ ) was added in the cell-conditioned medium in the upper compartment. BCECs were incubated in this radioactive medium for 48 h, and, under these conditions, cells were radiolabeled with phosphatidylcholine (PC) and sphingomyelin (SM). BCECs were washed with

CMF-PBS and incubated in DMEM supplemented with 5% (v/v) heat-inactivated calf serum and horse serum for 4 h. The cell monolayers were washed with CMF-PBS and incubated in transport buffer containing CDs or not (control) for 2 h. The control was incubated only with the transport buffer for 2 h. Then the endothelial cells were lysed with a solution of NaOH (0.1 N), and a mixture of chloroform/methanol (2:1) was added to extract lipids contained in the cells. The mixture was vigorously agitated and centrifuged at 2200 rpm at 4°C for 15 min. The organic phase was evaporated, and the residue was dissolved in a mixture of PC/SM (1:1) to facilitate the migration of the radiolabeled lipids. The lipid extracts were analyzed by thin-layer chromatography. Identification of lipids separated by thin-layer chromatography was accomplished by comigration with standard lipids. [<sup>3</sup>H]PC and [<sup>3</sup>H]SM were separated by monodimensional thin-layer chromatography carried out with the solvent system chloroform/methanol/formic acid (70:25:5) and revealed by using iodine. The concentration of [<sup>3</sup>H]PC and [<sup>3</sup>H]SM in BCECs in the presence and absence (control) of CDs after 2 h of experiment were determined in a liquid scintillation counter (Tri-Carb 2100TR). Results are expressed as a percentage of the total PCs or SMs released from BCECs in the presence of CD compared with the control.

## Results

**Monolayer Integrity.** Because the CDs can be employed as therapeutic agents for the brain disorders, the thresholds of toxicity of each CD have been determined. The integrity of the brain endothelial cell monolayer during exposure to CDs was checked by determination of the endothelial permeability coefficient (Pe) of [<sup>14</sup>C]sucrose across the BBB. Sucrose diffused very slowly across the BBB in physiological conditions both in vitro and in vivo (Dehouck et al., 1995). It was used as an indicator of the functional integrity of the tight junctions sealing the cells together, and a  $Pe_{(sucrose)}$  higher than  $1 \times 10^{-3} \text{ cm min}^{-1}$  was indicative of a leaky BBB. In our in vitro BBB model, the  $Pe_{(sucrose)}$  across the monolayer was inferior to  $1 \times 10^{-3} \text{ cm min}^{-1}$  (mean value of  $0.60 \pm 0.05 \times 10^{-3} \text{ cm min}^{-1}$ ) in the control conditions. Figure 1 shows the evolution of  $Pe_{(sucrose)}$  in the function of CD concentration and the CD concentration leading to a BBB breakdown. The addition of all CDs deeply modified the  $Pe_{(sucrose)}$  that increased with the CD concentration. The native CDs induced a toxicity in the order  $\alpha\text{-CD} > \beta\text{-CD} > \gamma\text{-CD}$ . The thresholds of toxicity were 1, 2.5, and 20 mM for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD, respectively (Fig. 1a). The toxicity effect followed the order  $\text{Me-}\alpha\text{-CD} \sim \text{Me-}\beta\text{-CD} > \text{Me-}\gamma\text{-CD}$  for the methylated CDs (2.5 mM for  $\text{Me-}\alpha\text{-CD}$  or  $\text{Me-}\beta\text{-CD}$  and 20 mM for  $\text{Me-}\gamma\text{-CD}$ ; Fig. 1b). HP- $\alpha$ -CD and HP- $\beta$ -CD were toxic at 2.5 mM, whereas HP- $\gamma$ -CD was not cytotoxic up to 50 mM (Fig. 1c). Finally, to summarize, for all CDs tested, the toxicity effect followed the order  $\alpha\text{-CD} > \text{Me-}\alpha\text{-CD} \sim \text{HP-}\alpha\text{-CD} \sim \beta\text{-CD} \sim \text{Me-}\beta\text{-CD} \sim \text{HP-}\beta\text{-CD} > \gamma\text{-CD} \sim \text{Me-}\gamma\text{-CD} > \text{HP-}\gamma\text{-CD}$ .

These results were confirmed by immunofluorescence stainings of occludin (a tight junction protein) after the BCECs had been incubated with or without CDs. For example, in Fig. 2, only the stainings with the native CDs were presented. Immunostaining of BCECs in control conditions revealed a reticular pattern at intercellular regions between all cells, indicating that the cells were sealed together by highly differentiated tight junctions (Fig. 2a). The other immunostainings have been realized in the presence of increasing concentrations of CD. No differences between control and BCECs in the presence of  $\alpha$ -,  $\beta$ -, or  $\gamma$ -CD at 0.5, 1, and 5 mM, respectively, were observed (Fig. 2, b, d, and f). When the

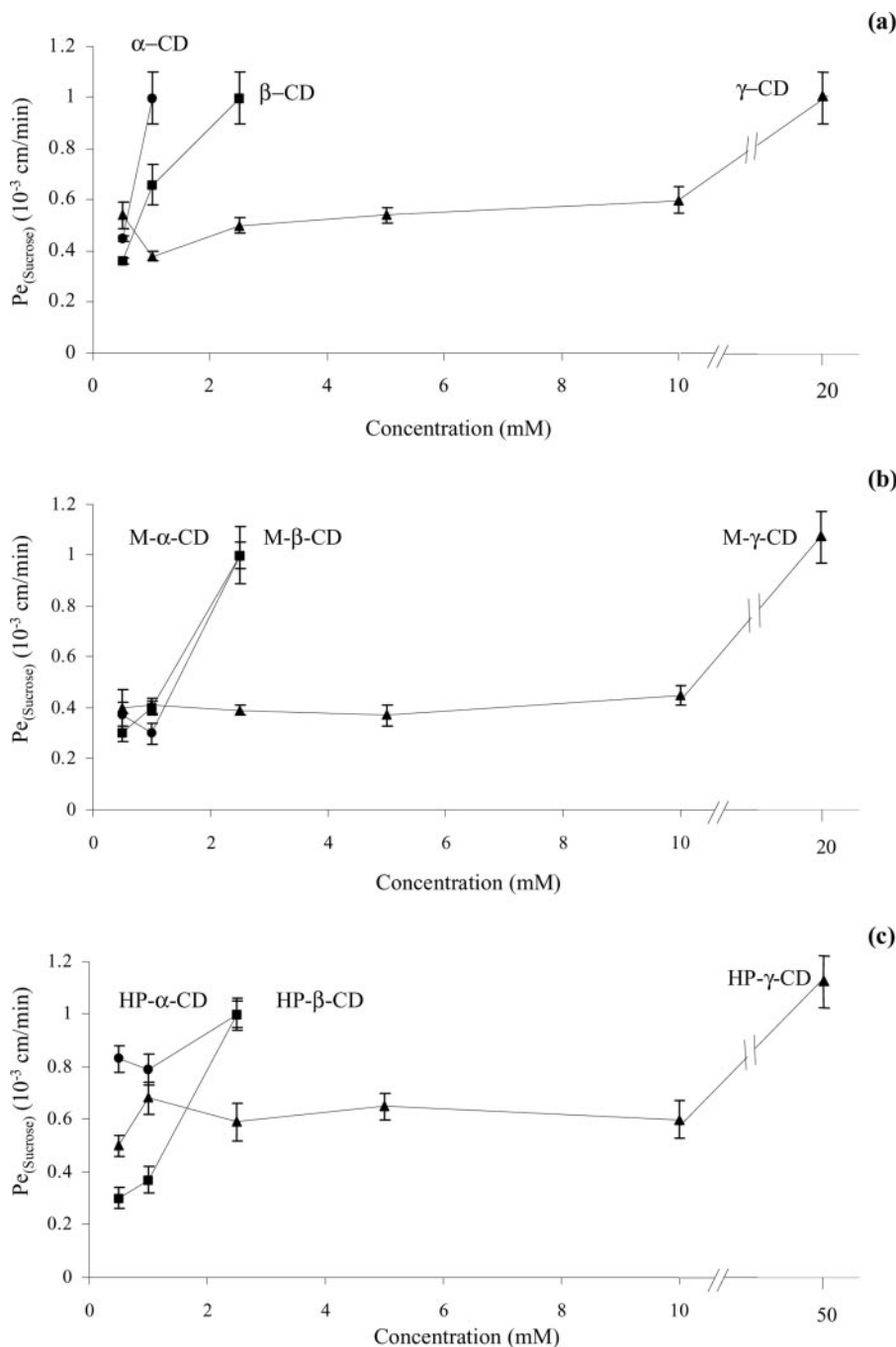
cells were in contact with  $\alpha$ -,  $\beta$ -, or  $\gamma$ -CD at 5, 5, and 50 mM, respectively, the occludin staining depicted disruptions and large gaps between cells (Fig. 2, c, e, and g). The results are in accordance with our sucrose permeability studies.

**Release of Membrane Components.** To gain an insight into the behavior of the CDs toward BCECs, we have examined whether the native CDs extracted lipids. Indeed, CDs are well known to release biological membrane components such as cholesterol or phospholipids (Irie and Uekama, 1997). Figure 3 represents the percentage of cholesterol extracted from endothelial cells by native CDs at various concentrations. Release of cholesterol from BCECs by  $\beta$ -CD was clearly dependent on CD concentration. At no toxic concentration for the BBB integrity (1 mM), the cholesterol efflux was equal to 12%, whereas at toxic concentrations (2.5 and 5 mM),  $\beta$ -CD extracted cholesterol up to 25 and 50%, respectively. In contrast, an increase in  $\alpha$ - and  $\gamma$ -CD concentration has a lower effect on the release of cholesterol. At toxic concentrations of  $\alpha$ -CD (5 mM) and  $\gamma$ -CD (50 mM), the efflux was 8 and 4 times less, respectively, compared with  $\beta$ -CD (5 mM). To further comprehend toxicity, release of phospholipids (phosphatidylcholine and sphingomyelin) was studied at toxic CD concentrations for the BBB integrity (5, 5, and 50 mM for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD, respectively). On the one hand,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CDs removed 63, 19, and 22% of phosphatidylcholine, respectively (Fig. 4, gray columns) of the BCECs. On the other hand, sphingomyelin (Fig. 4, black columns) was extracted at 64, 63, and 21% by  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD, respectively. Thus, the capacity of these CDs to extract these two phospholipids follows the order  $\alpha > \beta > \gamma$ -CD.

**Transport Studies of Cyclodextrins.** Because the permeability of the CDs through the BBB involves controversies, transport studies of CDs were performed on our model of BBB. To study the luminal-to-abluminal transport, CD was deposited on the luminal chamber of the coculture system. The concentrations used were lower than the limit of toxicity: 0.5 mM for  $\alpha$ -CD and 1 mM for  $\beta$ - and  $\gamma$ -CD, respectively. The percentage of transport was determined for each CD and is represented in Table 1. The value obtained depends on the nature of CD varying from 6.6 (HP- $\beta$ -CD at 1 mM) to 26.7% ( $\beta$ -CD at 1 mM). The methylation or hydroxypropylation did not modify the passage for the  $\alpha$ -CD (21.5 versus 20.4 and 16.5% for HP- $\alpha$ - and Me- $\alpha$ -CD, respectively), decreased it for the  $\beta$ -CD (26.7 versus 9.3 and 6.6% for HP- $\beta$ - and Me- $\beta$ -CD, respectively), and increased it for the  $\gamma$ -CD (11.1 versus 18.1 and 20.1% for HP- $\gamma$ - and Me- $\gamma$ -CD, respectively). The effect of the modification seems unforeseeable.

## Discussion

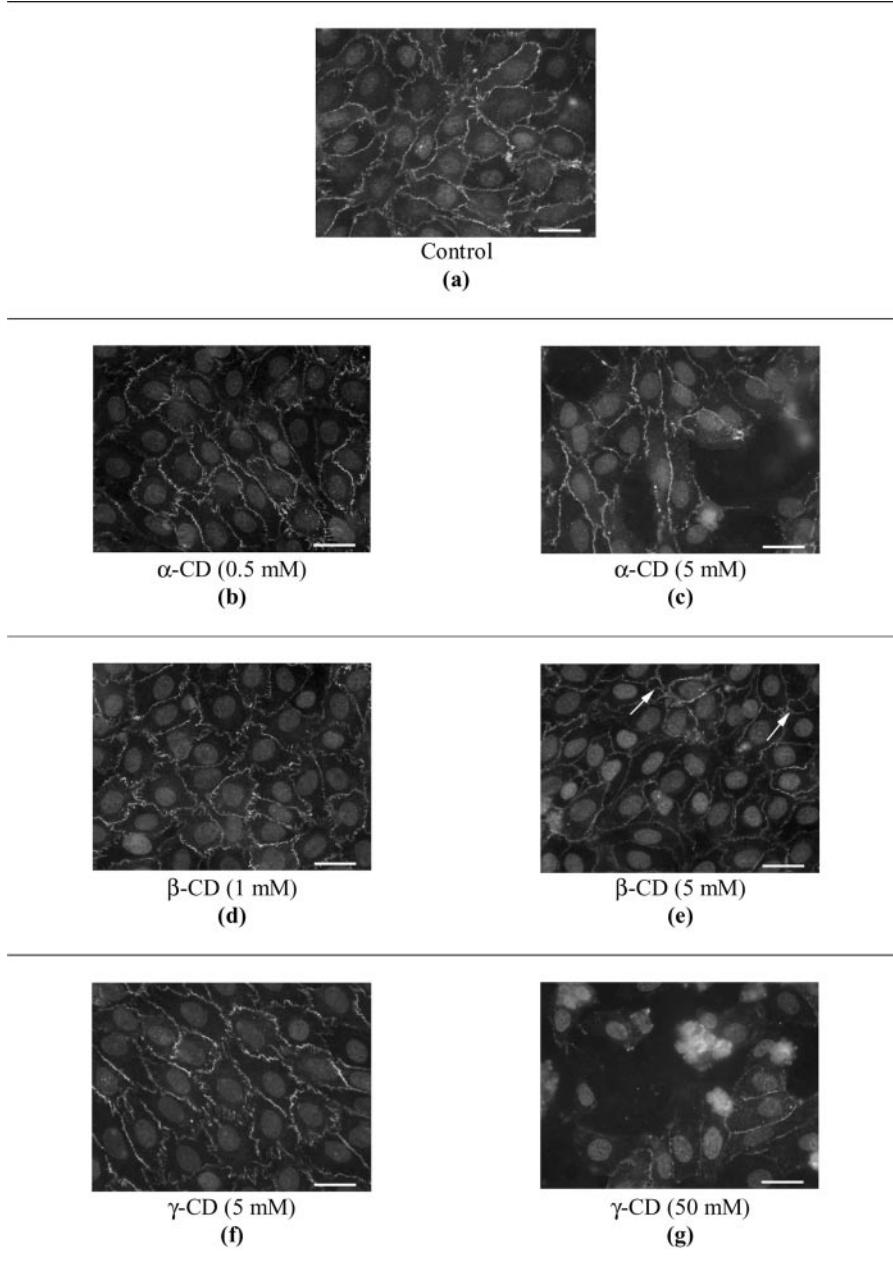
In the present work, we have summarized the toxicity and endothelial permeability for CDs on the BBB. The threshold of toxicity for each CD has been determined. The  $\alpha$ -CD series is the most toxic and is followed by the  $\beta$ -CD series, whereas the  $\gamma$ -CD series presents the lowest toxicities. The modified  $\alpha$ -CDs showed reduced toxicity compared with their natural parent, and the modification did not change the threshold of toxicity for the  $\beta$ -CD series. Finally, only the hydroxypropylation decreased the toxicity by a factor of two for the  $\gamma$ -CD. Leroy-Lechat et al. (1994) have also described a marked reduced toxicity for HP- $\gamma$ -CD compared with  $\gamma$ -CD toward P388 murine leukemic cells.



**Fig. 1.** Effect of native (a), methylated (b), and hydroxypropylated (c)  $\alpha$ -CD (●),  $\beta$ -CD (■), and  $\gamma$ -CD (▲) on the endothelial permeability coefficient for sucrose after 2 h of incubation. Transport studies were conducted at 37°C in buffered Ringer's solution at pH 7.4. Each point is the mean of three different filters and representative of three series of independent experiments.

To confirm the breakdown of the BBB, BCECs were stained for occludin to visualize the tight junctions. Occludin is a transmembrane component and forms tight junction strands. The results obtained are in accordance with the endothelial permeability for sucrose. The cortical repartition of occludin for an immunostaining realized at no toxic conditions [ $Pe_{(sucrose)} < 1 \times 10^{-3}$  cm min<sup>-1</sup>] showed that the integrity of BCEC monolayer is preserved. CD concentrations equal to 10, 2, and 2 times the threshold of toxicity for the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD, respectively, have been chosen to perform the immunostainings in toxic conditions [ $Pe_{(sucrose)} > 1 \times 10^{-3}$  cm min<sup>-1</sup>]. In these conditions, large gaps are observed between the cells corresponding to the disruption of the tight junctions. This breakdown of the BBB leads to an increase in the permeability for sucrose.

To determine the behavior of CDs on the BCECs, studies of lipid effluxes have been performed with the native CDs. Cholesterol efflux shows that  $\beta$ -CD, at various concentrations, is the most powerful cholesterol extractor compared with  $\alpha$ - and  $\gamma$ -CD. In contrast, it must be pointed out that, although the  $\alpha$ -CD is the most toxic among the three native CDs, only a slight efflux of cholesterol is observed. So, phospholipid effluxes (phosphatidylcholine and sphingomyelin) were investigated.  $\alpha$ -CD led to an important efflux of the two phospholipids, whereas  $\beta$ -CD preferentially extracted sphingomyelin.  $\gamma$ -CD induced only a small efflux of each. Similar investigations have been performed on human erythrocytes (Ohtani et al., 1989) or intestinal membrane (Nakanishi et al., 1992). These experiments suggested that (1) the acyl chain of phospholipids fits tightly into the hydrophobic cavity

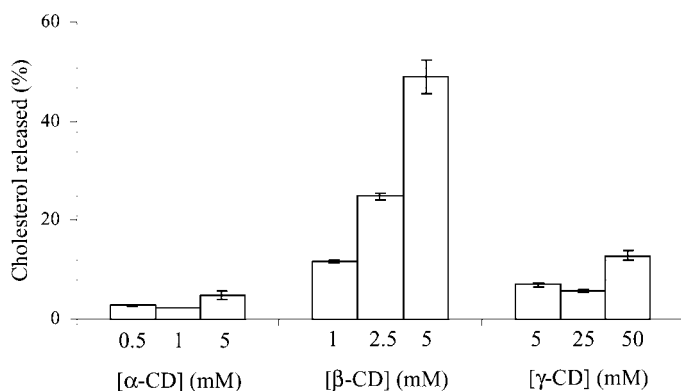


**Fig. 2.** Occludin immunofluorescent staining of BCECs after 2 h of incubation without CD (a) and with  $\alpha$ -CD at 0.5 (b) and 5 mM (c),  $\beta$ -CD at 1 (d) and 5 mM (e), and  $\gamma$ -CD at 5 (f) and 50 mM (g). DNA was stained using Hoescht reagent. Bar = 25  $\mu$ m.

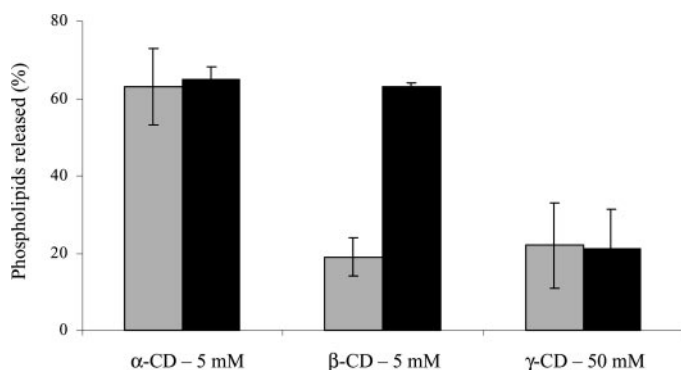
of the  $\alpha$ -CD and more loosely into the larger inner space of  $\beta$ - and  $\gamma$ -CD, (2) the side chain of cholesterol is preferably included in the  $\beta$ -CD cavity, and (3)  $\gamma$ -CD has the least lipid selectivity. It was also reported that the removal of cholesterol by CDs from cells may result in an increase in membrane fluidity, which would induce the membrane invagination through a loss of the bending resistance and consequently lead to cell lysis (Noji et al., 1982). In addition, other studies have shown that CDs removed phospholipids, especially phosphatidylcholine and sphingomyelin, from the outer half of the membrane bilayer, leading to an imbalance of the bilayer (Miyajima et al., 1987). The ability of Me- $\beta$ -CD to remove phospholipids, especially sphingomyelin, from a supported bilayer was also clearly demonstrated by atomic force microscopy experiments (Giocondi et al., 2004). The authors also concluded that the interpretation of Me- $\beta$ -CD effects on cell membrane only in terms of cholesterol move-

ments have to be treated with caution. Interestingly, Leppimäki et al. (1998) reported that the depletion of cholesterol by HP- $\beta$ -CD stimulates sphingomyelin synthesis in cultured human skin fibroblasts. Finally, it was also proposed that the release of membrane lipids in neuronal cells by Me- $\beta$ -CD leads to a deep reorganization of the overall structure of the cell membrane, eventually leading to cell death (Ottico et al., 2003). These data are thoroughly consistent with our results on the BCECs and suggest that the toxicity of CD toward the BBB is due to the different efflux of membrane components and/or reorganization of the membrane structure.

Another interesting point to discuss is the threshold of toxicity of the CDs on our model of BBB compared with other cellular types. Ohtani et al. (1989) reported that CDs induce hemolysis of human erythrocytes in the order  $\beta$ - >  $\alpha$ - >  $\gamma$ -CD. Leroy-Lechat et al. (1994) also reported the same order of cytotoxicity of CDs toward P388 murine leukemic cells. No



**Fig. 3.** Cholesterol released from BCECs after 2 h of incubation in the presence of various concentrations of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CDs. The quantity of [ $^3$ H]cholesterol in the BCECs for the control was equal to  $55400 \pm 2280$  dpm/well. Results are expressed as a percentage of cholesterol released from BCECs compared with the control. Each percentage is the mean of three different filters and representative of two series of independent experiments.



**Fig. 4.** Phosphatidylcholine (gray columns) and sphingomyelin (black columns) released from BCECs after 2 h of incubation in the presence of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CDs at 5, 5, and 50 mM, respectively. The quantity of [ $^3$ H]phosphatidylcholine and [ $^3$ H]sphingomyelin in the BCECs for the control was equal to  $9500 \pm 707$  dpm/well and  $4140 \pm 16$  dpm/well, respectively. Results are expressed as a percentage of phospholipids released from BCECs compared with the control. Each percentage is the mean of three different filters and representative of two series of independent experiments.

TABLE 1

Percentage of transport for native, methylated, and hydroxypropylated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CDs through the BBB after 2 h of incubation

All transport studies were conducted at 37°C in buffered Ringer's solution at pH 7.4. Results are expressed as a percentage of transport across the BCEC monolayer alone and were obtained from the transport across the inserts coated with collagen and seeded with BCECs and the transport across the inserts coated only with collagen. Each point is the mean of three different filters and representative of three series of independent experiments.

Nature	Concentration	Transport across the BBB	
		mM	%
$\alpha$ -CD	0.5		$21.5 \pm 1.1$
Me- $\alpha$ -CD	1		$20.4 \pm 2.2$
HP- $\alpha$ -CD	1		$16.5 \pm 2.7$
$\beta$ -CD	1		$26.7 \pm 1.2$
Me- $\beta$ -CD	1		$9.3 \pm 0.5$
HP- $\beta$ -CD	1		$6.6 \pm 0.1$
$\gamma$ -CD	1		$11.1 \pm 0.5$
Me- $\gamma$ -CD	1		$18.1 \pm 1.9$
HP- $\gamma$ -CD	1		$20.1 \pm 2$

toxic effect toward Caco-2 cells has been observed for  $\beta$ - and  $\gamma$ -CD (at 15 and 150 mM, respectively), whereas  $\alpha$ -CD started to decrease the viability of Caco-2 cells at 25 mM (Ono et al., 2001). Thus, the intensity and degree of toxicity

of CDs differ between the cell type, probably due to the difference in the membrane composition of the cells. In particular, these last data show that the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD are at least 25, 6, and 7 times more toxic for the BCECs compared with Caco-2 cells. Indeed, we have found that the native CDs were toxic for BCECs at 1, 2.5, and 20 mM for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD, respectively. These results and previous works (Lundquist et al., 2002) performed in our laboratory strongly reinforce the fact that all predictions in terms of toxicity or permeability for a drug toward BBB from a Caco-2 model (largely used in pharmaceutical industry) must be proscribed.

Finally, the percentage of passage of each CD was evaluated for concentrations where no toxic effect was observed. The  $\alpha$ -CD series derivatives showed the highest percentage, and the chemical modification did not markedly modify the passage across the BBB. Contradictorily, the modification decreased the percentage for the  $\beta$ -CDs and increased it for the  $\gamma$ -CDs. So, the effect of chemical modification is not foreseeable; for example, methylation leads to a relative invariability in the percentage of passage for the  $\alpha$ -CD, a decrease for the  $\beta$ -CD, and an increase for the  $\gamma$ -CD. No structure/permeability relationship has been observed according to the nature and chemical modifications of CDs. Concerning the percentage obtained for all the CDs, the highest values are situated at about 20%. For example, caffeine or nicotine, known to easily cross the BBB, show a percentage of 96% in our in vitro model. But, for vincristine, an antitumoral drug crossing the BBB with difficulty, the percentage is only 8.5% (unpublished results). Starting from the preceding percentages, we can conclude that some CDs cross the BBB slightly in normal conditions. It is worth mentioning that a similar conclusion was made on other membrane types. Indeed,  $\beta$ -CD and its methylated derivatives pass slowly through the intestinal wall by passive diffusion (Takakura and Hashida, 1996), or HP- $\beta$ -CD is poorly absorbed transdermally (Tanaka et al., 1995).

In conclusion, we have determined the threshold of toxicity for the integrity of the BBB for each CD studied. This loss of integrity in the presence of CDs seems to be due to the release of membrane components, as shown by lipid effluxes. The permeability of each CD through the BBB has been evaluated, and no structure/permeability relationship has been observed. These results explain the contradictory data of the literature, since the permeability is strongly dependent of the nature and/or chemical modification of the CD. More amphiphilic CDs, such as cholesteryl (Auzely-Velty et al., 1999) or phospholipid (Moutard et al., 2002) CDs, could be good candidates to cross the BBB more efficiently. Works are currently in progress to confirm this assumption.

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