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Visualization of Protein Penetration from an Aqueous Subphase into a Lipid Monolayer at an Air-Water Interface by Gold Marker

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Immunoelectron microscopic examinations provided a direct evidence that Staphylcoccus aureus α-toxin, an amphiphilic protein, could penetrate into a monolayer of L-α-dioleoyllecithin prepared on an aqueous subphase containing the toxin.

Proteins, together with lipids, form the membranes which surround and control the functioning of living cells. A growing field of research concerns with artificially created model systems of cells and cell membranes. Various attempts have been made to immobilize proteins into an organic film system. The so-called Langmuir-Blodgett (LB) film technique seems to have an important advantage over some of the complementary techniques of membrane mimetic chemistry. However, it has generally been recognized[1] that the technique for preparing LB films of proteins is not as simple as that of conventional insoluble monolayers. To our knowledge, there has been no direct evidence that protein molecules can be tightly immobilized in an LB film system. If protein molecules could penetrate into a lipid monolayer, they might be tightly immobilized in the film. In addition, there has been no profitable technique for providing direct evidence that protein molecules could penetrate into a lipid monolayer.

All the experiments were conducted at 25 ± 1 °C, unless otherwise stated. The instrument of Fromherz[2] (a circular trough with eight compartments to allow transfer of a monolayer from one subphase to another) was used in this study. This LB film technique seems to allow the study of protein penetration into a lipid monolayer by its transfer on an aqueous subphase without protein onto another with protein. Selection of protein for this study of protein penetration into a lipid monolayer is crucial. The α-toxin produced by Staphylcoccus aureus, which often causes infections leading to abscess formation, is an extracellular water-soluble protein.[3] When this toxin comes into physical contact with a hydrophobic environment such as membrane bilayer or sodium deoxycholate micell(s), the toxin enters and forms a lipid-soluble oligotoxin aggregate.[3] We assumed that when α-toxin was dissolved in an aqueous solution and a lipid monolayer was prepared on the protein solution, the toxin molecules might be able to penetrate into the monolayer. The

toxin was prepared according to the procedure of Ikigai and Nakae.[4] The toxin and its anti-α-toxin IgG were donated by Dr. Hajime Ikigai (Showa University, School of Medicine). Ikigai and Nakae[5] found that the presence of the unsaturated fatty acids in the target membrane is a prerequisite for the assembly of the toxin aggregate, but that this is not essential for toxin-membrane interaction. On the basis of their informations,[3-5] a monolayer of L-α-dioleoyllecithin (DOL) was used for the study of protein penetration. Each compartment of the Fromherz trough was fullfilled with Tris-HCl buffer (pH 8.5), in which the toxin molecules were found to be functionally stable.[3-5] DOL was obtained from Sigma Co., U.S.A. An appropriate volume of a DOL solution in chloroform (20mg/100 ml) was delivered onto the buffer solution without α-toxin to form a DOL monolayer. When necessary, the monolayer was transferred onto an aqueous subphase with α-toxin. A single DOL monolayer with or without α-toxin was deposited onto a copper grid (300 mesh, 3 μm diameter) covered with a plasma polymerization film, which was prepared according to the same procedure as described by Tanaka et al.[6] The apparatus for plasma polymerization (Model PNR-110, Ushio) consists of a high voltage power supply, a vacuum chamber containing naphthalene, and electrodes of an anode disk and a cathode of the specimen base. Plasma polymerization of naphthalene in negative glow phase on the cathode was carried out by gassing at 0.05-0.1 Torr and glow dischargeing at 1.5-3 kV D.C. Ionized hydrocarbon molecules deposited onto the surface of a single crystal of sodium chloride as a three dimensionally polymerized film of 10-50 nm in thickness. After the salt crystal was dissolved in water, the resultant film was then placed on the grid. The surface nature of the polymer film was hydrophilic. Thus, the surface nature of a single DOL-coated polymer film was hydrophobic (see Fig. 1 (a)). Distribution of the toxin molecules penetrated into a DOL monolayer was elucidated by the protein-A-gold immunoelectron microscopic method.[7] Protein A-Gold (PAG) was purchased from Jansen Life Science Products, Belgium. PAG was colloidal particles of 10 nm diameter. The gold particles was coated with Staphylococcus aureus Protein A. The method allowed to visualize the toxin distribution in a monolayer when used in conjugation with the specific antibody (IgG) coupled to colloidal gold. Each monolayer deposited on the polymer film was examined in a JEOL 100 S or 1200 EX electron microscope.

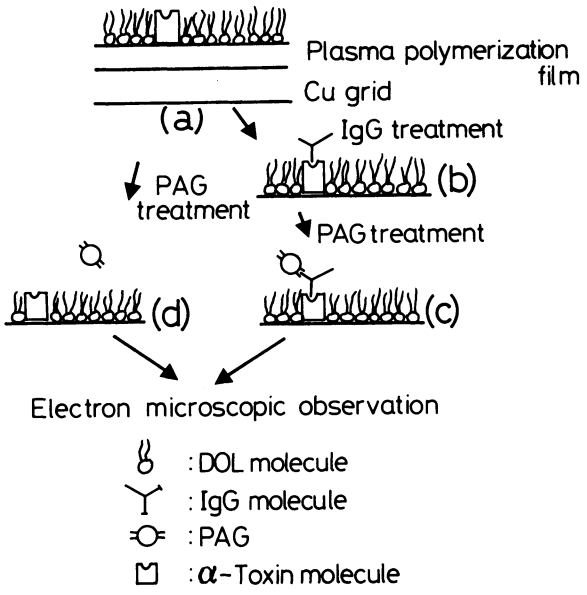

Fig. 1. Procedures for the immunochemical treatment of a monolayer on a plasma polymerization film.

As a preliminary study, plausible uptake and release of α-toxin into and from a DOL monolayer at an air-water interface were examined by measurements of force-area (F-A) characteristics (see Fig. 2). Curve (a) in Fig. 2 shows a

typical F-A curve of a DOL monolayer. A DOL monolayer at a surface pressure of 10 mN/m was transferred across a comartment onto a subphase containing 0.55 μg/ml α-toxin. Under a constant surface pressure controlled at 10 mN/m, the area per DOL molecule in the monolayer between the automated movable barriers was found to increase, probably due to the result of α-toxin uptake into the monolayer. After an appropriate interval, the proteo-lipid monolayer was compressed and curve (b) was obtained as its F-A characteristics. Curve (b) bore some expanded nature up to 38 mN/m and was found to coincide with that of curve (a) from 38 mN/m to its collapsing pressure suggesting the release of α-toxin from the monolayer. An F-A curve of the monolayer on the aqueous subphase containing 0.55 μg/ml α-toxin was recorded from a surface pressure of 50 mN/m upto that of 10 mN/m (see curve (c)). Curve (c) was found to coincide with curve (b). This finding suggests that the lowering of surface pressure given to the monolayer upto 38 mN/m seems to allow the re-uptake of α-toxin molecules into the DOL monolayer. In addition, an F-A curve of the monolayer transferred onto the subphase with the toxin and then returned onto the toxin-free subphase at a surface pressure of 50 mN/m was recorded by lowering the surface pressure given to the monolayer (see curve (d)). The feature of curve (d) was found to be similar to that of curve (a). This finding seems to suggest that the complete release of α-toxin molecules from the DOL monolayer at a surface pressure of 50 mN/m. Comparison of curves (a), (b), (c), and (d) in Fig. 1 indicates that the uptake and release phenomena of α-toxin into and from a DOL monolayer were reproducible under the present experimental conditions when the surface pressure given to the monolayer was well-controlled.

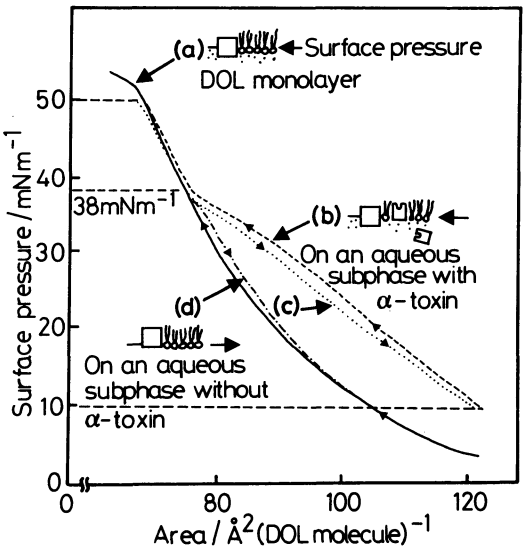

Fig. 2. F-A curves of DOL and proteo-DOL monolayers. For further explanations, see the text.

Figure 1 illustrates a procedure for the treatment of a proteo-DOL monolayer. A DOL monolayer prepared on the aqueous subphase containing 0.55 μg/ml α-toxin was deposited onto the plasma polymerization film-coated grid at a surface pressure of 20 mN/m. A drop of anti-α-toxin IgG in the Tris-HCl buffer was uniformly placed on the monolayer-coated side of the grid for 24 h at 4 °C (see (b) in Fig. 1). The antigen-treated monolayer was rinsed well with the buffer to remove non-specifically binding IgG. In addition, a drop of PAG solution was spread on the IgG-treated surface of the monolayer in order to form the product among α-toxin, IgG, and PAG (see (c) in Fig. 1). The reaction time was 24 h at 25 ± 1 °C. An exhausitively rinsed grid was then applied to transmission electron microscopy (TEM). As a reference, a DOL monolayer-coated grid, which was prepared under the same conditions as described above, was directly treated with PAG (see (d) in Fig. 1).

Figure 3 shows a typical TEM of the IgG and then PAG-treated specimen. As seen herein, a relatively high density of gold markers was observed. If the toxin molecules were unable to penetrate into the DOL monolayer, the IgG molecules were unable to react with the toxin molecules and the PAG molecules were unable to form any specific bond on the monolayer showing no gold marker in its TEM. The observed high density of gold markers of 10 nm mean diameter in Fig. 3 indicates that α-toxin molecules could penetrate into the DOL monolayer under the present experimental conditions. To our knowledge, this report seems to provide the first direct evidence that protein molecules can penetrate into a lipid monolayer.

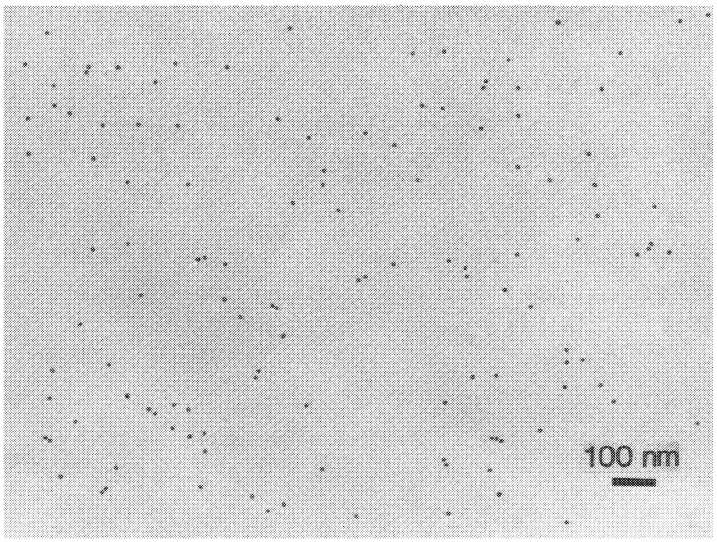

Fig. 3. Transmission electron microscopy of IgG and PAG treated specimen.

As demonstrated above, combination of the LB film technique developed by Fromherz,[2] the PAG immunoelectron microscopic method,[7] and the plasma polymerization method[6] seems to be widely applicable for the study of proteins in the well-controlled organic thin-film systems.

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