Monatsh Chem (2011) 142:431–437
DOI 10.1007/s00706-011-0471-0

ORIGINAL PAPER

Aluminum enhances the toxic effects of amyloid β-peptide on cell membranes and a molecular model

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Received: 29 October 2010 / Accepted: 21 February 2011 / Published online: 18 March 2011

Abstract The amyloid β-peptide (Aβ) and aluminum are found, among other components, in the senile plaques of patients with Alzheimer's disease. Aggregated Aβ and aluminum are toxic to neurons but the mechanism of accumulation and toxicity remains poorly understood. It has been proposed that Aβ and aluminum toxicity results from Aβ– and aluminum–membrane interactions. It was therefore of interest to study the effect that Aβ and aluminum could have on cell membranes. Thus, the interactions of Aβ(1-40), Aβ(1-42), and Al(III) with the human erythrocyte membrane and a molecular model of the erythrocyte membrane were examined by electron microscopy and X-ray diffraction, respectively. The molecular model consisted of bilayers built up of dimyristoylphosphatidylcholine and dimyristoylphosphatidylethanolamine, phospholipid classes located in the outer and inner monolayers of most cell membranes, respectively. Aβ(1-40) and Aβ(1-42) in the presence of Al(III) altered the erythrocyte membrane morphology and the structure of dimyristoylphosphatidylcholine bilayers, effects that were different and stronger than those induced by Aβ and Al(III) separately.

Keywords Alzheimer's disease · Aluminum · Membrane · Molecular model · Monolayers

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Introduction

Alzheimer's disease (AD) is an age-related disorder characterized by progressive cognitive decline and neurodegeneration. One of the key features of this disease is the presence of amyloid plaques associated with neuritic degeneration [1]. The amyloid plaques are composed predominantly of 40- to 42-residue peptides, the amyloid β-peptide (Aβ), the most toxic isoform being Aβ(1-42) [2]. The Aβ peptides are proteolytically cleaved from the transmembrane amyloid precursor protein (APP) by the β- and γ-secretases with the γ-cleavage site within the membrane bilayer [3]. Soluble Aβ peptides interact with cell membranes and have been proposed to affect membrane integrity leading to apoptosis [4]. The molecular mechanisms of these interactions are still unclear. It has been suggested that membrane alterations may proceed to cell death by either an oxidative stress mechanism, caused by the Aβ peptide and synergized by transition metal ions, or through formation of ion channels by peptide interfacial self-aggregation [5]. It has also been suggested that Aβ peptides modulate membrane functions by a non-receptor-mediated mechanism, potentially as a result of altering the physico-chemical properties of membrane lipids and proteins [6]. Shin et al. [7] found that the Aβ42 is essential for the early development of AD but not sufficient to promote the formation of plaques unless succeeded by Aβ40 deposition. Studies performed in rat synaptic plasma membrane indicated that soluble Aβ40 was located in its hydrophobic core, whereas the aggregated form was associated with the phospholipids head group area; however, both soluble and aggregated Aβ42 interacted only with the membrane lipid bilayer hydrocarbon core [6]. Furthermore, it was reported that both Aβ40 and Aβ42 decrease the fluidity of the acyl chains and head groups of human brain

plasma, endosomal and lysosome cell membranes, whereas that of the Golgi bilayer fluidity increased [8]. On the other hand, studies indicated that the ability of both Aβ40 and Aβ42 to disrupt and/or aggregate phospholipids vesicles was mediated through electrostatic interactions with the phospholipids head groups [9]. However, Müller et al. [10] reported that Aβ specifically disturbs the acyl-chain layer of cell membranes, while by contrast membrane properties at the level of the polar head groups are much less affected. Given that Aβ is generated in a membrane environment, that its pathological behavior may be due to interactions with membranes, and the unclear nature of these interactions, their understanding are important for deciphering the biological role of Aβ.

Aluminum, an environmentally abundant non-redox cation, has long been implicated as a potential risk factor in AD [11–15]. The involvement of Al as a risk factor for AD is suggested by its presence in a high focal concentration in senile plaques and hyperphosphorylated neurofibrillary tangles; however, others attributed the presence of the metal to contamination of the tissue samples by Al from fixatives and staining reagents, a point of view refuted by the use of more precise techniques (reviewed by Zatta et al. [15], Exley and Korchazhkina [16], and Gupta et al. [17]).

With the aim to better understand the molecular mechanisms of the interaction of Aβ and aluminum with cell membranes, we have utilized two paradigmatic well-established models: intact human erythrocytes and molecular models of the erythrocyte membrane. Erythrocytes were chosen because, although less specialized than many other cell membranes, they perform enough functions in common with them, e.g., active and passive transport, and the production of ionic and electric gradients, to be considered representative of the plasma membrane in general. On the other hand, circulating blood cells are exposed to soluble Aβ, with binding detected at physiological levels of 5 ng/cm^3 [18]. It was also reported that erythrocytes of subjects with AD show increased levels of lipid peroxidation, perturbations in the physical state of membrane proteins, and irregular shape distortions [19–22]. Intact human erythrocytes incubated with Aβ and aluminum were observed by scanning electron microscopy (SEM). The molecular model consisted of bilayers built up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively [23, 24]. The capacity of Aβ and aluminum to perturb the bilayer structures of DMPC and DMPE was determined by X-ray diffraction. These systems and techniques have been used in our laboratories to determine the interaction with and the membrane-perturbing effects of other metal ions such as Pb^{2+} [25], Cd^{2+} [26], Au^{3+} [27], Ti^{4+} [28], Fe^{2+} and Fe^{3+} [29], Cr^{3+} and Cr^{6+} [30], and Zn^{2+} [31].

Results and discussion

SEM studies on the effects of Al(III) on human erythrocytes

Figure 1 shows the SEM image of intact human erythrocytes, and Fig. 2 the results observed after incubating the red cells with different concentrations of Al(III). As can be observed, 1 and 10 μM Al(III) ions did not induce significant morphological changes to erythrocytes, whereas 25 and 100 μM Al(III) ions induced cell adhesion.

SEM studies on the effects of Aβ(1-40) and Aβ(1-42) on human erythrocytes

Figures 3 and 4 show the SEM images of human red cells incubated with Aβ(1-40) and Aβ(1-42). Both Aβ isoforms in the maximum concentration assayed (20 μM) did not produce any significant morphological change to the red cells.

SEM studies on the effects of Al(III)/Aβ(1-40) and Al(III)/Aβ(1-42) on human erythrocytes

Erythrocytes incubated with the mixtures of 20 μM Aβ(1-40) and 100 μM Al(III) (Fig. 5), and 20 μM Aβ(1-42) and 100 μM $AlCl_3$ (Fig. 6) show echinocytosis, a shape alteration characterized by the development of blebs and/or protuberances on the red cell surfaces.

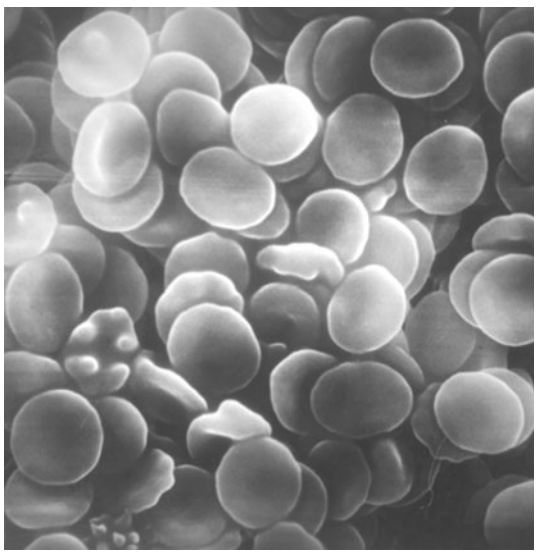

Fig. 1 Scanning electron microscopy image (×2,500) of intact human erythrocytes

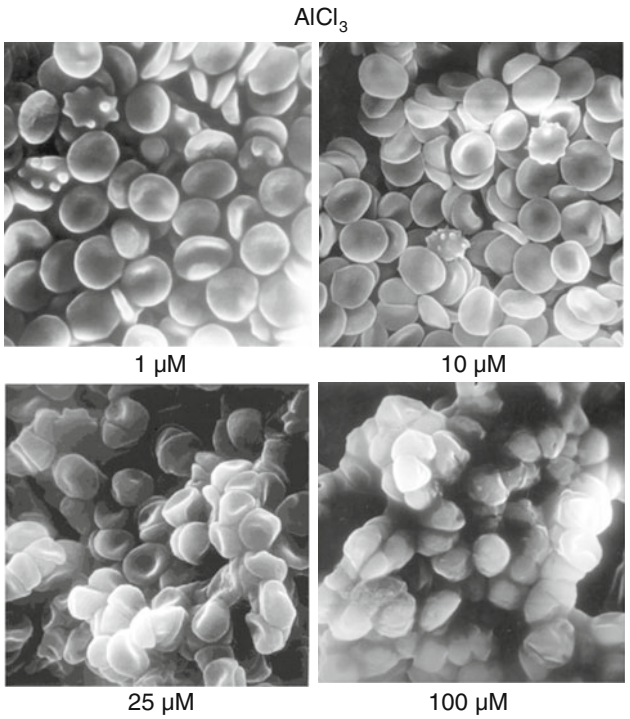

Fig. 2 Scanning electron microscopy images (×2,500) of human erythrocytes incubated with $AlCl_3$ in a range of concentrations

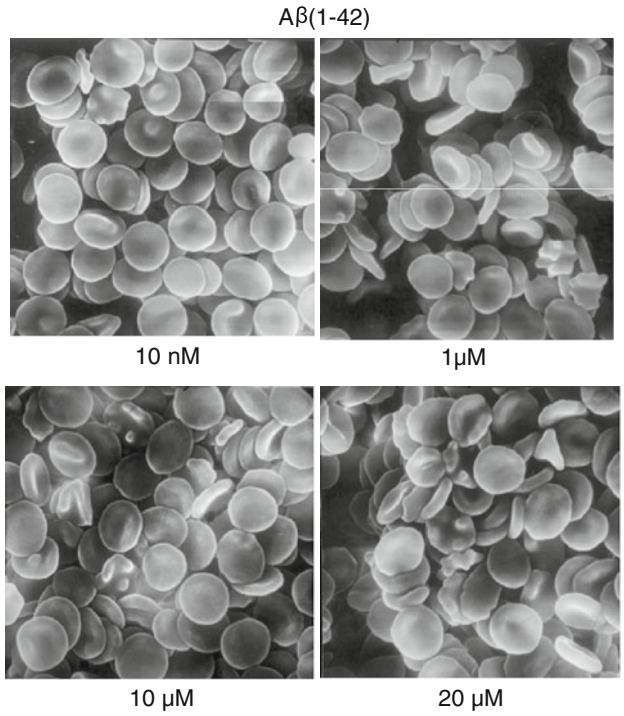

Fig. 4 Scanning electron microscopy images (×2,500) of human erythrocytes incubated with Aβ(1-42) in a range of concentrations

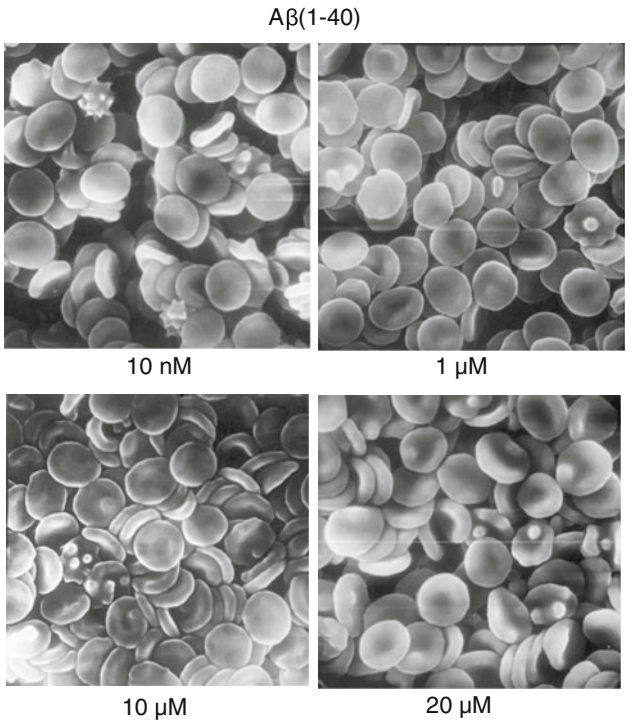

Fig. 3 Scanning electron microscopy images (×2,500) of human erythrocytes incubated with Aβ(1-40) in a range of concentrations

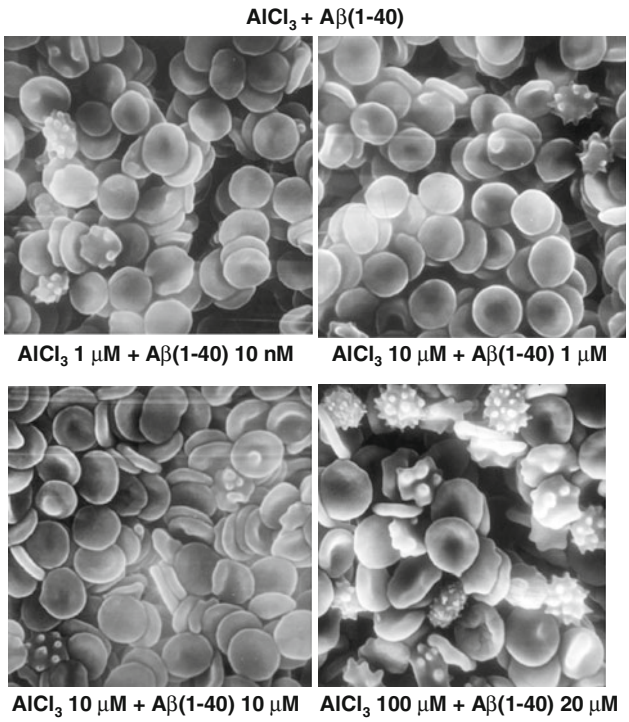

Fig. 5 Scanning electron microscopy images (×2,500) of human erythrocytes incubated with $AlCl_3$ and Aβ(1-40) in a range of concentrations

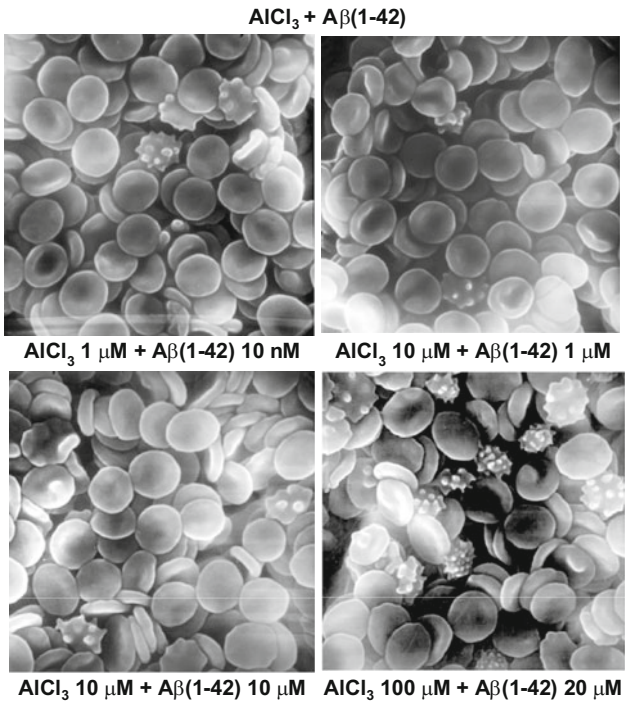

Fig. 6 Scanning electron microscopy images (×2,500) of human erythrocytes incubated with $AlCl_3$ and Aβ(1-42) in a range of concentrations

X-ray diffraction studies on the interaction of Al(III) and Aβ(1-40) alone and of their mixtures with DMPC bilayers

Figure 7a shows the results obtained after DMPC bilayers were mixed and had interacted with water and aqueous solutions of Al(III). As expected, pure water altered the structure of DMPC. In fact, its bilayer repeat (bilayer width plus the width of water layer between bilayers) expanded from about 55 Å when dry [32] to 64.5 Å when immersed in water, and the reflections were reduced to only the first two orders of the bilayer repeat. On the other hand, the structure became more fluid as indicated by the presence of a new and strong 4.2-Å reflection, which corresponds to the average separation of the fully extended acyl chains rotationally disordered in an hexagonal arrangement. Figure 7a also shows that a considerable weakening of the lipid reflection intensities occurred after DMPC was exposed to 1 and 10 μM Al(III). From these results it can be concluded that Al(III) produced a significant structural perturbation of the DMPC bilayers. Figure 8a shows the results obtained after DMPC was mixed and had interacted with aqueous solutions of Aβ(1-40). The figure shows that increasing concentrations of Aβ(1-40) from 10 nM up to 10 μM gradually induced a decrease of all the reflection intensities, which means that the polar head and acyl chain groups were perturbed by the peptide. The fact that the reflections completely disappeared at a 20 μM Aβ(1-40) concentration indicated a total destruction of the organized lipid structure. The combined effect of Al(III) and Aβ(1-40) is presented in Fig. 8b, which implies that the DMPC structure was completely destroyed when Aβ(1-40) and $AlCl_3$ concentrations were 1 and 10 μM, respectively. This effect was stronger, and achieved with lower concentrations of

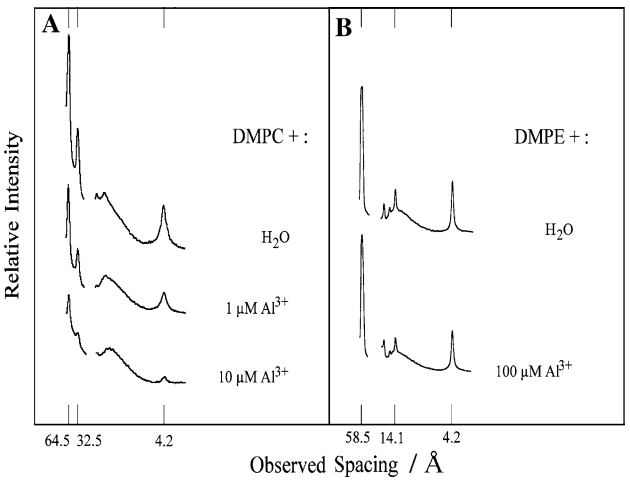

Fig. 7 Microdensitograms from X-ray diffraction of DMPC (**a**) and DMPE (**b**) incubated with aqueous solutions of $AlCl_3$

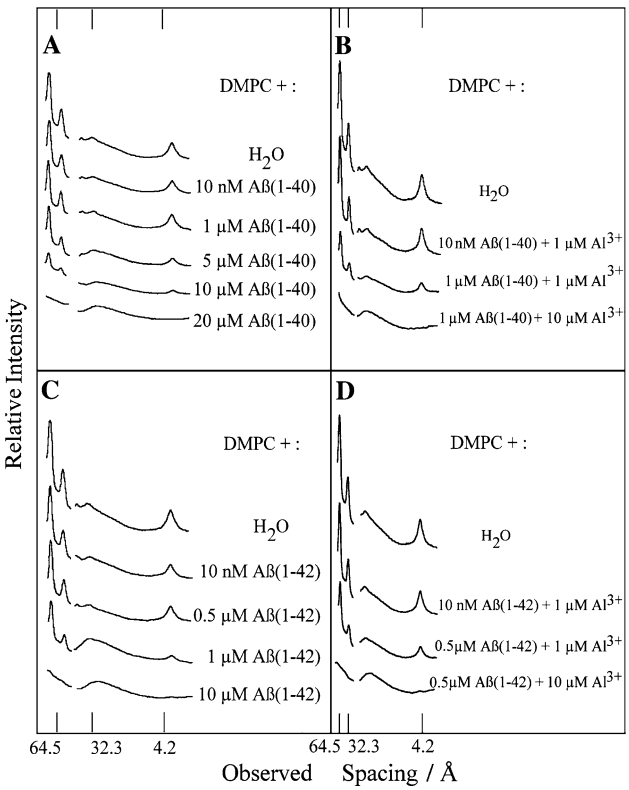

Fig. 8 Microdensitograms from X-ray diffraction of DMPC incubated with Aβ(1-40) (**a**); Aβ(1-40) + $AlCl_3$ (**b**); Aβ(1-42) (**c**); Aβ(1-42) + $AlCl_3$ (**d**)

Aβ(1-40) and $AlCl_3$, than that induced by each compound interacting separately with DMPC.

X-ray diffraction studies on the interaction of Aβ(1-42) alone and in the presence of Al(III) with DMPC bilayers

Figure 8c shows the effects of Aβ(1-42) on DMPC. This peptide induced about the same results observed with Aβ(1-40). However, in this case the complete destruction of the lipid structure was achieved when the peptide concentration was 0.5 μM, i.e., half that of Aβ(1-40). This finding implies that the interaction of Aβ(1-42) with DMPC is stronger than with Aβ(1-40). When this experiment was repeated in the presence of Al(III) (Fig. 8d) it was again observed that the complete destruction of DMPC bilayer arrangement was achieved at lower concentrations than those separately induced by Aβ(1-42) and Al(III).

X-ray diffraction studies on the interaction of Al(III) and Aβ(1-40) alone and of their mixtures with DMPE bilayers

Figure 7b shows the results of the interaction of Al(III) with DMPE. As reported elsewhere, water did not significantly affect the bilayer structure of DMPE [32]. Figure 7b also shows that an Al(III) concentration as high as 100 μM did not affect the bilayer structure of DMPE. Results from similar experiments performed on DMPE incubated with Aβ(1-40) are exhibited in Fig. 9a. A concentration of the peptides as high as 20 μM, that destroyed DMPC structure, did not affect that of DMPE. Figure 9b shows the combined effect of the highest assayed concentrations of Aβ(1-40) and $AlCl_3$ (20 and 100 μM, respectively) upon DMPE bilayers. The DMPE structure was considerably less affected than that of DMPC incubated with 1 μM Aβ(1-40) and 10 μM $AlCl_3$.

X-ray diffraction studies on the interaction of Aβ(1-42) alone and in the presence of Al(III) with DMPE bilayers

Results from similar experiments performed on DMPE incubated with Aβ(1-42) are exhibited in Fig. 9c. The highest assayed concentration of this peptide (20 μM) did not induce any perturbation of the lipid structure. The addition of 10 and 100 μM $AlCl_3$ (Fig. 9d) did not produce a significant alteration of the DMPE bilayer structure. This result can be compared with that induced on DMPC by much lower concentrations of both Aβ(1-42) and $AlCl_3$ (0.5 and 10 μM, respectively). The results of the X-ray diffraction analysis of DMPC and DMPE with Aβ in the presence of Al(III) allow one to conclude that (a) the

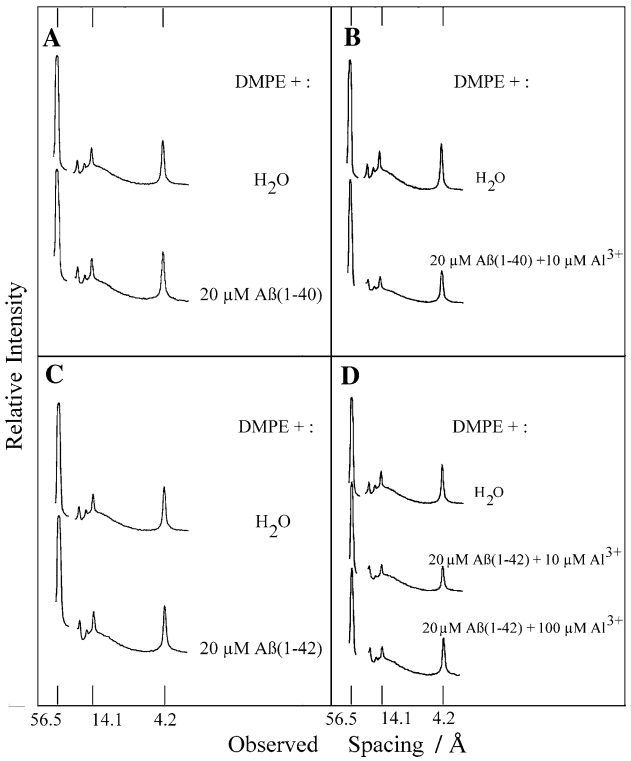

Fig. 9 Microdensitograms from X-ray diffraction of DMPE incubated with Aβ(1-40) (**a**); Aβ(1-40) + $AlCl_3$ (**b**); Aβ(1-42) (**c**); Aβ(1-42) + $AlCl_3$ (**d**)

DMPC bilayer structure is more susceptible than that of DMPE to be altered by both Aβ(1-40) and Aβ(1-42), (b) Aβ(1-42) induces higher structural perturbations than Aβ(1-40), and (c) Al(III) enhances the perturbing capacity of both Aβ isoforms.

The SEM results indicate that none of the assayed amyloid peptides significantly affected the shape of the human erythrocytes. On the other hand, $AlCl_3$ induced cellular adhesion of these cells. However, the incubation of erythrocytes with Aβ(1-40) and Aβ(1-42) in the presence of $AlCl_3$ resulted in relevant morphological alterations. In fact, SEM observations of specimens showed that the erythrocytes changed their normal biconcave shape to an echinocytic form, characterized by the formation of blebs and/or protuberances over the cell surface. According to the bilayer couple hypothesis [33], shape changes induced in erythrocytes by foreign molecules are due to a differential expansion of the two monolayers of the membrane. Thus, the spiculated shape (echinocyte) arises when the added compound locates in the outer monolayer, whereas a cup shape (stomatocyte) is induced when the compound is inserted in the inner monolayer. The fact that echinocytes were observed is an indication that the peptides and Al(III) ions accumulated in the outer moiety of the red cell membrane. This conclusion is supported by the X-ray diffraction experiments performed in bilayers built up of

DMPC and DMPE. They represent phospholipid classes located in the outer and inner monolayers of the human erythrocyte and many other cell membranes, respectively [23, 24]. Chemically the two lipids only differ in their terminal amino groups, these being NH_3^+ in DMPE and $N(CH_3)_3^+$ in DMPC. Moreover, both molecular conformations are very similar in their dry crystalline phases; their acyl chains are mostly parallel and extended with the polar groups lying perpendicularly to them; however, DMPE molecules pack tighter than those of DMPC. This effect, which is due to the DMPE smaller polar group and higher effective charge, makes for a very stable multilayer arrangement which is not significantly perturbed by the presence of water [32] or several metal ions [25–31]. On the other hand, the gradual hydration of the DMPC bilayers leads to water filling the highly polar interbilayer spaces. Consequently, there is an increase in its bilayer repeat from 54.5 Å when dry up to 64 Å when fully hydrated at a temperature below that of its main transition. This condition promoted the incorporation of the Al(III) ions and peptides into the DMPC highly polar interbilayer space and the ensuing molecular perturbation of the phospholipid bilayer structure.

These results allow us to postulate a simplified mechanism of action of Aβ isoforms and aluminum at the erythrocyte membrane level. The incorporation of Al(III) ions into the outer monolayer and the ensuing interaction with phosphatidylcholine phosphate groups [34] disrupts their arrangement resulting in a more fluid condition. On the other hand, Aβ interactions with Al(III), with at least four aluminum ions bound to each peptide molecule [35], stabilize certain conformational states of the peptides; this allows them a greater degree of penetration in the bilayer with which the effect of alteration of the molecular order is much greater. This interleaving of the peptide includes the hydrocarbon chain region of the bilayer producing a net disorder in both bilayer zones (polar heads and the hydrophobic chains). The amphoteric and amphiphilic characteristics of the Aβ peptides endow these molecules with a capacity to interact with lipids [18]. The insertion of Aβ in the lipid bilayers is relatively easy. However, there are differences in the conformation that the peptide adopts once inserted. The Aβ1-42 has two additional hydrophobic residues, isoleucine and alanine, at the C-terminus [36]. These last two residues add significantly to the hydrophobicity of the C-terminus because both of them are nonpolar, and isoleucine is strongly hydrophobic. Therefore, Aβ1-42 has a greater tendency than Aβ1-40 to get only partially inserted in the bilayer, i.e., the last several residues are not anchored in the lower head region of the bilayer. That means that its conformation is more flexible than that of Aβ1-40. According to Mobley et al. [37], this situation with the 1-42 peptide hanging up in the upper leaflet facilitates the formation of harmful pores or channels. As indicated by Curtain et al. [38] the interaction of Aβ with the lipid matrix of neuronal cell membranes plays an important role in the pathogenesis of AD. On the basis of these results, the controversy surrounding the participation of aluminum in the pathology of AD is partly clarified: aluminum enhances the injurious effects of Aβ peptides in cell membranes [39].

Experimental

SEM studies on human erythrocytes

Blood (0.05 cm^3) was obtained from healthy male volunteers by aspiration into tuberculin syringes containing 1 cm^3 heparinized saline/buffered solution (154 mM NaCl, 10 mM Tris–HCl pH 7.4; 50 UI/cm^3 heparin); this mixture was centrifuged at 1,000 rpm for 10 min, the supernatant extracted, and the pellet was washed three times with 1 cm^3 of saline. The following specimens were prepared from the pellet: (a) control, by mixing it with 0.1 cm^3 of saline, and (b) Aβ samples by adding 0.1 cm^3 of adequate concentrations. Samples were incubated at 37 °C for 1 h and then fixed overnight at 5 °C by adding one drop of each sample to plastic tubes containing 1 cm^3 of 2.5% glutaraldehyde in saline, reaching a final fixation concentration of about 2.4%. The samples were centrifuged at 1,000 rpm for 10 min, and the fixed samples were directly placed on Al stubs, air dried at 37 °C for 30 min to 1 h, and gold-coated for 3 min at 10^{-1} Torr in a sputter device (Edwards S150, Sussex, England). Resulting specimens were examined in an Etec Autoscan SEM (Etec Corp., Hayword, CA, USA).

X-ray diffraction studies of phospholipid multibilayers

The capacity of Aβ and Al(III) to perturb the structures of DMPC and DMPE multilayers was determined by X-ray diffraction. Synthetic DMPC (lot 80H-8371 A grade MW 677.9), DMPE (lot 68F-8350 A grade MW 635.9) from Sigma, Aβ(1-40) (lot Q9640C, MW 4331), and Aβ(1-42) (lot Q9644, MW 4515) from Biosource International Inc. (CA, USA) and $AlCl_3$ (Merck) were used without further purification. About 6 mg of each phospholipid mixed with 0.2 cm^3 of (a) distilled water, aqueous solutions of (b) $AlCl_3$, (c) Aβ, and (d) mixtures of $AlCl_3$ and Aβ in a range of concentrations was incubated for 1 h at 37 °C (DMPE was previously incubated at 58 °C for 20 min). The reported concentrations of aluminum in the human brain of patients suffering from Alzheimer's disease range between 18.5 and 37 μM, and those of Aβ range between 0.6 and 185 μM [40]. On the basis of this information, we studied

first the effects of aluminum on lipid bilayers in the 1–100 μM range. Afterwards, a range of Aβ concentrations were used, 1 nM being the minimum concentration and 20 μM the maximum. An aliquot of 0.1 cm^3 of each sample was transferred to 1.5-mm-diameter special glass capillaries (Glas Technik and Konstruktion, Berlin, Germany), and centrifuged at 2,000 rpm for 10 min. The specimens were immediately subjected to X-ray diffraction in flat plate cameras provided with rotating devices. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered Cu Kα radiation from a Philips PW 1140 X-ray generator (The Netherlands) was used. The reflection intensities on films versus observed spacing were obtained using a Bio-Rad GS-700 (CA, USA) microdensitometer and Bio-Rad Molecular Analyst/PC image software. The experiments were performed at 17 $\pm$ 2 °C, which is below the main phase transition temperature of both DMPC and DMPE. Each experiment was repeated three times and in case of doubts additional experiments were carried out.

Acknowledgments The authors thank Fernando Neira for his valuable technical assistance. This work was supported by a grant from FONDECYT (1090041).

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