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INTERACTION OF RICIN AND ITS CONSTITUENT POLYPEPTIDES WITH DIPALMITOYLPHOSPHATIDYLCHOLINE VESICLES

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The interaction of ricin and of its constituent polypeptides, the A- and B-chain, with dipalmitoylphosphatidylcholine (DPPC) vesicles was investigated. The A- and B-chain were individually associated with DPPC vesicles, although the intact ricin was not associated. The maximum binding and association constants were evaluated to be 154 μg per mg of DPPC and $K_a = 2.30 \cdot 10^5\ M^{-1}$ for the A-chain, and 87 μg per mg of DPPC and $K_a = 14.5 \cdot 10^5\ M^{-1}$ for the B-chain, respectively. The A-chain could induce the phase transition release of carboxyfluorescein from DPPC vesicles to a greater extent than the B-chain, whereas the release induced by the intact ricin was negligible. The evidence indicated that the hydrophobic regions on the A-chain and on the B-chain were buried inside when the two chains constituted the intact ricin molecule through one interchain disulfide bond, and that the A-chain caused perturbation of the DPPC bilayer at the phase transition temperature with consequent leakage of carboxyfluorescein.

Introduction

Recently, several proteins including cytoskeletal proteins [1–4] and toxic proteins [5–8] have been shown to interact specifically with phospholipid vesicles and planar lipid bilayers. A few papers on the hydrophobic nature of toxic proteins, in particular, have been published so far in relation to their cytotoxicity [9–11]. For example, it has been apparent that the localization of a hydrophobic moiety in toxic proteins such as cholera toxin [6], diphtheria toxin [5] and tetanus toxin [10] is very important to manifest their toxicity.

Ricin is a potent plant toxin from *Ricinus communis*, and consists of two polypeptide chains (i.e., A-chain and B-chain) held together through only one disulfide linkage [12]. The function of the A-chain is to inhibit irreversibly protein synthesis in eukaryote cells by attacking the 60S subunit in ribosomes by an unknown mechanism [13,14]. The B-chain has the capacity to recognize and bind to a galactose residue in a receptor glycoprotein on the cell surface [15,16]. For its cytotoxicity against susceptible cells, the events are essentially as follows: the ricin molecule is endocytosed into the cell by receptor mediated pinocytosis with subsequent transfer into the cytoplasm; alternatively, the ricin may transport either itself or a constituent A-chain directly into the cytoplasm through the plasma membrane after binding to cell surface receptors.

In any event, either ricin or a constituent A-chain must cross the plasma membrane or the pinosome membrane to exert its toxicity in the cytoplasm, with the reduction of a disulfide bond between the A-chain and the B-chain by a specific enzyme or reductant either in a series of events of membrane transportation or in the cytoplasm.

In order to further explore the mechanism of A-chain translocation through the lipid membrane, the elucidation of the hydrophobic nature of the ricin molecule would be helpful. Within this

framework, we focussed our attention on the respective interaction of the ricin, A-chain and B-chain with DPPC vesicles, and clarified the fashion of interaction of those polypeptides with lipid vesicles both by detailed analysis of binding of polypeptides to DPPC vesicles and by a novel method utilizing DPPC vesicles trapping of carboxyfluorescein [4].

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine (DPPC), lactoperoxidase and pronase were purchased from Sigma Chemical Co. Trypsin and chymotrypsin were obtained from Worthington Biochemical Co. Carboxyfluorescein was obtained from Eastman-Kodak Co. and purified as described in the report [4]. Ficoll 400 and Sepharose 4B were obtained from Pharmacia Fine Chemicals. Ricin and its constituent polypeptide chains (i.e., A-chain and carboxymethylated-B-chain (CM-B-chain)) were isolated by the methods established in our laboratory [12,17,18] and were homogeneous by SDS-polyacrylamide gel electrophoresis. $Na^{125}I$ (carrier free, specific activity 2 mCi/nmol) was purchased from Amersham International Ltd. All other reagents were of the highest analytical grade.

Preparation of DPPC vesicles

DPPC (136 μmol) was dissolved in 10 ml of a chloroform/methanol (2 : 1, v/v) mixture. 20 μmol of DPPC in 1.5 ml of the mixture were dried under vacuum in a conical glass tube. After addition of 3 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4), the dried DPPC was hydrated with repeated vortexed-mixing at 50°C for 30 min. The suspension was sonicated at 50°C for 60 min using a Branson Sonifier Model W-185 and then centrifuged at $1500 \times g$ for 10 min to remove titanium particles. The mixture of multi- and unilamellar vesicles was used for the study of protein-lipid interaction without further purification. When the unilamellar vesicles trapping carboxyfluorescein were prepared, the same method as described above was used except that the dried DPPC (15 μmol) was hydrated in 1.0 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4), containing 100 mM carboxyfluorescein. The mixture of the multilamellar and unilamellar vesicles trapping carboxyfluorescein was subjected to gel-filtration through a Sepharose 4B column (1×20 cm) in 0.1 M NaCl/20 mM phosphate buffer (pH 7.4). The separated small unilamellar vesicles, with a phase transition of 37–38°C, were utilized in phase-transition release measurements.

Labeling of ricin, A-chain and CM-B-chain

The labeling of the ricin and its constituent polypeptides was carried out by the lactoperoxidase method in a manner similar to that described by Marchalonis [19]. To 1 mg of the polypeptide was added 0.5 mCi of carrier free-$Na^{125}I$ in the presence of unlabeled NaI to give an average of 1 mole of iodine per mole of protein. Lactoperoxidase (10 μg) and H_2O_2 (200 μg) were added and the mixture was incubated at 20°C for 60 min. The mixture was subjected to gel-filtration through a Sephadex G-25 column to remove free iodine. ^{125}I-Ricin, ^{125}I-A-chain and ^{125}I-CM-B-chain were precipitable to an extent greater than 95% in 10% trichloroacetic acid. The specific activities of ^{125}I-ricin, ^{125}I-A-chain and ^{125}I-CM-B-chain were 136.0 μCi, 165.2 μCi and 60.2 μCi per mg, respectively. Finally, each of the ^{125}I-labeled polypeptides was diluted with its respective unlabeled carrier polypeptide to a specific radioactivity of $8 \cdot 10^5$ cpm per mg before use.

Interaction of polypeptide with DPPC vesicles

A certain amount of ^{125}I-labeled polypeptide in the range of 25 μg to 500 μg was mixed with DPPC vesicles (1.0–2.5 mg) in 1 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) at 20°C, and incubated further at 20°C for 30 min. The incubation mixture was brought to the density of 1.05 by addition of an equal volume of Ficoll 400 solution with a density 1.10. The mixture was layered on 2 ml of a 1.08 density solution, and then 2-ml portions of the different density solutions were layered over in turn to produce a discontinuous density gradient, 1.08, 1.05, 1.03 and 1.01. All density solutions were prepared with Ficoll 400 in 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) [20]. The mixture was subjected to the discontinuous density gradient centrifugation at $100000 \times g$ for 60 min at 20°C by using a

Hitachi Model 55P-7 ultracentrifuge, and fractionated in 0.5 ml portions. Radioactivity in each fraction was determined in a γ-well counter (Aloka ARC-501).

Fluorescence measurement

Fluorescence measurements were made on a Shimazu Spectrophotofluorometer RF-510 equipped with a Haake PK-10 water bath to maintain the temperature in the cuvette holder. The temperature of the fluid in the cuvette was monitored by using a Kyoritsu temperature probe Model 7045. Carboxyfluorescein was excited at 470 nm and emission at 515 nm was recorded.

Phase-transition release

Self-quenching of the fluorescence of carboxyfluorescein at a high concentration is derived from dye-dye interaction [21]. Apparently, the total fluorescence intensity of the dye trapped within DPPC vesicles at 100 mM concentration is only 5% of that obtained when the dye is released from the vesicles into the entire suspension volume [1]. Thus the release of carboxyfluorescein could be easily monitored by measuring the increase in fluorescence intensity. The following protocol was employed for phase transition release measurements. DPPC vesicles, the individual polypeptides and 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) were individually pre-chilled on ice. to 2 ml of phosphate buffer in the cuvette on ice, were added 50 μl of DPPC vesicles containing 100 mM carboxyfluorescein to give a final concentration of 20 μM DPPC. 50 μl of an appropriate dilution of polypeptide in phosphate buffer were added to the mixture. The cuvette was placed in the heated (42°C) cuvette holder of the fluorometer equipped with mechanical stirring, and both the fluorescence intensity and the temperature were continuously recorded. For determination of the fluorescence intensity derived from 100% dye-release, 10 μl of Triton X-100 solution (20% in phosphate buffer) were added to dissolve the vesicles. The percentage of dye release caused by the polypeptide was evaluated by equation, $100 \times (F - F_o)/(F_t - F_o)$, where F is the fluorescence intensity achieved by polypeptides, F_o and F_t are those of post-transition fluorescence without polypeptides and post-Triton X-100 treatment, respectively.

Results

Interaction of ricin, A-chain and CM-B-chain with DPPC vesicles

Individual interactions of ^{125}I-ricin, ^{125}I-A-chain and ^{125}I-CM-B-chain with DPPC vesicles were examined quantitatively by means of Ficoll 400 discontinuous density gradient centrifugation. Fig. 1 shows the typical profiles of the separation of stable ^{125}I-polypeptide-DPPC vesicle complex free of unbound ^{125}I-labeled polypeptide. The ^{125}I-polypeptide-DPPC vesicle complexes were floated upward to the layer with a density of 1.03, and DPPC vesicles alone upward to the interface between 1.03 and 1.01, as shown by arrows in Fig. 1. An appropriate amount of ^{125}I-A-chain, as well as ^{125}I-CM-B-chain associated with DPPC vesicles when each of the ^{125}I-labeled polypeptides was

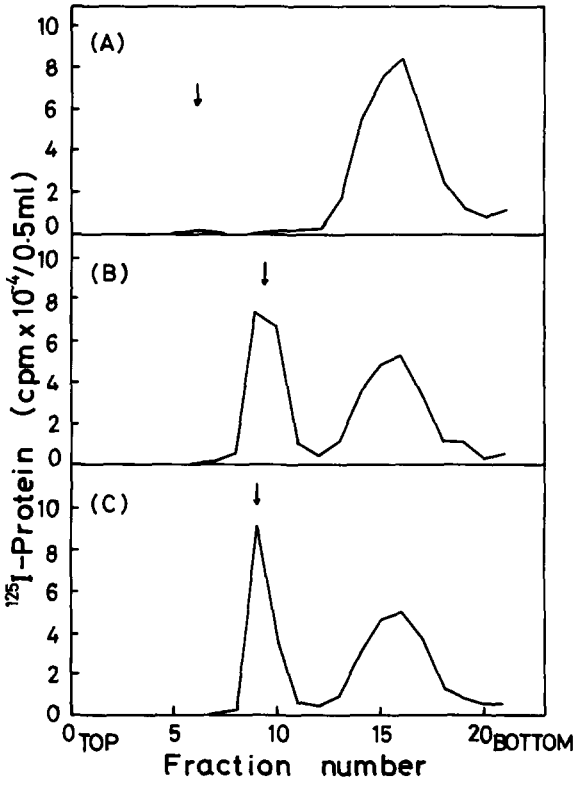

Fig. 1. Ficoll 400 discontinuous density gradient centrifugation of DPPC vesicles with ^{125}I-ricin, ^{125}I-A-chain or ^{125}I-CM-B-chain. Arrows indicate the position of vesicles alone in (A), and vesicles with ^{125}I-A-chain in (B) and with ^{125}I-CM-B-chain in (C), respectively. Details are in the text.

incubated at 20°C for 30 min, as can be seen in Figs. 1B and 1C. The association of ^{125}I-ricin with DPPC vesicles, on the contrary, was negligible under the same incubation conditions as those used for the ^{125}I-A-chain and the ^{125}I-CM-B-chain, as shown in Fig. 1A. Therefore, interaction of either ^{125}I-A-chain or ^{125}I-CM-B-chain with DPPC vesicles was analyzed in detail by this established technique.

In Fig. 2A, the amount of ^{125}I-A-chain bound to 1 mg of DPPC vesicles is plotted as a function of increasing protein concentration. The same data are shown in the inset as a double reciprocal plot. From the intercepts on the vertical and horizontal axes, a maximum binding of ^{125}I-A-chain was evaluated to be 154 μg per mg of DPPC vesicles

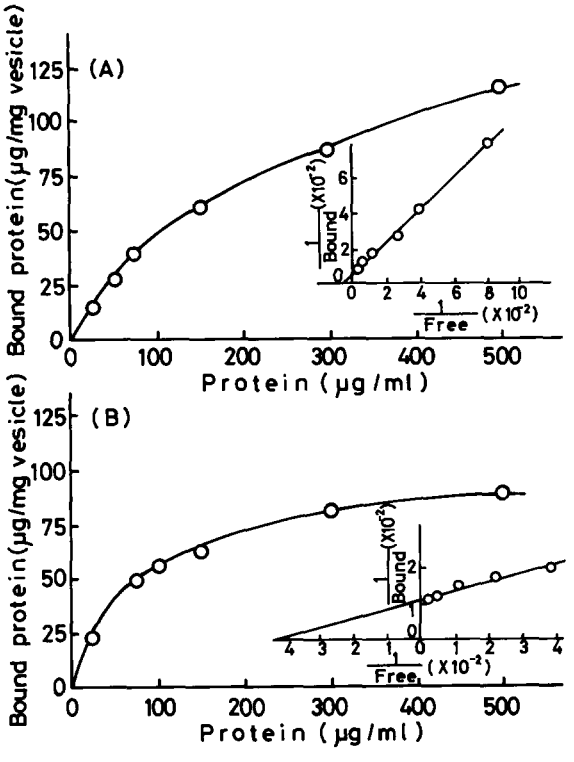

Fig. 2. Binding of ^{125}I-A-chain or ^{125}I-CM-B-chain to DPPC vesicles as a function of protein concentration. Individual ^{125}I-labeled chains at different concentrations were incubated with 1 mg of DPPC vesicles in 1 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) at 20°C for 30 min. The reaction mixture was subjected to Ficoll 400 discontinuous density gradient centrifugation to separate the bound ^{125}I-labeled chain from the free one. (A) ^{125}I-A-chain, (B) ^{125}I-CM-B-chain.

with an association constant, $K_a = 2.30 \cdot 10^5$ M^{-1}. The curve and the double reciprocal plot shown in Fig. 2B are drawn to calculate a maximum binding and an association constant for the ^{125}I-CM-B chain in the same manner as that for the ^{125}I-A-chain. A maximum binding of ^{125}I-CM-B-chain was evaluated to be 87 μg per mg of DPPC vesicles with an association constant, $K_a = 14.5 \cdot 10^5$ M^{-1}.

After incubation of ^{125}I-A-chain (100 μg) or ^{125}I-CM-B-chain (100 μg) with 2.5 mg of DPPC vesicles at 20°C for 30 min, the mixtures were individually processed, in turn, with 10 μg of trypsin for 1 h, 10 μg of chymotrypsin for 1 h and 20 μg of pronase for 2 h at 20°C. The resulting mixtures were followed by the discontinuous density gradient centrifugation along with a protease-untreated mixture as a control experiment, and then fractionated in 0.5 ml portions for analyzing the radioactivity. In this experiment, it was observed that 27.5% of the total radioactivity of the ^{125}I-A-chain molecules initially associated with the vesicles was recovered in the vesicle fraction, whereas 9.6% of the total radioactivity in the case of the ^{125}I-CM-B-chain was recovered.

Effects of ricin, A-chain and CM-B-chain on release of carboxyfluorescein from DPPC vesicles

The typical profiles of the caroxyfluorescein release from DPPC vesicles as a function of temperature in the presence and in the absence of polypeptide are shown in Fig. 3. Temperature was scanned over the range of 0 to 40°C within 3 min following the completion of the dye leakage. In the absence of polypeptide, a slight increase in the release of carboxyfluorescein was produced, if the temperature was raised through the DPPC-phase transition. Non-detectable leakage, however, was observed below the phase transition temperature with and without polypeptides. On one hand when the temperature was raised through the phase transition, A-chain at 50 μg/ml induced a striking dye release from the vesicles, whereas CM-B-chain and intact ricin at 50 μg/ml caused 20% and 8% dye release, respectively, as seen in Fig. 3. DPPC-phase transition in temperature was apparently required for the dye release, although the extents of the dye release induced by polypeptides were different from one another.

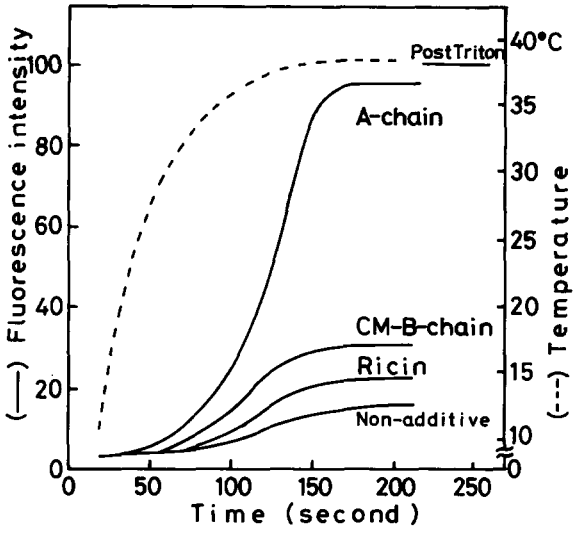

Fig. 3. Profiles of carboxyfluorescein release from DPPC vesicles with ricin, A-chain and CM-B-chain. Individual polypeptides (100 μg) were incubated in 2 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) containing 20 μM DPPC vesicles at temperatures from 0 to 40°C for the indicated scanning time.

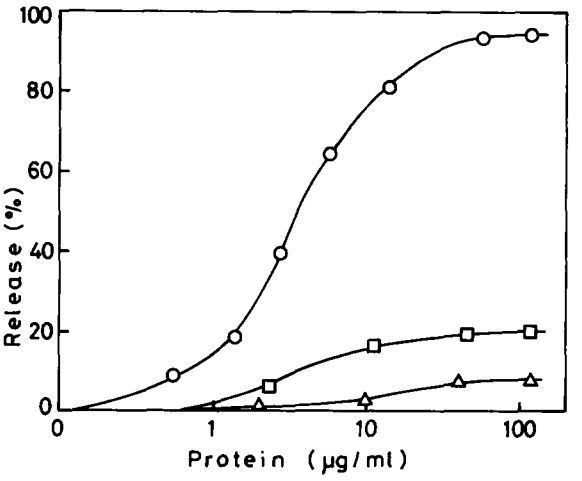

Fig. 4. Dependence of phase-transition release on the concentration of polypeptide. Different concentrations of polypeptides were incubated in 2 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) containing 20 μM DPPC vesicles at temperatures from 0 to 45°C for 2 min scanning time. △——△, Ricin; ○——○, A-chain; □——□, CM-B-chain.

Effect of polypeptide concentration on phase-transition release

The curves of phase-transition release as a function of increasing concentrations of polypeptides are shown in Fig. 4. The concentration required to induce 50% leakage for A-chain was approximately evaluated to be 4 μg/ml, and almost complete leakage was achieved with A-chain at concentrations higher than 20 μg/ml. The dye leakage potency of either CM-B-chain or ricin was much less than that of A-chain, at concentrations from 1 to 100 μg/ml. The evidence indicates that the induction of the dye release from the vesicles is due to the specific nature of the A-chain. In this experiments, the heating rate was changed by setting the temperature of the water bath at 45°C, as the dye leakage was completed during the 2-min temperature scan. This change in heating rate caused a nondetectable difference in carboxyfluorescein leakage compared to the 3-min temperature scan as described above.

Effects of temperature and pH on carboxyfluorescein leakage induced by A-chain

To confirm that carboxyfluorescein was released through phase transition at the transition temperature of the DPPC, the leakage induced by A-chain was measured at different starting temperature for 3 min of scanning. Almost the same degree of carboxyfluorescein release was observed, when the scanning was started at a lower temperature than the phase transition temperature, as can be seen in Fig. 5. Alternatively, negligible or no leakage was detected in the scanning temperature range from either 36.1°C or 37.1°C to 39°C, which were the temperatures higher than the phase-tran-

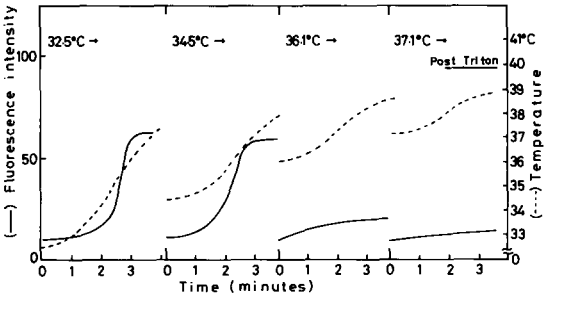

Fig. 5. Dependence of phase-transition release induced by A-chain on temperature. A-chain (10 μg) was incubated in 2 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4), containing 20 μM DPPC vesicles in the different ranges of scanning temperature.

sition temperature of DPPC. In adition, no change in the carboxyfluorescein release induced by A-chain was detected in the pH range of 6.5 to 8.0 in 0.1 M NaCl/20 mM phosphate buffer. These results demonstrate that the carboxyfluorescein release from the vesicle-A-chain complex arose from the passage through the phase transition temperature of DPPC, the pH having no effect.

Discussion

An attempt to explore the hydrophobic nature of ricin and its constituent polypeptides was made by analyzing their interaction with DPPC vesicles.

From the lack of association of ricin with DPPC vesicles, it was apparent that the intact ricin has no hydrophobic region on the surface of the molecule which is accessible to the lipid vesicles. In contrast, it was of interest that both A-chain and CM-B-chain could evidently associate with the lipid vesicles. This hydrophobic nature of the A-chain was coincident with the findings of Uchida et al. [11] that the A-chain could associate with phosphatidylcholine/cholesterol vesicles, but there was no description of the hydrophobicity of the B-chain. The CM-B-chain had 6.3-times the association constant of the A-chain, indicating that the CM-B-chain had a greater affinity to DPPC vesicles than the A-chain. The amount of maximum binding of the A-chain to the vesicles, however, was 1.8-times that of the CM-B-chain. From these findings that there were differences in their tendencies to associate with the vesicles, it was suggested that the A-chain and CM-B-chain have their individual characteristics in the fashion of polypeptide/lipid vesicles interaction. In addition, these differences in hydrophobic characteristics between A-chain and CM-B-chain were in harmony with the observation of Ishida et al. [22] on interaction individually of ricin, A-chain and B-chain with the membrane envelop of virus. With respect to the hydrophobicity of ricin, A-chain and B-chain, Houston et al. [23] reported a similar observation; the A-chain was more hydrophobic than the ricin or the B-chain (using 8-anilino-1-naphthalene sulfonic acid as a hydrophobic probe). This interpretation on the hydrophobic nature of the A-chain was supported by the fact that the A-chain bound to the vesicles was less susceptible to digestion with proteases than the CM-B-chain, suggesting the possibility that either the A-chain associated tightly with the surface of vesicles or it was incorporated into the lipid bilayer.

To examine this possibility, the phase-transition release of carboxyfluorescein from the vesicles was utilized, since this novel technique could provide an appropriate perspective on the fashion of physiological protein insertion into lipid bilayer, as pointed out [4]. The experiments indicated that the A-chain has an ability to induce complete dye-release from DPPC vesicles at temperatures through the phase transition of DPPC. Neither CM-B-chain nor ricin could induce the phase-transition release, even at concentrations of 5-times that required for the complete effect with A-chain.

Furthermore, it should be noted that no change in carboxyfluorescein release was detected in the presence of 1 M NaCl in the reaction mixture (data not shown), at different pH values as well. These findings suggested that carboxyfluorescein release depends mostly on the interaction of the hydrophobic regions, instead of that of the ionized groups, between the A-chain and the lipid bilayer.

On the basis of the experimental results that an incubation temperature lower than that of the phase transition of DPPC at the start of scanning was essential for the phase transition release, it was reasonable that the A-chain initially associated with the vesicles at lower temperature and then induced the carboxyfluorescein release, as a subsequent event, through the phase transition of DPPC.

In view of the specific carboxyfluorescein release with the A-chain, it is possible to conclude that the A-chain, different from the CM-B-chain and intact ricin, has an ability to penetrate through DPPC bilayers with sufficient perturbation of both leaflets of the bilayer. This interpretation is reasonably supported by the description of Weinstein et al. [4] that phase-transition release probably reflects a stable insertion of polypeptide into lipid bilayers with a concomitant increase in lateral compressibility of the lipid structure. Therefore, it is apparent that the very little induction of the dye leakage with CM-B-chain, despite its appreciably strong interaction with vesicles, is due to its hydrophobic region(s) which cause no perturbation of the lipid bilayer.

With in vitro and in vivo inhibition of protein synthesis with ricin, it is pointed out that the reduction of one interchain disulfide bond between the A-chain and the B-chain is necessary to reveal the inhibitory activity [24–26]. In this case, probably the subsequently exposed hydrophobic region(s) on the A-chain plays an important role in the processes which insert A-chain itself into the cytoplasm where it exhibits inhibitory activity for protein synthesis.

To obtain more detailed information about the physiological function of the A-chain at the molecular level, the topography of the hydrophobic domains on the A-chain molecule, which seem to be involved in penetration of the molecule through the lipid bilayer, is under investigation.

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