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Aluminum effects on brain microvessel endothelial cell monolayer permeability

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Summary

The permeability of primary cultures of brain microvessel endothelial cell (BMEC) monolayers has been shown to be sensitive to micromolar concentrations of aluminum. Aluminum increased BMEC permeability to a biomembrane impermeant molecule, fluorescein sodium, in a concentration-dependent manner with maximal effects observed after a 5 min preincubation. Aluminum attenuated BMEC pinocytosis as quantitated by the uptake of Lucifer yellow, a fluorescent marker for fluid-phase pinocytosis. Following labeling BMEC membranes with either diphenylhexatriene or trimethylammonium diphenylhexatriene, fluorescence anisotropy measurements showed that aluminum has no effect on membrane lipid order neither at the core of BMEC membranes nor near the surface of the BMEC membrane. The absence of aluminum-induced increases in BMEC pinocytosis, the absence of aluminum effects on BMEC lipid order, and the rapid onset of concentration-dependent increases in BMEC monolayer permeability suggests the potential site of aluminum-induced alterations of BBB permeability may originate at the luminal BMEC surface. Results confirm and support in vivo findings of aluminum-induced increases in blood–brain barrier (BBB) permeability to peptides and non-peptides. We conclude that primary cultures of BMEC monolayers provide an appropriate in vitro model for molecular/cellular level elucidation of mechanisms modulating BBB permeability to blood-borne factors.

Introduction

Aluminum is a ubiquitous environmental substance found in water (Sorenson et al., 1974), food additives (Lione, 1983), cosmetics (Gosselin et al., 1976), and non-prescription drugs (Lione, 1985). Ingested orally, 0.3–25% of an aluminum salt may be absorbed with primary distribution in the plasma compartment (Gupta et al., 1986). Aluminum is recognized as a potential neurotoxin or contributing factor in the development of several central nervous system (CNS) disorders including Alzheimer's disease (Crapper et al., 1980; King et al., 1981; Bjorksten, 1982), Parkinsonism dementia in Guam (Perl et al., 1982), and dialysis encephalopathy and dementia (King et al., 1981; Bjorksten, 1982). In experimental animal models, aluminum has been shown to increase the permeability of the blood–brain barrier (BBB) to peptides and non-peptides (Banks and Kastin, 1983; 1985; Kim et al., 1986). The mechanism by which

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aluminum induces increases in BBB permeability is not precisely known. However, it has been suggested that the increased permeability would, perhaps, allow passage of aluminum and other potential blood-borne toxins into the CNS contributing to the development of CNS dysfunction (Banks and Kastin, 1983; 1985; 1987; Kim et al., 1986).

The development of in vitro models has facilitated molecular/cellular level characterization of the BBB (Joo, 1985). In this report we have examined the utility of an in vitro model comprised of primary cultures of brain microvessel endothelial cell (BMEC) monolayers (Audus and Borchardt, 1986a; 1988) in studying the sensitivity of the BBB to aluminum. Demonstration of BMEC monolayer sensitivity to aluminum would provide an alternative model for elucidation of aluminum effects on the BBB integrity at the molecular/cellular level.

Materials and Methods

Cell culture

BMECs were isolated and grown up in primary culture according to the protocol of Audus and Borchardt (1986a; 1988). These cells have been extensively characterized as an appropriate in vitro BBB model (Audus and Borchardt, 1986a and b; 1987; Baranczyk-Kuzma et al., 1986; Rim et al., 1986). Briefly, BMECs were seeded at a density of 50,000 cells/cm^2 into 100 mm plastic culture dishes or multi-well plates, pretreated with rat-tail collagen and fibronectin, in culture medium comprised of minium essential medium/Eagle's modified, F-12 nutrient mix, 10 mM HEPES, 13 mM sodium bicarbonate, pH 7.4, 10% plasma derived equine serum, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 100 μg/ml heparin, and 20 μg/ml endothelial cell growth factor. Cells were refed every 3 days after seeding. Monolayers were generally formed by 8–10 days after seeding.

Transendothelial transport studies

BMECs were grown to monolayers in primary culture as described above except that monolayers were grown on permeable translucent polycarbonate (PC) discs (Nucleopore; 13 mm diameter with 3 μm pore) placed in 100 mm culture dishes. After formation of a monolayer on the PC discs, the PC discs were lifted out of the culture dishes and placed for various time periods at 37 °C in a culture dish containing culture medium with either saline or different concentrations of aluminum chloride. Control PC discs, collagen-coated, but not containing BMEC monolayers were treated in a similar manner. Following the incubation period, discs with and without BMEC monolayers were rinsed with a modified phosphate-buffered saline (in mM: NaCl 129, KCl 2.5, Na_2HPO_4 7.4, KH_2PO_4 1.3, $CaCl_2$ 0.63, $MgSO_4$ 0.74, glucose 5.3, and ascorbic acid 0.1, pH 7.4 (PBSA). The discs were then placed in a Side-Bi-Side diffusion cell (Crown Glass Co.) with 3 ml of PBSA in each of the donor and receptor chambers. The use of the Side-Bi-Side diffusion cells for studying drug transport across BMEC monolayers has previously been detailed (Audus and Borchardt, 1986b; 1987; Rim et al., 1986). The water-jacketed diffusion cells were thermostated at 37 °C with an external water bath and the contents of the donor and receptor chambers constantly stirred at 600 rpm with a diffusion cell console (Crown Glass Co.). An aliquot of fluorescein sodium (Sigma) dissolved in PBSA was added at time 0 (final concentration of 1 μM) to the donor chamber. At various times after time 0, 100 μl sample aliquots were removed from the receptor chamber, placed in disposable microcuvets, diluted to 1 ml with PBSA, and amount of fluorescein present analyzed by fluorescence emission (EX = 490 nm; EM = 520 nm) on an SLM-AMINCO Model 4800 fluorometer. Fluorescein sodium fluorescence emission of prepared standards was linear over a concentration range of 0.1 to 5 nM ($r = 0.99$). After removal of sample aliquots, fresh PBSA aliquots were added to the receptor chamber to maintain equal volumes in donor and receptor chambers.

Apparent permeability coefficients were estimated by the following relationship:

$$P(\text{cm/s}) = X/(A \times t \times C_D)$$

Where P is the apparent permeability coefficient,

X is the amount of fluorescein in mol in the receptor chamber at time t (in s), A is the diffusion area (i.e., 0.636 cm^2), and C_D the concentration of fluorescein in the donor chamber in mol/cm^3 (in the studies of BMEC monolayer permeability, C_D remains $> 90\%$ of the initial value over the time course of the experiments).

BMEC pinocytosis studies

The protocol of Swanson et al. (1985) was used to characterize the sensitivity of BMEC fluid-phase pinocytosis of aluminum. Briefly, Lucifer yellow (LY; Molecular Probes) was dissolved in culture medium to a concentration of 0.5 mg/ml. Aliquots, 0.45 ml, of LY in culture medium were added to each well of 24-well plates for varying periods of time following a 1 h preincubation of the cells in culture medium containing different concentrations of aluminum, or 1 μM vincristine, or saline. The 24-well plates were then drained and quickly washed 3 times with phosphate-buffered saline, pH 7.4 (PBS), containing 1 mg/ml bovine serum albumin (BSA) at 4°C. Following the wash, the plates were drained and 0.50 ml of PBS containing 0.05% Triton X-100 (Sigma) added to each well. Cell lysate samples, 0.35 ml, were removed from each well of the culture plates, placed in an acrylic cuvet (Fisher Scientific) and diluted up to 1.9 ml with PBS. Fluorescence was measured with an SLM-AMINCO Model 4800 fluorometer (EX = 430 nm; EM = 540 nm). LY fluorescence, in prepared standards, was linear over a concentration range of 0.1 to 100 ng/ml. Protein was determined by a modified Lowry method using 1% sodium dodecyl sulfate (Markwell et al., 1981). The characteristics and detailed kinetics of LY uptake by primary cultures of BMECs has been previously described (Guillot et al., 1987).

BMEC membrane studies

BMECs grown to monolayers in 100 mm culture dishes were washed 3 times with PBS and exposed to 3 ml solution of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA-Na (Sigma) in PBS for 2 min at 37°C. Excess trypsin/EDTA solution was aspirated off and the cells incubated for another 2 min at 37°C. Following the second incubation, PBS containing 1% BSA (w/v) was added to the dish and the cells were suspended by gentle agitation. Suspended BMECs were washed 3 times by centrifugation in PBS for 10 min at 200 g. After the final wash the BMECs were suspended in PBS. BMECs were counted by Crystal violet staining and diluted to a final concentration of 2×10^5 cells/ml. Cell viability determined by Trypan blue exclusion was $> 90\%$ during the time course of subsequent labeling and the experiments.

BMECs were labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH; molecular probes) by adding 2.5 μl of a 1 mM freshly prepared stock of DPH in tetrahydrofuran (Fisher Sci.) to 2.5 ml of 2×10^5 cells/ml (Bronner et al., 1986). Alternatively, BMECs were labeled with 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH; Molecular Probes) by adding 2.5 μl of a 5×10^4 M freshly prepared stock of TMA-DPH in dimethylformamide (Fisher Sci.) to 2.5 ml of 2×10^5 cells/ml (Bronner et al., 1986).

The fluorescence anisotropy of DPH and TMA-DPH labeled BMECs was measured with an SLM-AMINCO Subnanosecond Lifetime Fluorometer, Model 4800 essentially as described by Audus and Gordon (1984). Photomultiplier tubes were placed to the right and left of the dual chamber sample cell with Glans-Thompson polarizers inserted in the emission and excitation beams. A Schott KV-489 cut on filter was inserted into the horizontally polarized emission beam and the SLM MC320 monochromator set at 430 nm placed in the vertically polarized emission beam. Fluorescence intensity was first measured with the sample excited (i.e., source was 450 W Xenon arc lamp) at 360 nm with a horizontally polarized beam, and then measured a second time with the sample excited with a vertically polarized beam. Corrected fluorescence anisotropy data were calculated from the relationships

$$P = (A - B)/(A + B)$$

$$r = (2 \times P)/(3 - P)$$

where P is the fluorescence polarization, A is the ratio of fluorescence intensities parallel and perpendicular to the plane of polarized excitation

light with the excitation polarizer in the vertical position, B is the ratio of fluorescence intensities parallel and perpendicular to the plane of polarized light with the excitation polarizer in the horizontal position, and r is the fluorescence anisotropy.

Light scattering due to turbidity of the BMEC suspension was insignificant. The temperature of the dual chamber sample cell was controlled by an external circulating water bath and maintained at either 25 °C or 37 °C and the BMECs maintained in suspension by stirring the contents of the sample cell with a magnetic stirrer. Two sample cells (i.e., quartz cuvets) containing BMEC suspensions were always used, one which was exposed to various concentrations of aluminum chloride and the other which received equivalent aliquots of saline as a control.

Results and Discussion

We have previously demonstrated that the primary cultures of BMEC monolayers exhibit permeability characteristics similar to those observed for the BBB in vivo (Audus and Borchardt, 1986a and b, 1987; Rim et al., 1986). In this study we found the time-dependent flux of fluorescein across collagen-coated PC discs supporting BMEC monolayers was linear (r ranged from 0.989 to 0.995) and sensitive to increasing concentrations of aluminum chloride (Fig. 1). The apparent permeability coefficient calculated for fluorescein flux across collagen-coated PC discs was approximately 1.6×10^{-4} cm/s without BMEC monolayers and 2.7×10^{-5} cm/s with BMEC monolayers present. The apparent permeability coefficient of collagen-coated PC discs supporting BMEC monolayers, for fluorescein sodium (ca. 2.7×10^{-5} cm/s), was similar to that for sucrose (Rim et al., 1986), also a biomembrane-impermeant molecule. Following a 15 min preincubation of the BMEC monolayers with increasing concentrations of aluminum chloride, a 5–6 fold maximal increase in the permeability coefficient of the BMECs was observed (Fig. 2). In contrast, the apparent permeability coefficient for fluorescein flux across collagen-coated PC discs without monolayers (i.e., approximately 1.6×10^{-4} cm/s),

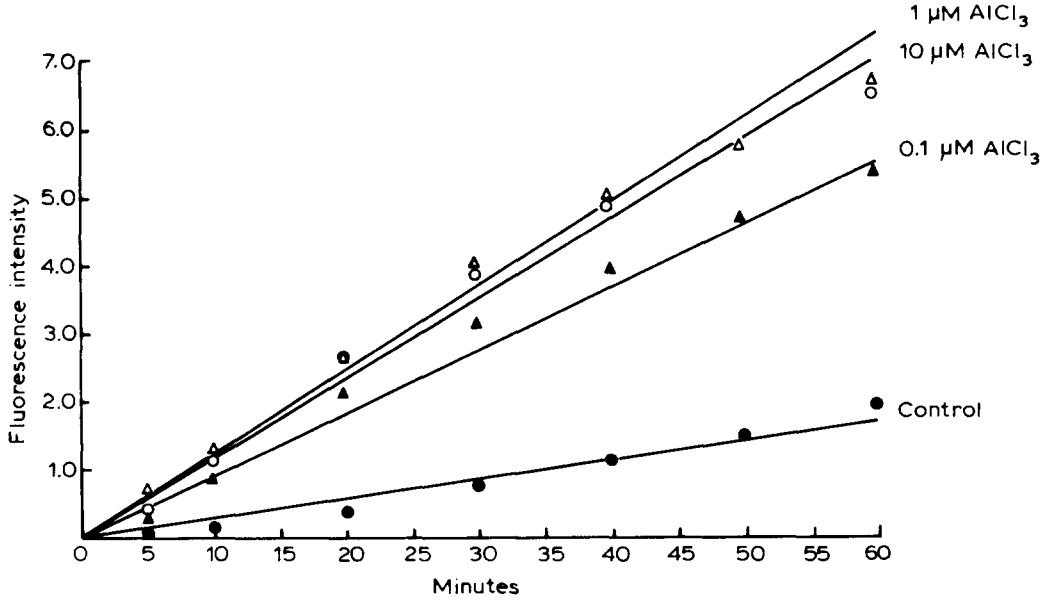

Fig. 1. Effect of aluminum chloride on the time-dependent flux of fluorescein sodium across BMEC monolayers. The fluorescence intensity of fluorescein sodium in samples removed from the receptor chamber of a Side-Bi-Side (Crown Glass Co.) diffusion cell was recorded at various times in the indicated concentrations of aluminum chloride as described in Materials and Methods. Data points represent the means from at least 3 different monolayers. S.D.s were approximately 10% of the mean.

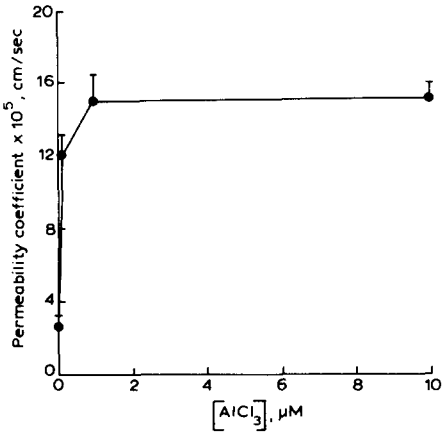

Fig. 2. Concentration-dependent effects of aluminum chloride on the apparent permeability coefficient of BMEC monolayers for fluorescein sodium. Permeability coefficients were calculated from the time-dependent flux of fluorescein sodium across BMEC monolayers following a 15 min preincubation at 37 °C with indicated concentrations of aluminum chloride as described in Materials and Methods. Data points represent the means from at least 3 different monolayers ± S.D.

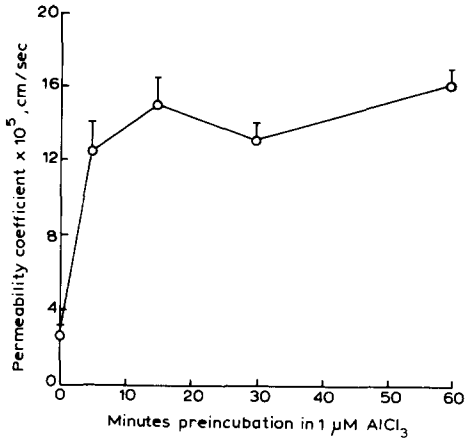

Fig. 4. Effects of preincubation time with aluminum chloride on the apparent permeability coefficient of BMEC monolayers for fluorescein sodium. Permeability coefficients were calculated from the time-dependent flux of fluorescein sodium across BMEC monolayers following preincubation with 1 μM aluminum chloride for indicated times as described in Materials and Methods. Data points represent the means ± S.D. from at least 3 different monolayers.

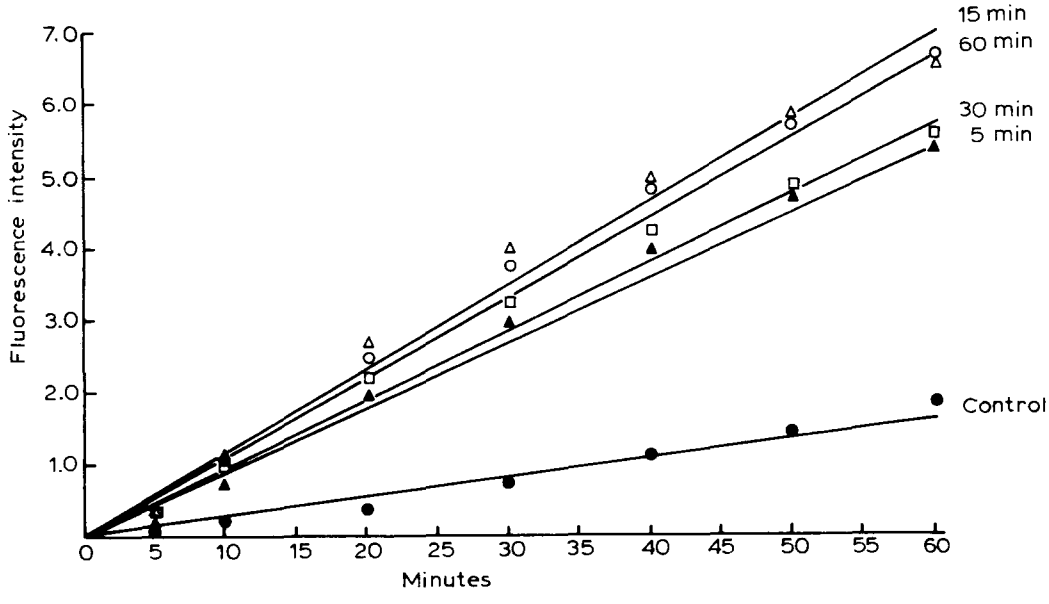

Fig. 3. Effect of preincubation time with aluminum chloride on the time-dependent flux of fluorescein sodium across BMEC monolayers. The fluorescence intensity of fluorescein sodium in samples removed from the receptor chamber of a Side-Bi-Side (Crown Glass Co.) diffusion cells was recorded at various times following preincubation for indicated times with 1 μM aluminum chloride as described in Materials and Methods. Data points represent the means from at least 3 different monolayers. S.D.s were approximately 10% of the mean.

when preincubated with increasing aluminum chloride concentrations, was not altered (data not shown). By comparison, the magnitude of the aluminum-induced changes in vitro were similar to the reported magnitude of aluminum-induced permeability changes to sucrose at the BBB in vivo (Kim et al., 1986). That the BMEC monolayer's permeability to fluorescein sodium can be altered by addition of a test substance and used to quantitate permeability changes was consistent with the use of fluorescein sodium to characterize BBB permeability changes in vivo in the presence of hypertonic saline and alcohols (Gulati et al., 1982, 1985).

The time-dependent flux of fluorescein across collagen-coated PC discs supporting monolayers was sensitive to varying preincubation times with 1 μM aluminum chloride (Fig. 3). The changes in permeability of the BMECs to fluorescein were rapid with near maximal changes in the apparent permeability coefficients occurring within 5 min (Fig. 4). In control experiments, the apparent permeability coefficient for fluorescein flux across collagen-coated PC discs (i.e., approximately 1.6 $\times 10^{-4}$ cm/s) was not altered after preincubation with 1 μM aluminum chloride for various times (data not shown).

The concentration-dependence is consistent with the effects of aluminum chloride and sulfate salts on BMEC growth (Audus et al., 1988) and, as indicated above, with observed permeability changes in vivo (Banks and Kastin, 1983, 1985; Kim et al., 1986). The concentrations of aluminum effective in producing changes in BMEC growth and permeability are similar to those resulting in aluminum intoxication acutely (Cummings et al., 1982) and chronically (Smith et al., 1980). However, the rapid onset of aluminum effects in this in vitro system is in contrast to the 2 h time period required for maximal effects of aluminum on BBB permeability are observed in vivo (Banks and Kastin, 1985; Kim et al., 1986). This difference may reflect a tissue distribution process associated with administration of aluminum into the whole animal.

Characteristics of fluid-phase pinocytosis in primary cultures of BMEC monolayers have been previously described both quantitatively and qualitatively. Findings from those studies indicate that fluid-phase pinocytosis occurs in BMECs but at a reduced level typical of the BBB in vivo (Guillot et al., 1987). Under normal conditions then, fluid-phase transcytosis has a minor contribution in overall BBB permeability. To determine if aluminum enhances fluid-phase transcytosis, thus increasing BMEC monolayer permeability, we examined the effect of aluminum on LY uptake. Fluid-phase endocytic activity of the BMEC monolayers was attenuated in the presence of increasing concentrations of aluminum chloride (Fig. 5). A known inhibitor of BBB fluid-phase pinocytosis (Larsson et al., 1979), vincristine, was shown to similarly attenuate BMEC fluid-phase endocytosis by ca. 30% (Fig. 5). The contribution of the fluid-phase endocytic pathway to the aluminum-increased permeability of the BMEC monolayers would seem to be negative. This direct

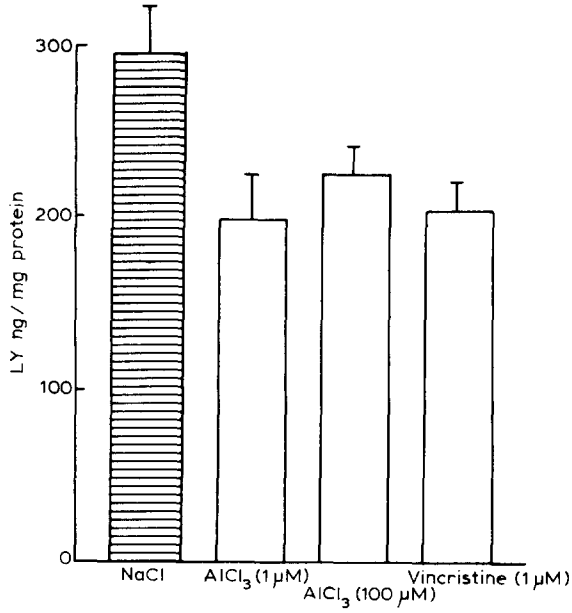

Fig. 5. Effects of aluminum chloride and vincristine on the uptake of the fluid-phase pinocytosis marker LY by BMEC monolayers. BMEC monolayers were preincubated with indicated concentrations of aluminum chloride, saline, or 1 μM vincristine in culture medium for 1 h at 37°C. Following exposure to test substances, 0.5 mg/ml LY in culture medium, cells were incubated for 1 h at 37°C, washed, and the amount of LY quantitated by fluorescence spectroscopy as described in Materials and Methods. Data represent means from at least quadruplicate monolayers ± S.D.

evidence confirms the supposition of others that aluminum enhancement of pinocytosis does not contribute to aluminum-induced increases in BBB permeability (Banks and Kastin, 1985; 1987; Kim et al., 1986).

The effects of aluminum on membrane transport processes have been observed to be both inhibitory (King et al., 1983) and stimulatory (Banks and Kastin, 1985). Interactions with specific components of the carrier (King et al., 1983), cell surface charge (Deleers, 1985) and, rigidification of membranes (Deleers et al., 1986) have been proposed as mechanisms for aluminum effects on membrane transport. Aluminum chloride, in increasing concentrations, was not effective in altering the fluorescence anisotropy of either DPH (i.e., a marker for lipid order in the core of the cell membrane (Heyn, 1979; Van Blitterswijk et al., 1981; Audus and Gordon, 1984)) or TMA-DPH (i.e., a marker for lipid order near the surface of the cell membrane (Prendergast et al., 1981; Bronner et al., 1986)) labeled BMECs either at 25°C or 37°C (Figs. 6 and 7). Had aluminum resulted in rigidification of the BMEC membrane, as observed in DPH labeled artificial membranes (Deleers et al., 1986), an increase in the fluorescence anisotropy would have been observed. The absence of effects of aluminum on BMEC membrane lipid order suggest that interactions important for aluminum-induced increases in BBB permeability do not originate in rigidification of hydrophobic regions of the BMEC membrane.

At the molecular/cellular level we have been able to provide direct evidence that neither increased pinocytic activity, nor changes in hydrophobic regions of the BMEC membrane are involved in aluminum-induced changes in BBB permeability. Yet, after short time exposures, large aluminum-induced increases in the permeability of BMEC monolayers to a negatively charged (i.e., at physiological pH), membrane-impermeant molecule, fluorescein sodium, was observed. This confirms and supports findings in previous reports on aluminum-induced changes in BBB function in vivo (Banks and Kastin, 1983; 1985; 1987; Kim et al., 1986). Moreover, these observations are consistent with growing evidence that permeability of the BBB might be manipulated by neutralization

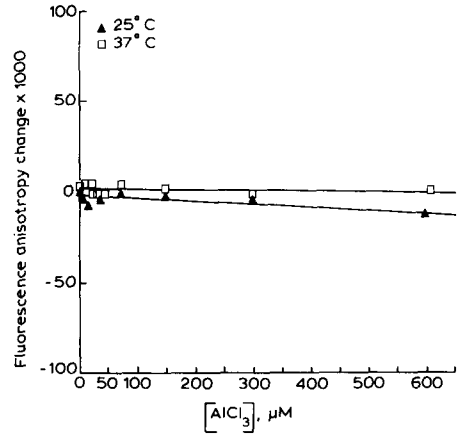

Fig. 6. Concentration-dependent effects of aluminum chloride on the fluorescence anisotropy (r) of TMA-DPH-labeled BMEC membranes. Fluorescence anisotropy change was calculated by subtracting r for TMA-DPH-labeled BMEC membranes exposed to saline (controls) from r for TMA-DPH-labeled BMEC membranes exposed to indicated concentrations of aluminum chloride for 5 min at either 25°C or 37°C, as described in Materials and Methods. The mean r for TMA-DPH-labeled BMEC membranes exposed to saline was 0.2764 ± 0.0037 at 25°C and 0.2596 ± 0.0016 at 37°C for the time period of the experiments.

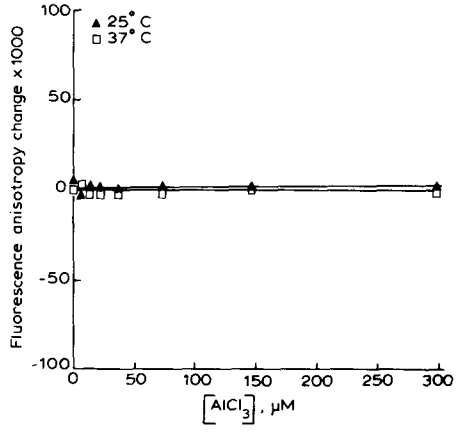

Fig. 7. Concentration-dependent effects of aluminum chloride on the fluorescence anisotropy (r) of DPH-labeled BMEC membranes. Fluorescence anisotropy change was calculated by subtracting r for DPH-labeled BMEC membranes exposed to saline (controls) from r for DPH-labeled BMEC membranes exposed to indicated concentrations of aluminum chloride for 5 min at either 25° or 37°C, as described in Materials and Methods. The mean r for DPH labeled BMEC membranes exposed to saline was 0.2047 ± 0.0017 at 25°C and 0.1650 ± 0.0019 at 37°C for the time period of the experiments.

of the cell surface charge by cationic molecules such as aluminum (Kim et al., 1986; Banks and Kastin, 1987), protamine sulfate, and poly-L-lysine (Strausbaugh, 1986). Further evidence indicates that aluminum readily associates with blood components (Gupta et al., 1986), the cell surface as well as the nuclei of endothelial cells (including BMECs), pericytes and smooth muscle of blood vessels in animals treated with aluminum salts (De Boni et al., 1976; Wen and Wisniewski, 1983). Aluminum-induced increases in BBB permeability in vivo have also been demonstrated to occur without physical disruption of the barrier (Banks and Kastin, 1985; Kim et al., 1986). While we can not rule out aluminum-induced changes in cell–cell junctions in the absence of direct evidence, cumulatively present considerations strongly suggest that rapid onset aluminum-induced increases in BMEC monolayer permeability are referrable to the cell surface. Future work with the in vitro model will be aimed at providing additional molecular/cellular level evidence (e.g., examination of cell–cell junctional integrity, aluminum binding to the BMEC surface, and the reversibility of aluminum effects) for the mechanism by which aluminum alters BBB permeability.

In summary, we have shown that aluminum increases BMEC permeability to fluorescein sodium in a concentration-dependent manner, inhibits BMEC fluid-phase pinocytosis, and has no effect on lipid order in BMEC membranes. Results suggest that interaction of aluminum with BMEC membranes occurs at the cell surface. Our results are consistent with present knowledge of the effects of aluminum on the BBB in vivo and suggest that BMEC monolayers represent a useful in vitro model for studying the effects of aluminum on BBB function at the molecular/cellular level. More generally, the findings of this study provide a further indication of the utility of primary cultures of BMEC monolayers for investigation of blood-borne factors implicated in modulation of BBB permeability to circulating substances.

Acknowledgements

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