Direct Evidence that Polysorbate-80-Coated Poly(Butylcyanoacrylate) Nanoparticles Deliver Drugs to the CNS via Specific Mechanisms Requiring Prior Binding of Drug to the Nanoparticles

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Purpose. It has recently been suggested that the poly(butylcyanoacrylate) (PBCA) nanoparticle drug delivery system has a generalized toxic effect on the blood–brain barrier (BBB) (8) and that this effect forms the basis of an apparent enhanced drug delivery to the brain. The purpose of this study is to explore more fully the mechanism by which PBCA nanoparticles can deliver drugs to the brain.

Methods. Both *in vivo* and *in vitro* methods have been applied to examine the possible toxic effects of PBCA nanoparticles and polysorbate-80 on cerebral endothelial cells. Human, bovine, and rat models have been used in this study.

Results. In bovine primary cerebral endothelial cells, nontoxic levels of PBCA particles and polysorbate-80 did not increase paracellular transport of sucrose and inulin in the monolayers. Electron microscopic studies confirm cell viability. *In vivo* studies using the antinociceptive opioid peptide dalargin showed that both empty PBCA nanoparticles and polysorbate-80 did not allow dalargin to enter the brain in quantities sufficient to cause antinociception. Only dalargin preadsorbed to PBCA nanoparticles was able to induce an antinociceptive effect in the animals.

Conclusion. At concentrations of PBCA nanoparticles and polysorbate-80 that achieve significant drug delivery to the brain, there is little *in vivo* or *in vitro* evidence to suggest that a generalized toxic effect on the BBB is the primary mechanism for drug delivery to the brain. The fact that dalargin has to be preadsorbed onto nanoparticles before it is effective in inducing antinociception suggests specific mechanisms of delivery to the CNS rather than a simple disruption of the BBB allowing a diffusional drug entry.

KEY WORDS: CNS drug delivery; blood–brain barrier; nanoparticles; poly(butylcyanoacrylate).

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INTRODUCTION

A number of studies have shown that drug delivery to the brain is considerably enhanced by prior binding of a drug to poly(butyl cyanoacrylate) nanoparticles (PBCA nanoparticles) overcoated with polysorbate-80, followed by intravenous injection (1–5). The presence of the blood–brain barrier (BBB) prevents the entry of many drugs and blood-borne substances into the CNS (6). The BBB consists of a physical barrier of tight junctions between the cerebral endothelial cells that abolishes all aqueous paracellular diffusional pathways, a biochemical barrier consisting of enzymes that specifically metabolize many drugs, and the presence of specific efflux mechanisms (P-glycoprotein and multidrug resistance protein) that transport many of the more penetrant lipophilic compounds across the BBB and out of the CNS (6,7).

The mechanism of drug transport enhancement mediated by nanoparticles into the brain, however, is still not fully understood. Recently, Olivier *et al.* (8) suggested that the PBCA nanoparticle delivery system had a generalized toxic effect on the BBB as a result of breakdown of the PBCA nanoparticles by ubiquitous esterases, thus allowing the brain entry of drugs simultaneously intravenously injected with the particles. In addition, they also suggested that the nanoparticles might secondarily have the effect of nonspecifically opening the tight junctions between endothelial cells in the brain microvasculature, thus creating a paracellular pathway through the BBB. They base their arguments to a large extent on observations in an *in vitro* model of the BBB consisting of a coculture of bovine brain endothelial cells and rat astrocytes.

If a mechanism of general toxicity involving an opening of the BBB were the major facilitator of drug entry, then prior binding of drug to the nanoparticles would not be necessary, as the drug would have free diffusional access to the brain if the circumferential tight junctions were opened. In this case, administration of free drug together with or shortly after injection of nanoparticles should lead to similar central nervous effects as does prior binding. In order to test this hypothesis *in vivo,* we injected free dalargin alone intravenously into mice or 5 and 30 min after the injection of polysorbate-80-coated empty nanoparticles. Dalargin, an opioid analgesic, does not penetrate into the brain via the BBB and thus does not exert an antinociceptive effect after intravenous administration of the peptide alone (1,9). However previous studies have shown that, after prior binding of dalargin to polysorbate-80-coated nanoparticles, a highly significant antinociceptive effect is evident, as tested with the tail-flick or hot-plate test in mice (1,10).

MATERIALS AND METHODS

Preparation of Poly(Butyl Cyanoacrylate) Nanoparticles

Poly(butyl cyanoacrylate) (PBCA) nanoparticles were produced as described before (1). Specifically, 1% (v/v) butyl cyanoacrylate monomer (Sichelwerke, Hannover, Germany)

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was added slowly dropwise to a 1% (w/v) solution of dextran 70,000 (Fluka, Buchs, Switzerland) in 0.01 N hydrochloric acid (E. Merck, Darmstadt, Germany). This mixture was stirred for 4 h with a magnetic stirrer to promote polymerization. After this time the polymerization was completed by neutralization of the mixture with 1 N sodium hydroxide (E. Merck, Darmstadt, Germany). After filtration through a G3 glass filter (Schott AG, Mainz, Germany), the particles were separated from residual monomers by threefold centrifugation (L8-60M, Beckman, Hannover, Germany) at 90,000 *g* for 1 h and washing with distilled water after resuspension by ultrasonication. The particle diameters were determined by dynamic light scattering (photon correlation spectroscopy, PCS) using a digital correlator BI-2030 (Brookhaven Instruments Corporation, Holtsville, New York). An average diameter of 300 nm was found with a polydispersity index of 0.177 before lyophilization. The particles were stored after addition of an equal weight of D(+)-glucose to the preparation (Fluka, Buchs, Switzerland) and lyophilization in a LYOVAC GT2 (Leybold AG, Köln, Germany).

Polysorbate-80-coated nanoparticles were prepared by the addition of 1% (v/v) polysorbate-80 (ICI Chemikalien, Essen, Germany) to the reconstituted nanoparticles and stirred at 400 rpm for 30 min.

Dalargin Nanoparticles

Dalargin-loaded polysorbate-80-coated nanoparticles were prepared as previously described (1). Lyophilized PBCA nanoparticles were resuspended in phosphate-buffered saline (PBS) under constant stirring. The concentration of PBCA nanoparticles was 20 mg/ml. After this, 0.75 mg/ml dalargin was added, and the mixture was stirred for 4 h. Then (relative to the total suspension volume) polysorbate-80 was added to give a final solution of 1% polysorbate-80, and the mixture was incubated for 30 min.

Human and Bovine Brain Capillary Endothelial Cells

The isolation of human and bovine capillary endothelial cells was performed as described by Ramge *et al.* (12). Endothelial cells were isolated from human brain tissue recovered at autopsy, and bovine brain tissue was obtained from the slaughterhouse as previously described (13). Briefly, the tissue samples were chopped into 1- to 2-mm pieces with a sterile scalpel and then incubated for 30–45 min at 37°C in PBS containing 0.0375% collagenase type II (Biochrom, Berlin, Germany). After centrifugation of the homogenized tissue through a 25% BSA (Merck, Darmstadt, Germany) gradient in PBS, the pellet was removed and digested with 0.1% collagenase/dispase (Boehringer Ingelheim, Germany) and 1% bovine serum albumin in PBS, followed by a 1- to 2-min incubation with 1 μg/ml DNase (Boehringer Ingelheim). This mixture was centrifuged through a percoll gradient, and the part of the gradient containing the microvessels was collected (density 1.065 to 1.055 g/ml), washed twice, and resuspended in Dulbecco's modified Eagle medium DMEM (BioWhittaker, Verviers, Belgium). The medium contained 4 nM L-glutamine (Biochrom), 1× MEM nonessential amino acids (Biochrom), 100 U/ml penicillin (Biochrom), 100 μg/ml streptomycin (Biochrom), and 1% retinal factor (12). Fifteen percent fetal calf serum was added for the culture of bovine endothelial cells, and human cells were cultured in medium containing 15% pooled human serum plus 75 μg/ml endothelial cell growth supplement (ECGS) (Sigma, Deisenhofen, Germany). Cells were plated onto collagen-coated (Collagen G, Biochrom) plastic cell culture plates (Costar, Corning, Corning, New York). Cultures were incubated at 37°C in 5% CO_2. The medium was replaced every 2–3 days. Confluent monolayers of endothelial cells that have grown out from capillary fragments are obtained after 5 to 10 days of culture. Cells were passaged with trypsin onto collagen G–coated SlideFlasks® (Nunc, Wiesbaden, Germany). The cells were used for studies when confluence was reached.

Characterization of Cells

Endothelial cell characterization was carried out, as described by Ramge *et al.* (12), by immunocytochemical staining with a polyclonal antibody for von-Willebrand factor (anti-von-Willebrand factor supplied by DAKO, Glostrup, Denmark), anti–glial fibrillary acid protein (GFAP)-specific monoclonal antibody (DAKO), and anti–LDL-receptor (Dianova, Hamburg, Germany). All antibodies reacted with human and bovine material. For immunocytochemistry cells were grown until confluent and fixed with cold paraformaldehyde (4%) (Merck, Darmstadt, Germany) for 15 min. Blocking reagents, secondary antibody (biotinylated goat antimouse IgG), and enzyme–avidin complex were part of the Vectastain ABC mouse IgG-Kit; substrate for the enzyme was AEC (Vector Laboratories, Petersborough, UK). Cells stained for GFAP were fixed with acetone. Primary antibodies used were of murine origin and were incubated for 45 min at 37°C. Biotinylated *Ulex europaeus* agglutinin (UEA)-1 purchased from DAKO was incubated under identical conditions. Murine IgG1 antibody was used as negative control (isotype control). A fibroblastoid cell line (MRC-5) was used in parallel experiments as negative control.

In Vitro Blood-Brain Barrier Model

Bovine Brain Capillary Endothelial Cells (BBCE)

Endothelial cells were isolated as described by Méresse *et al.* (14). The use of selected and subcloned endothelial cells allowed a pure endothelial cell population to be obtained without contamination by pericytes. The cells were cultured in gelatin-coated dishes in the presence of DMEM (glucose concentration 1 g/l) supplemented with 10% (v/v) heat-inactivated newborn calf serum (CS) and 10% (v/v) horse serum (HS) (Life Technologies, Karlsruhe, Germany), 2 mM glutamine, 50 μg/ml gentamycin, and 1 ng/ml basic fibroblast growth factor (bFGF, Sigma, Deisenhofen, Germany).

Rat Astrocytes

Primary cultures of mixed astrocytes were prepared from newborn rat cerebral cortex. After removal of the meninges, the brain tissue was forced gently through a nylon sieve, as described by Boother and Sensenbrenner (15). Astrocytes were plated on six-well plates (Greiner, Frickenhausen, Ger-

many) at a concentration of 1.2×10^5 cells/ml in 2 ml of DMEM (glucose concentration 1 g/l) supplemented with 10% (v/v) fetal calf serum (PAN, Nürnberg, Germany), 2 mM glutamine, and 50 μg/ml gentamycin; the medium was changed twice a week. Three weeks after seeding, the purity of the astrocyte cultures was confirmed by immunofluorescent staining for glial fibrillary acidic protein (GFAP). More than 95% of the astrocyte population was found to be GFAP positive.

Preparation of Filters for Coculture

Culture plate inserts (Millicell® CM, 0.4 μm, 30 mm diameter, Millipore, Eschborn, Germany) were coated on the upper side with rat tail collagen. The following solution was prepared on ice: 1 part 0.34 N NaOH, 2 parts 10 × nonbuffered DMEM, and 9 parts collagen solution (rat tail collagen dissolved in 0.1% acetic acid); 150 μl of this solution was spread evenly over each insert. After 1 h of incubation at 37°C, the collagen gel was washed with H_2O and PBS.

Coculture of BBCE Cells and Rat Astrocytes in a Two-Chamber System

Cultures of astrocytes were prepared as described above. After 3 weeks, collagen-coated inserts were set in the six-well plates containing the astrocytes, and BBCE cells were plated on the upper side of the inserts at a concentration of 4×10^5 cells/ml. The coculture medium was DMEM (glucose concentration 1 g/l) supplemented with 10% (v/v) CS, 10% (v/v) HS, 2 mM glutamine, 50 μg/ml gentamycin, and 1 ng/ml basic fibroblast growth factor (bFGF, Sigma, Deisenhofen, Germany). The medium was changed every other day. Within 3 to 4 days, BBCE cells formed a confluent monolayer. The transendothelial transport studies were performed after 10 days of coculture. BBCE cells were used from passage 3 to 7 (split ratio 1/15).

Transendothelial Permeability Studies

In order to study the influence of nanoparticles on the BBB permeability in this *in vitro* BBB model, culture plate inserts with or without BBCE cells were transferred after 10 days of coculture into six-well plates containing 2 ml of the fresh coculture medium (lower compartment); 2 ml of the coculture medium was supplemented with uncoated or polysorbate-80-coated PBCA nanoparticles (10–20 μg/ml). For permeability measurements, the radiolabeled tracers [^{14}C]sucrose (0.05 μCi/ml) and [^{3}H]inulin (0.05 μCi/ml) were added, and this supplemented medium was applied in the upper compartment. Every 15 min the inserts were transferred into new six-well plates containing 2 ml coculture medium. The permeability assay was performed in triplicate at 37°C. For each time point aliquots were taken from the lower compartments, and radioactivity was determined with a β-scintillation analyzer (Canberra Packard, Frankfurt-am-Main, Germany).

The diffusion of sucrose and inulin across the BBCE monolayer was expressed as a clearance according to the method of Siflinger-Birnboim *et al.* (16) by dividing the accumulated amounts of the substances detected in the lower compartment by the initial concentrations of these substances in the upper compartment. The mean cumulative cleared volumes were plotted against time, which under constant conditions gives a linear relationship. The slope of the curve calculated by linear regression analysis corresponds to the clearance (ml/min) of the tracer substance.

Electron Microscopy

Following 10 days of coculture, culture plate inserts with BBCE cells were further incubated with coculture medium in which in the upper compartment of the two-chamber system was supplemented with nanoparticles with and without polysorbate-80 at a concentration of 10 μg/ml. After 60 min of incubation at 37°C, cells were fixed with 2.5% glutaraldehyde in PBS for 2 h, washed with 0.1 M cacodylate buffer, and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer. Specimens were dehydrated in a series of ethanol. The 70% ethanol step was saturated with uranyl acetate for contrast enhancement. Dehydration was completed in propylene oxide, and specimens were embedded in araldite (Serva, Heidelberg, Germany). Ultrathin sections were mounted on pioloform-coated copper grids and contrasted with lead citrate. Specimens were observed and documented with an EM 10A electron microscope (Zeiss, Oberkochen, Germany).

Animal Testing

Male ICR mice 20–22 g were kept at an ambient temperature of 22 ± 3°C with a 12 h light and 12 h dark cycle (light at 6.00 h). Water and standard laboratory feed were freely available. All animal experiments were conducted in accordance with institutional guidelines and were approved by local ethics authorities.

The mice were divided into six groups. Group 1 was injected, 200 μl into the tail vein, with a suspension of empty PBCA nanoparticles. Group 2 was injected, 200 μl, with dalargin (7.5 mg/kg) solution. Group 3 was injected, 200 μl, with polysorbate-80-coated dalargin-loaded nanoparticles (dose of dalargin 7.5 mg/kg). Group 4 was injected, 200 μl, with mixture of polysorbate-loaded PBCA nanoparticles and dalargin solution (7.5 mg/kg). Animals in groups 5 and 6 were injected twice. The first injection consisted of polysorbate-loaded PBCA nanoparticles, and these were injected into animals of both of these groups. For the second injection, animals of group 5 received dalargin solution (7.5 mg/kg) 5 min after the first injection, and animals of group 6 received dalargin 30 min after the first injection.

The nociceptive threshold was measured by the "tail flick test" using a Tail Flick Analgesia Meter (Model 33, Iitic Inc.,Woodland Hills, CA). Antinociceptive procedures were carried out according to a previously published protocol (1). Tail-flick latency was tested 15, 30, 45, and 60 min after injection.

Six animals per group were used. Tail flick response latencies are presented as a mean ± standard deviation. Response latencies were converted to percent maximal possible effect (MPE) using Eq. (1):

$$\%MPE = \frac{post.drug.latency - pre.drug.latency}{cut.off.time - pre.drug.latency} \cdot 100 \quad (1)$$

RESULTS

BBB Permeability Studies *in Vitro*

The influence of PBCA nanoparticles on BBB permeability was studied in an *in vitro* BBB model consisting of BBCE cells and rat astrocytes (17). The coculture of these two cell types (11) for 10 days was shown to establish a permeability barrier across the endothelial monolayer as measured by sucrose and inulin permeability. Permeability in the *in vitro* BBB model was evaluated by measuring the flux of the radioactively labeled extracellular markers [^{14}C]sucrose and [^{3}H]inulin. These substances normally cross a cell monolayer by the paracellular route. When uncoated and polysorbate-80-coated PBCA nanoparticles at concentrations of 10 or 20 μg/ml were coincubated for 1 h, the permeability of [^{14}C]sucrose and [^{3}H]inulin did not change significantly in comparison to the control untreated cells (Figs. 1 and 2). The sucrose and inulin permeabilities recorded for these cultures are similar to those found in other studies (18,19; see Discussion).

Viability of the BBCE cells in the presence of nanoparticles was further examined by electron microscopy. Therefore BBCE cells were incubated with uncoated and polysorbate-80-coated PBCA nanoparticles for 1 h and fixed for electron microscopy. In contrast to untreated control cells (Fig. 3A), after incubation with polysorbate-80-coated PBCA nanoparticles, BBCE cells showed a number of protrusions of their luminal plasma membrane (Fig. 3C). These changes could not be detected to the same degree with uncoated PBCA nanoparticles (Fig 3B). Furthermore, we observed that a wide electron-dense layer, probably a thick filament network, was located at the luminal plasma membrane when BBCE cells were incubated with polysorbate-80-coated PBCA nanoparticles. These observations indicate that the polysorbate-80-coated PBCA nanoparticles are inducing changes at the cell membranes. However, the cell morphology, the number of visible intracellular endocytic vesicles, and the structure of the cell-to-cell contacts (tight junctions) were all normal and of unchanged appearance, demonstrating that the PBCA nanoparticles had exerted no generalized toxic effect on the BBCE cells.

In Vivo Studies

The results of the antinociceptive testing following intravenous injection of free dalargin mixed with empty polysorbate-80-coated PBCA nanoparticles and immediately injected or free dalargin injected 5 and 30 min after the injection of empty polysorbate-80-coated PBCA nanoparticles clearly demonstrate that free dalargin does not penetrate into the brain in amounts that are able to induce an antinociceptive effect (Table I). The maximum possible effects (%MPE) of these treatments were identical to those of a dalargin solution or empty uncoated nanoparticles alone. In contrast, dalargin bound to nanoparticles prior to polysorbate-80-coating and then injected i.v. exhibited a strong and statistically significant antinociceptive effect, indicating that prior binding of dalargin to the nanoparticles is necessary for a delivery to the brain.

DISCUSSION

This study provides additional *in vivo* and *in vitro* evidence that PBCA nanoparticles loaded with dalargin and overcoated with polysorbate-80 can deliver pharmacologically significant amounts of the hexapeptide to the CNS in quantities sufficient to produce a statistically significant analgesic effect. These results support previous studies (1–5) that

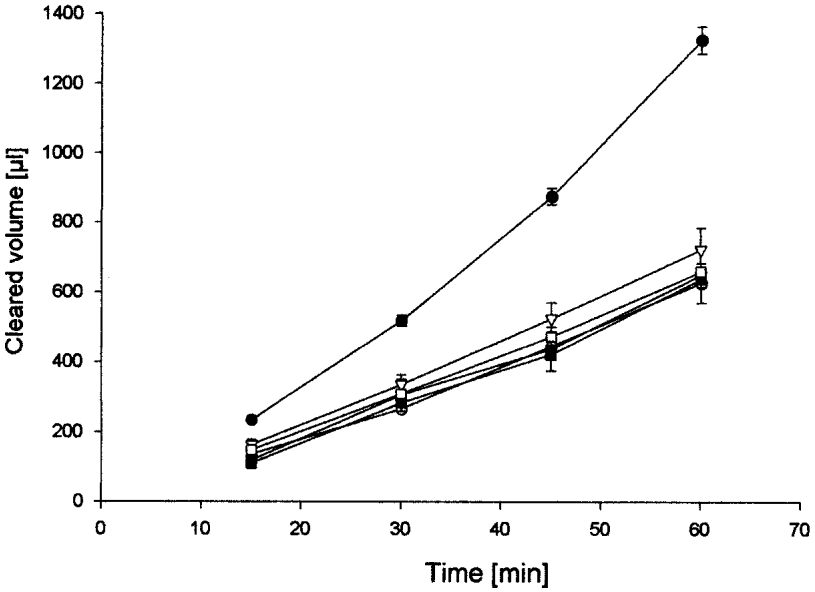

Fig. 1. Sucrose permeability in the coculture model after incubation with poly(butyl cyanoacrylate) nanoparticle preparations or controls. ●, Filter holder without cells; ○, brain endothelial cell coculture with astrocytes without addition of nanoparticles; ▽, addition of 10 μl/ml poly(butyl cyanoacrylate) nanoparticles; ▼, addition of 20 μl/ml poly(butyl cyanoacrylate) nanoparticles; □, addition of 10 μl/ml poly(butyl cyanoacrylate) nanoparticles overcoated with polysorbate-80; ■, addition of 20 μl/ml poly(butyl cyanoacrylate) nanoparticles overcoated with polysorbate-80.

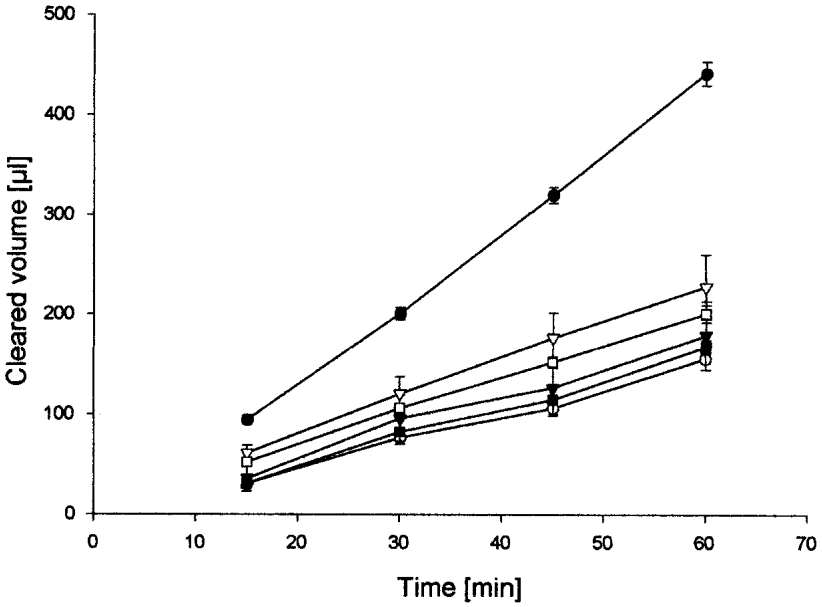

Fig. 2. Inulin permeability in the coculture model after incubation with poly(butyl cyanoacrylate) nanoparticle preparations or controls. ●, Filter holder without cells; ○, brain endothelial cell coculture with astrocytes without addition of nanoparticles; ▽, addition of 10 μl/ml poly(butyl cyanoacrylate) nanoparticles; ▼, addition of 20 μl/ml poly(butyl cyanoacrylate) nanoparticles; □, addition of 10 μl/ml poly(butyl cyanoacrylate) nanoparticles overcoated with polysorbate-80; ■, addition of 20 μl/ml poly(butyl cyanoacrylate) nanoparticles overcoated with polysorbate-80.

have demonstrated the delivery of a number of drugs to the CNS using this nanoparticle delivery system.

The results of the present study do not support the recent suggestion that PBCA nanoparticles deliver drugs to the brain by a nonspecific disruption of the BBB either by opening of tight junctions or by general toxic effects on the BBB endothelium as a result of nanoparticle degradation (8). In addition, electron micrographs prepared after 60 min of incubation of uncoated and polysorbate-80-coated nanoparticles in an *in vitro* model of the BBB showed a normal morphology of the endothelial cells, again emphasizing a lack of toxicity. Moreover, the permeability to the extracellular markers sucrose and inulin of the endothelial monolayer in the same system (11,14,17) was not significantly changed compared to that of controls in the presence of 10 or 20 μg/ml of PBCA nanoparticles with and without polysorbate-80, indicating no facilitation of the paracellular route by disruption of tight junctions. The bovine endothelial cells appear intact, with no evidence of open paracellular pathways. The lateral cell membranes remain opposed, and junctional complexes can be clearly seen. Thus, a nonspecific disruptive or toxic effect at these concentrations of nanoparticles or polysorbate-80 is not found. Also, the *in vivo* experiments in mice clearly demonstrated that an analgesic effect of dalargin is obtained only when the drug is preadsorbed to the PBCA nanoparticles (Table I), whereas a mixture of dalargin and PBCA nanoparticles did not show any analgesic effect. Thus, we obtained no evidence that the nanoparticles and/or the polysorbate-80, singly or in combination at the concentrations tested, are nonspecifically opening the BBB, either by a disruption of the tight junctions or by generalized damage to the endothelium, thus allowing free dalargin in solution to enter the brain and thereby to induce the observed antinociceptive effect.

Our earlier studies have shown small effects on brain intravascular inulin space in a rat *in vivo* model, where pretreatment of the animals with polysorbate-80-coated nanoparticles increased the inulin space from 1.03% to 2.05% (19,20). However, earlier experiments (1,20) in which a simple mixture of nanoparticles, polysorbate-80, and dalargin does not produce an antinociceptive effect indicate that under these conditions, the small observed increase in the inulin space does not reflect a major opening of tight junctions. The increase in the inulin space might be produced by (a) recruitment of cerebral capillaries, (b) stimulation of endocytosis in the endothelium, (c) modulation rather than disruption of tight junction permeability, or (d) a combination of these factors. Thus, it is clear that the dalargin must be adsorbed by the PBCA nanoparticles in order to facilitate its transport across the BBB. Because the diameter of the nanoparticles is 300 nm, the movement of the dalargin–nanoparticle–polysorbate-80 complex must be the result of a process other than that of simple tight junction disruption or modification followed by diffusion, which might promote the entry of the much smaller dalargin molecule alone; or administered in a mixture of PBCA nanoparticles, polysorbate-80 and dalargin; or following injection of polysorbate-80-coated PBCA nanoparticles. Earlier studies (12,22) have shown internalization of polysorbate-80-coated PBCA nanoparticles by cerebral endothelial cells *in vitro,* which may be a critical step in the delivery of adsorbed drug to the brain.

Our present findings are in contrast to the recent observations made by Olivier *et al.* (8). These authors suggested that nanoparticles are able to increase brain uptake of drugs simply by nonspecifically opening the tight junctions between the brain endothelial cells. Moreover, they postulate a general toxic effect as a result of breakdown products of the nano-

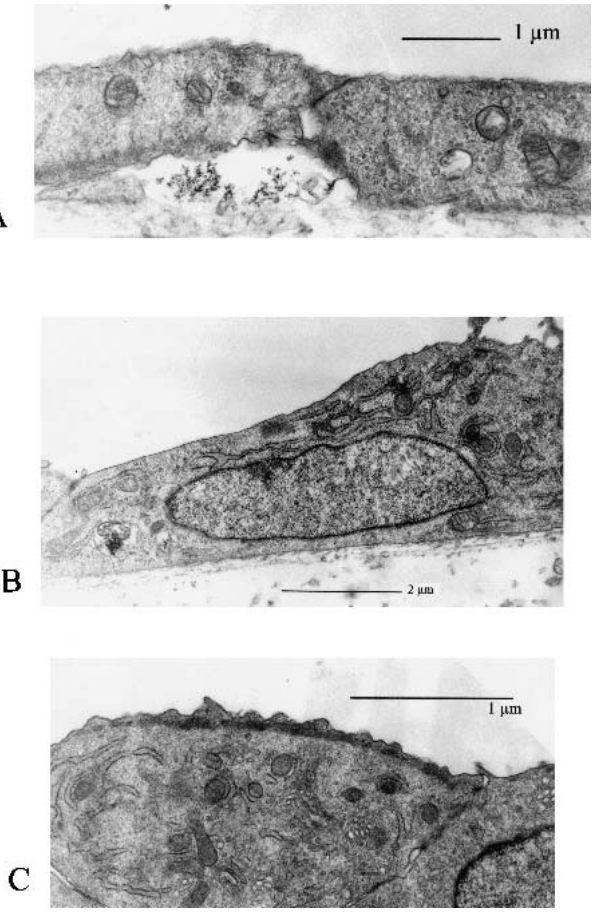

Fig. 3. Electron micrographs of primary bovine brain endothelial cells. Cells were incubated for 1 h before fixation with 4% paraformaldehyde. (A) Control cells without nanoparticles; (B) cells with 10 μg/ml PBCA nanoparticles without polysorbate-80; (C) cells with 10 μg/ml PBCA nanoparticles coated with polysorbate-80.

particle polymer caused by esterases in body fluids. The experiments in the present study do not support these suggestions. Indeed, the quantity of nanoparticles injected, 200 mg/kg in the present study, is without apparent toxic effect in the intact animal and is below the suggested LD_{50} of 230 mg/kg (23), although it is greater than the 135 mg/kg injected in the Olivier *et al.* (8) study where a generalized toxic effect is claimed. Death following tail vein injection of the nanoparticle suspension is, in our hands, a very rare phenomenon and invariably follows a faulty injection. Indeed, in a recent study (24) in rats, no animals died after intravenous injection of up to 400 mg/kg of PBCA nanoparticles.

A major difference between our *in vitro* BBB model and the one used by Olivier *et al.* (8) was that we did not alter the culture conditions before the experiments. Olivier used serum-free medium on the donor side, whereas we used serum on both sides of the endothelium. Thus, differences between the two studies might reflect the medium in which the PBCA nanoparticles are presented to the *in vitro* BBB. In our experience, short-term changes in the coculture system such as a change of medium or a change to serum-free conditions immediately before an assay result in a change in morphology of the endothelial cells and a variable permeability of the monolayer. This may explain why Olivier *et al.* (8) found no difference in sucrose and inulin flux between 10 μg/ml and 500 μg/ml concentrations of the nanoparticles and also no flux differences between the two markers.

In addition, Olivier *et al.* (8) found no differences between uncoated and polysorbate-80-coated nanoparticles in the above *in vitro* BBB model but obtained an antinociceptive effect with the coated and no effect with the uncoated nanoparticles in mice after injection of the dalargin nanoparticles. The latter finding (i.e., an antinociceptive effect with the coated and no effect with the uncoated nanoparticles in mice) is in agreement with previous observations by Kreuter *et al.* (20,21), Alyautdin *et al.* (1,22), and Schroedinger and Sabel (25). Olivier *et al.* (8) are not able to convincingly explain the differences between their *in vivo* and *in vitro* results. The results of Olivier *et al.* (8) suggest a disruption of the BBB by the PBCA nanoparticles, thus facilitating dalargin entry into the brain only in the presence of polysorbate-80.

A further difference between the findings reported here and those of Olivier *et al.* might arise from the use of selected clones of cerebral endothelial cells. It might be possible to select a sensitive clone, which exhibits toxic effects at lower concentrations of nanoparticles and polysorbate-80 than we obtained or pertain *in vivo*. Our present study used two clones of cerebral endothelial cells *in vitro*, bovine and human, with no observed toxicity. We also observed no toxicity in the *in vivo* studies or in further *in vitro* studies with the immortalized rat brain endothelial cells, RBE4. The P_e for sucrose in the bovine endothelial cell cultures used for this study was $3.1 \pm 0.2 \times 10^{-3}$ cm/min compared to $0.63 \pm 0.2 \times 10^{-3}$ cm/min in a study by Dehouck *et al.* (18) and 1.47 ± 0.2

Table I. Antinociceptive Effect in Percentage of the Maximum Possible Effect (% MPE) in Mice ($n = 6$) after Intravenous Injection of Polysorbate 80-Coated PBCA Nanoparticles and Dalargin (7.5 mg)

Intravenous administration of	% MPE (mean ± SD)			
	15 min	30 min	45 min	60 min
Empty PBCA nanoparticles	3.8 ± 3.3	1.5 ± 9.0	0.75 ± 3.2	3.9 ± 4.3
Dalargin solution	2.3 ± 4.6	10 ± 9.8	9.3 ± 2.8	4.7 ± 5.1
Polysorbate 80-coated dalargin-loaded PBCA nanoparticles	8.6 ± 6.2	35 ± 11.7[a]	52 ± 20.2[b]	26 ± 13.4[a]
Polysorbate 80-coated nanoparticles mixed with dalargin immediately before injection	6.1 ± 8.3	3.3 ± 7.0	4.5 ± 5.1	10.3 ± 4.3
Polysorbate 80-coated nanoparticles followed by dalargin 5 min later	3.7 ± 8.4	5.2 ± 6.9	3.9 ± 4.6	7.8 ± 11.4
Polysorbate 80-coated nanoparticles followed by dalargin 30 min later	4.9 ± 5.9	5.2 ± 11.1	0.7 ± 4.8	3.5 ± 7.0

[a] $2p < 0.05$, [b] $2p < 0.01$ compared to dalargin solution.

$\times 10^{-3}$ cm/min in a study by Raub *et al.* (19). The bovine monolayers used in this present study did have a greater sucrose permeability than in the studies quoted above, and the rate of sucrose clearance was also approximately eight times that of the Olivier study (8). However the critical point is that, although the basal sucrose permeabilities of the cultures used are higher than for some other studies, the nanoparticle and polysorbate treatments do not increase the *in vitro* sucrose permeability significantly over 60 min of exposure, indicating that there is no significant modification of the integrity of the tight junctions between cells, and there is no generalized disruption of the endothelial monolayer via a toxic effect compared to the untreated state.

In conclusion, the results presented here show that the facilitation of drug entry into the brain that is obtained by the absorption of a number of drugs onto PBCA nanoparticles that are then overcoated with polysorbate-80 cannot be explained by a simple toxicity-related disruption or opening of the blood–brain barrier. Clearly, for drug delivery to occur, the drug has to be physically associated with the nanoparticles in order to enter the brain.

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