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Membrane-damaging action of ricin on DPPC and DPPC-cerebrosides assemblies

A Raman and FTIR analysis

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Abstract. Perturbations induced by a toxic lectin (ricin) on lipid organisation of model membranes prepared with DPPC and DPPC-cerebrosides mixtures have been analysed by Raman and infrared spectroscopy, two powerful and non-invasive methods. Our approach involves the observation of changes in the vibrational spectra of liquid multilayers in the PO_2^-, C=O and CH_2 spectral regions for two lipid : ricin molar ratios (225 : 1, 75 : 1).

The interfacial and polar regions of the multilayers, analysed by FTIR, appear to be perturbed by the protein. With both kinds of membranes, ricin mainly perturbs the C=O ester groups of the sn-2 acylchain of DPPC. In the PO_2^- stretching region, the frequency shifts are correlated with changes in polar group hydration.

In the hydrophobic core of the multilayer membrane studied by Raman spectroscopy, the interaction of ricin is associated with changes in lipid packing. These perturbations depend upon the lipid composition of the membrane. With DPPC membranes, an affect is detected at temperatures lower than T_m. It corresponds to a decrease of the lipid ordering. With DPPC-cer membranes, the protein increases the acylchain packing order regardless of the temperature of the experiments ($10\,°C < T < 75\,°C$). No perturbation of T_m is observed after addition of ricin to either DPPC or DPPC-cer membranes.

The different perturbations detected by Raman and FTIR suggest that ricin mainly interacts with the interfacial domains of the membranes.

Key words: Ricin, lipid–protein interactions, Raman, infrared

Introduction

Ricin is a cytotoxic protein with a molecular weight of approximately 60,000. This lectin consists of two subunits linked by a disulphide bridge. The A-chain inhibits protein synthesis by a modification of the 28S ribosomal RNA (Endo and Tsurugi 1987). Its internalisation is mediated by the B-chain which binds cell membranes via glycoproteins and/or glycolipids that contain terminal non-reducing galactose residues (Olsnes and Sandvig 1985). For several years, it has been realized that the molecule enters the cell via an endocytotic process and may cross the endosomal membrane to reach its cytosolic target. Although several groups have studied the interaction of ricin with model membranes (Surolia and Bacchawat 1978; Baenzinger and Fiete 1979; Utsumi et al. 1987), little information is available on the effects induced on the lipid organisation as the toxin interacts either with the plasma membrane or the endosomal membrane.

With the aim of elucidating the membrane damage resulting from the interaction with the protein we have undertaken, by infrared and Raman spectroscopy a comparative analysis of model membranes containing DPPC or [DPPC + cerebrosides] and ricin. To better mimic the "in vivo" membrane interaction with ricin, we have used cerebrosides extracted from bovine brain i.e. material containing nearly 80% of galactocerebrosides (Norton and Cammer 1984).

Using FTIR spectroscopy, the analysis of the stretching modes of carboxyl and phosphate groups of DPPC multilayers has allowed us to characterize the perturbations induced by cerebrosides or ricin in the polar and interfacial regions of the membrane. The results obtained show that the same spectral perturbation is observed in the C=O stretching region (1,800–1,600 cm^{-1}) whether we add the cerebrosides and ricin separately, or the two components simultaneously. In the PO_2^- stretching region (1,400–1,000 cm^{-1}), the DPPC spectrum is modified differently by cerebrosides and ricin.

Raman spectra were studied in the 3,000–2,800 cm^{-1} and 1,160–1,050 cm^{-1} ranges. Analysis of the 3,000–2,800 cm^{-1} region, which includes the con-

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tribution of the methyl and methylene CH stretching modes and Fermi resonances arising from interactions of the CH_2 stretching modes with the overtones of the CH_2 bending modes (1,430–1,460 cm^{-1}), provides information on the perturbations arising in the hydrophobic core of the membrane. The 1,160–1,050 cm^{-1} region includes the antisymmetrical and symmetrical C–C stretching modes (skeletal modes) and gives information on the presence of gauche conformers. Addition of ricin to DPPC multilayers decreases the acyl chain packing order at temperatures below T_m. In contrast, addition of ricin to DPPC-cer multilayers has a stabilizing effect on the lipid packing characteristics at temperatures below and above T_m. With both model membranes, the same result is observed for L : P ratios of either 75 : 1 or 225 : 1.

Materials and methods

L-α Phosphatidylcholine dipalmitoyl (DPPC), *N*-palmitoylcerebrosides (cer) extracted from bovine brain (thus containing a high content of galactocerebrosides) were purchased from Sigma. Ricin was obtained from *Ricinus communis* seeds according to the procedure of Nicolson and Blaustein (1972). After dialysis against distilled water, the ricin concentration was determined by absorbance measurements at 280 nm using $a = 1.41 \cdot g^{-1} \cdot cm^{-1}$ (Zentz et al. 1978).

Reconstitution procedure

DPPC or [DPPC + cerebrosides] (at a molar ratio equal to 3 : 1) multilamellar structures were prepared by hydratation of the powders in distilled water, heating and then vortexing. Ricin is added to these preformed lipid multilayers to yield final lipid : protein molar ratios of 225 : 1 and 75 : 1. Since the number of toxin molecules entering the cell is not yet determined, these L : P ratios were chosen so as to detect any membrane perturbation. The samples were lyophilised in order to improve the protein–lipid interaction, as previously described (Lavialle et al. 1985). This technique also allows one to reach the high concentrations required for vibrational analysis. For comparison, DPPC and DPPC-cer multilamellar structures were also lyophilised.

Raman experiments

Raman spectroscopic experiments were performed on a Jobin-Yvon Ramanor HG2S double spectrometer, using a Spectra Physics Ar^+ laser. We used the 514.5 nm laser line with a power level of 400 mW.

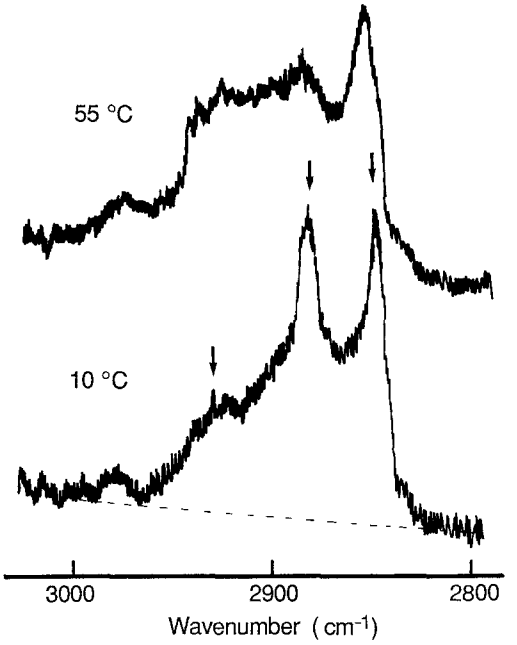

Fig. 1. Raman spectra of the C–H stretching modes at 10 °C (*lower*) and 55 °C (*upper*) of a DPPC-cer aqueous solution

Lyophilised lipid-protein mixtures were resuspended in H_2O. The final lipid concentration was about 120 $mg \cdot ml^{-1}$. After sealing, capillaries containing the multilayer samples were placed within a cryostat thermostatically controlled to ±1 °C. The temperature curves were determined from low to high temperatures; the samples were equilibrated for nearly 15 min at each temperature before scanning.

The scattered light, detected at right angles to the incident light, was collected on the photocathode of a cooled photomultiplier and amplified by a DC operational amplifier.

The spectral resolution is 5 cm^{-1}. The estimated experimental error in the calculation of the peak height intensity ratios made by amplitude measurement is ±0.03 for I_{2880}/I_{2850} and I_{2935}/I_{2880} peak intensity ratios and ±0.1 for the I_{1069}/I_{1099} ratio. The baseline was taken by drawing a straight line between 2,800 and 3,000 cm^{-1} as illustrated in Fig. 1. The size of the symbols on Figs. 2 and 4 takes into account the experimental error.

Infrared measurements

Samples were obtained by dissolving 2 mg of the lyophilised mixture in 40 µl of distilled water. Films were then cast directly on CaF_2 windows by drying under vacuum. Spectra were recorded on a Nicolet 5DX spectrometer equipped with a TGS detector. In order to eliminate spectral contributions of atmospheric water vapour, the instrument was purged with

dry air. For each spectrum, 100 scans at 4 cm^{-1} resolution were collected, co-added, apodized with a Happ-Genzel function and Fourier transformed.

Experimental results

1) DPPC, DPPC-cerebroside assemblies

Figure 1 displays representative Raman spectra for the CH stretching mode region of DPPC-cerebrosides membranes at 10° and 55 °C. They are quite similar to those obtained with DPPC. The 2,850 and 2,880 cm^{-1} features correspond respectively to the symmetric and antisymmetric methylene CH stretching modes of the lipid acyl chains. When melting occurs, the antisymmetrical mode intensity decreases in relation to the symmetrical one. The peak height intensity ratio I_{2880}/I_{2850} measures lateral interactions of adjacent chains (Yellin and Levin 1977; Wallach et al. 1979) and allows one to characterise the perturbations induced in the lipid acyl chain organisation.

Figure 2a shows the temperature profiles for DPPC and DPPC-cerebrosides formed from the ratio I_{2880}/I_{2850}. As shown in this figure and according to previous results (for review see Levin 1984), pure DPPC bilayers undergo two transitions at 35 °C (pretransition) and 40°–41 °C (main transition). At 10 °C, the I_{2880}/I_{2850} ratio is equal to 1.12 and decreases to 0.95 just before the onset of the transition. Taking into account the experimental error, this difference is significant. From 42 °C to 75 °C the ratio value remains constant and equal to 0.75.

At low temperatures (10°–39 °C) addition of cerebrosides leads to a large decrease of the I_{2880}/I_{2850} ratio value which drops at 10 °C from 1.12 to 0.95. Simultaneously, the I_{1069}/I_{1099} ratio, which reflects the trans/gauche isomerisation arising along the lipid acyl chain, decreases from 1.4 to 1.2 (Table 1). At 39 °C, just before the transition, the I_{2880}/I_{2850} ratio value obtained with DPPC-cer is equal to 0.85 i.e. significantly lower than the value calculated for pure DPPC. The transition still occurs at 40°–41 °C. The absence of perturbation of the transition temperature may result from the fact that DPPC and cerebrosides used in this study (*N*-palmitoylcerebrosides) have the same acylchain length. Above this temperature, no significant perturbation of the I_{2880}/I_{2850} ratio is detected after addition of cerebrosides.

Figure 2b presents the temperature profiles based on the peak height intensities for the methyl and methylene stretching modes at 2,935 and 2,880 cm^{-1}. This ratio is used to probe intrachain disorder in the core of the bilayer. At 10 °C the ratio value I_{2935}/I_{2880} is equal to 0.35 for pure DPPC. Just before the transition it reaches 0.5. Above T_m, a constant value (0.83) is calculated. Up to 50 °C, no significant perturbation of the profile is detected after addition of cerebrosides. Above 50 °C, a slight but significant change of the ratio value is observed. At 70 °C, the $I(2935)/I(2880)$ index is found to be equal to 0.95 for DPPC-cer instead of 0.80 found for pure DPPC.

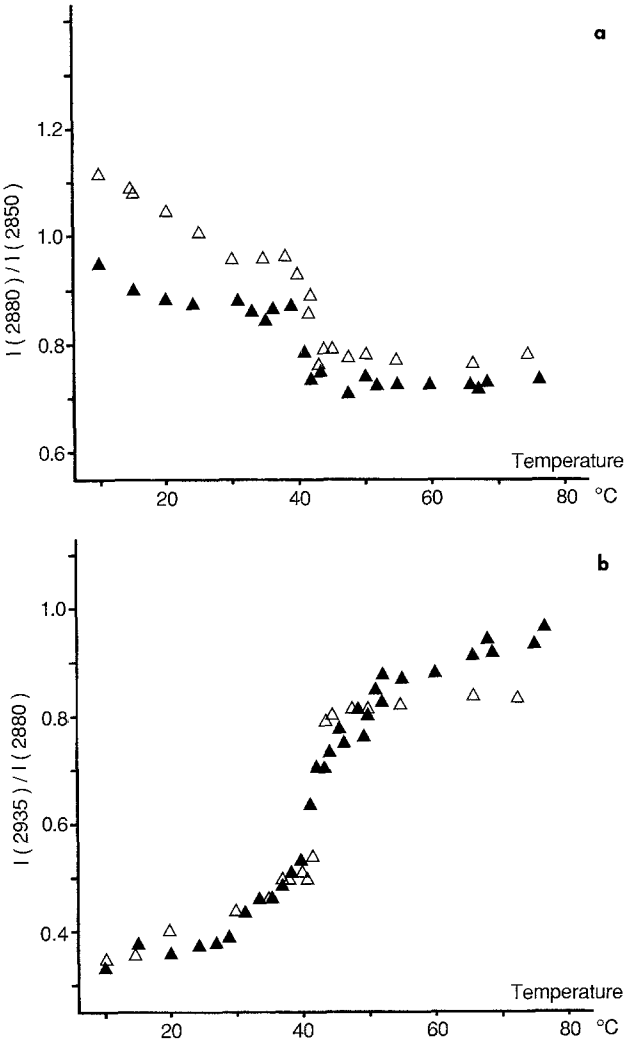

Fig. 2a and b. Temperature profile ratio of DPPC (*open triangles*) and DPPC-cer (*full triangles*) constructed from **a** I_{2880}/I_{2850} ratio and **b** I_{2935}/I_{2880} ratio

Table 1. I_{1069}/I_{1099} and I_{2880}/I_{2850} peak height intensity ratios measured at 10 °C. All the samples were lyophilised except those noted

Samples	I_{1069}/I_{1099} ±0.1	I_{2880}/I_{2850} ±0.03
Non lyophil. DPPC	1.8	1.34
Non lyophil. sonic. DPPC	1.3	1.07
DPPC	1.4	1.12
DPPC-cer	1.2	0.95
DPPC-ricin	1.3	1.03
DPPC-cer-ricin	1.2	1.10

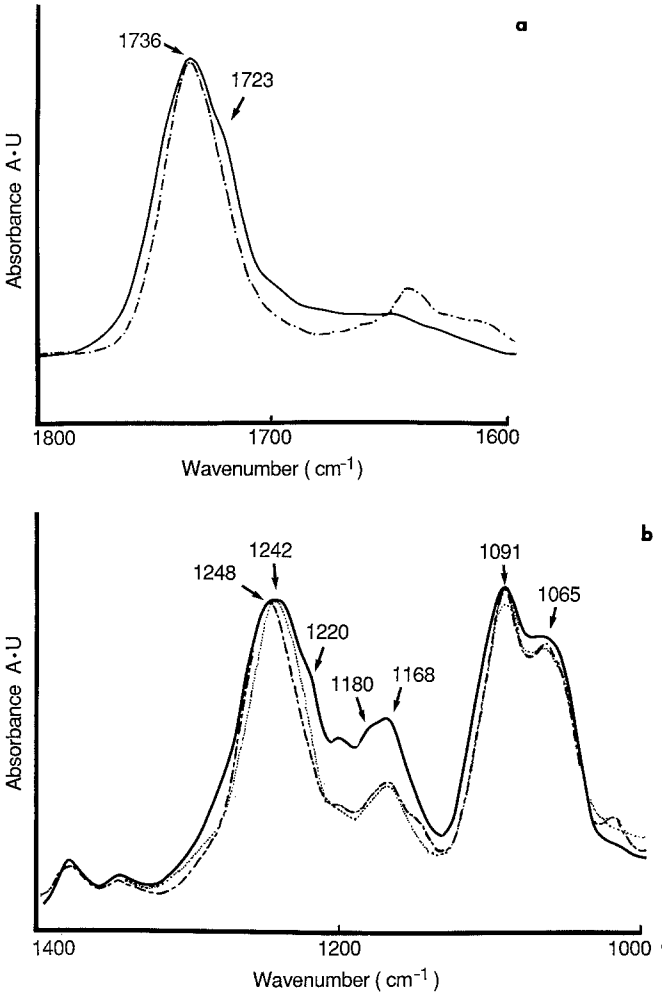

Fig. 3. **a** Infrared spectra of the C=O stretching mode at room temperature for DPPC (*full line*) and DPPC-cer, DPPC-ricin, DPPC-cer-ricin (*full-dotted line*). **b** Infrared spectra of the phosphate group at room temperature for DPPC (*full line*), DPPC-cer (*full-dotted line*) and DPPC-ricin (*dotted line*). The contribution of CaF_2 is subtracted

The infrared spectrum of DPPC in the 1,800–1,600 cm^{-1} region (Fig. 3a) has a single band centered at 1,736 cm^{-1} with a shoulder at 1,723 cm^{-1}. Those frequencies are attributed to the sn-1 and sn-2 C=O stretching modes respectively (Mushayakarara and Levin 1982). Addition of cerebrosides only changes the band contour: the shoulder at 1,723 cm^{-1} is not observed and the bandwidth decreases from 38 cm^{-1} to 31 cm^{-1}.

The 1,400–1,000 cm^{-1} spectral range is used to monitor the vibrations associated with the phosphate groups (Fig. 3b). The DPPC spectrum exhibits two main features with maxima wavenumbers at 1,242 cm^{-1} and 1,091 cm^{-1}. These bands are attributed to the antisymmetrical and symmetrical stretching modes of phosphate groups respectively. Another signal appears as a shoulder at 1,065 cm^{-1} and is probably due to the R–O–P–O–R′ vibration (Arrondo et al. 1984). The band at 1,168 cm^{-1} and the shoulder at 1,180 cm^{-1} are due to the C–O single bond stretching modes in the non-planar conformations of the C–C(=O)–O–C group respectively (Fringeli and Günthard 1981). Upon addition of cerebrosides no significant effect is observed on the symmetrical stretching modes of the phosphate group. In contrast large changes are detected in the antisymmetrical PO_2^- stretching mode region: the bandwidth decreases from 61 cm^{-1} to 46 cm^{-1}, the maximum of the vibration shifts from 1,242 to 1,248 cm^{-1} and the 1,220 cm^{-1} band vanishes. It should be noted that the decrease in intensity of the C–O single bond mode at 1,180–1,168 cm^{-1} is not due to perturbations induced by the cerebrosides, since it arises from the smaller concentration of C–C(=O)–O–C– groups in DPPC-cer mixtures compared to pure DPPC.

2) DPPC-ricin assemblies

Figure 4a shows the temperature profiles constructed from the I_{2880}/I_{2850} ratio of DPPC multilayers in the absence and in the presence of the protein. Addition of ricin (225 : 1 or 75 : 1) results at low temperatures in a significant decrease of the I_{2880}/I_{2850} ratio value from 1.12 for pure DPPC to 1.03 (Table 1). The main effect of the protein is to eliminate the pretransition. No perturbation of the temperature of the main transition is detected.

Above T_m, the I_{2880}/I_{2850} ratio value is close to 0.75 for pure DPPC and both DPPC-ricin recombinants (225 : 1, 75 : 1) indicating that in the liquid-crystalline phase the lipid chain ordering is not modified by the protein irrespective of the L : P ratios studied.

Because of the participation of the CH_2 and CH_3 groups of ricin in the Raman signal at 2,935 cm^{-1}, the I_{2935}/I_{2880} ratio is not useful for investigating ricin-lipid mixtures.

Infrared data indicate that addition of ricin to DPPC perturbs the C=O and PO_2^- stretching feature. In the 1,800–1,600 cm^{-1} region, the spectra obtained with DPPC-ricin recombinants are similar to those of the DPPC-cer mixture (Fig. 3a). In contrast, ricin induces a different perturbation in the PO_2^- stretching mode region from that observed after addition of cerebrosides. The bandwidth of the antisymmetrical mode is reduced. This is not associated with a frequency shift of the maximum which remains at 1,242 cm^{-1} (Fig. 3b).

3) DPPC-cer-ricin assemblies

Comparison of the temperature profiles obtained for DPPC-cer and DPPC-cer-ricin from the I_{2880}/I_{2850} ratio (Fig. 4b) shows that ricin (75 : 1, 225 : 1) signifi-

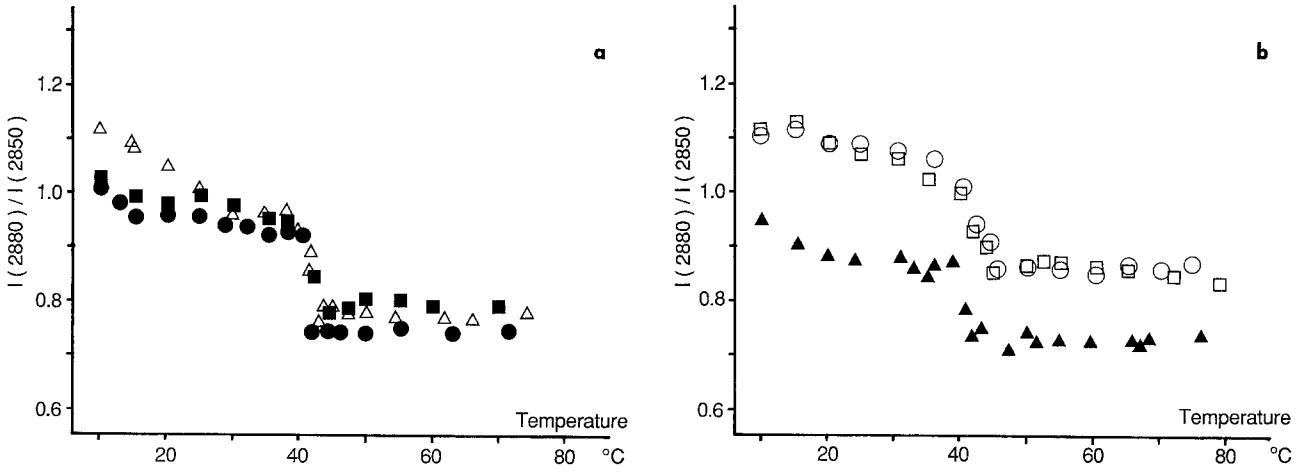

Fig. 4a and b. I_{2880}/I_{2850} intensity ratios versus temperature: **a** DPPC (*open triangles*), DPPC-ricin (225 : 1) (*full dotts*), DPPC-ricin (75 : 1) (*full squares*); **b** DPPC-cer (*full triangles*), DPPC-cer-ricin (225 : 1) (*open dotts*), DPPC-cer-ricin (75 : 1) (*open squares*)

cantly modifies the DPPC-cer lipid ordering. At low temperature the ratio value is increased from 0.95 to 1.10. After completion of the transition, whose temperature does not change, the I_{2880}/I_{2850} ratio value increases from 0.75 to 0.85 for both L : P mixtures.

In the C=O stretching region no spectral change is observed as we add ricin to DPPC-cer (Fig. 3a): DPPC-cer and DPPC-cer-ricin exhibit the same feature. In contrast, in the PO_2^- spectral region (Fig. 3b), a perturbation induced by ricin is detected: as observed with DPPC-ricin, the bandwidth of the antisymmetrical mode is reduced to 46 cm^{-1} and the maximum is located at 1,242 cm^{-1}.

Discussion

The lyophilisation process

In a previous paper (Lavialle et al. 1985) we used lyophilisation techniques to prepare the lipid-protein recombinants. Analysis by differential scanning microcalorimetry and fluorescence polarisation did not indicate perturbations to the membrane organisation. Our present spectroscopic investigation throws a new light on this problem. Differences are actually observed in the I_{2880}/I_{2850} and I_{1069}/I_{1099} intensity ratio values measured at low temperature (10 °C) (Table 1). Before lyophilisation, the I_{2880}/I_{2850} and I_{1069}/I_{1099} ratio values were found to be equal to 1.34 and 1.8 for DPPC, in agreement with Gaber et al. (1978) and Picquart (1986). After lyophilisation these ratios decrease to 1.12 and 1.4 (Table 1). These last values are quite similar to those obtained in the present work with sonicated DPPC (1.07 and 1.3) (Table 1) and those previously reported by Mendelsohn et al. (1976) and Spiker and Levin (1976). This suggests that a significant number of small vesicles are present after lyophilisation. Taking into account the I_{1069}/I_{1099} and I_{2880}/I_{2850} ratio values obtained with DPPC-cer mixtures (Table 1) the same conclusions are drawn for these model membranes.

Another interesting aspect of the lyophilisation step is the result obtained with DPPC-cer complexes. Before lyophilisation (data not shown), the temperature profile constructed from the I_{2880}/I_{2850} ratios shows two transition temperatures at 41° and 71 °C attributed to the chain melting of DPPC-cer and non-interacting cerebrosides (Ruocco et al. 1983). After lyophilisation of DPPC-cer mixtures, the upper transition disappears and the amplitude of the transition at 41 °C decreases (Fig. 2a). A possible explanation is that lyophilisation allows a complete insertion of cerebroside molecules within the DPPC bilayer without the formation of cerebroside clusters (Ruocco et al. 1983). The analysis of the I_{2880}/I_{2850} ratio suggests that the cerebrosides induce an increase of the interchain distance. The temperature effect observed above T_m (Fig. 2b) on the I_{2935}/I_{2880} ratio indicates that this perturbation reaches the CH_3 termini of the lipid acyl chains in the central part of the bilayer. It also probes a less regular lateral arrangement of the acyl chains when the cerebrosides are intercalated. This result is in agreement with Poss et al. (1979), who show that cerebrosides increase the membrane permeability to CrO_4^{2-} ions. It is worth noting that this chain disordering could be partly due to the C=C double bond of the sphingosine or to a rearrangement of the polar region of the membrane.

As expected from previous data (Lavialle et al. 1985), lyophilisation also favours ricin-lipid interactions. On DPPC-ricin mixtures prepared by simple mixing of the two components, no perturbation of the DPPC bilayer (data not shown) is detected even at low

temperatures, suggesting that, for these experimental conditions no lipid–protein interaction takes place. In contrast, with lyophilised and non-lyophilised DPPC-cer-ricin mixtures the same perturbing effect is obtained. This indicates that the interaction is completed before lyophilisation, as expected from the ability of ricin to bind galactosyl residues. These data also show that the dehydration step via lyophilisation does not perturb this specific interacting process.

In this discussion we have already suggested that lyophilisation induces the formation of vesicles from multilayer structures; it is now interesting to note that the presence of ricin does not seem to prevent this process. This is deduced from the comparison of the I_{1069}/I_{1099} ratios (Table 1) calculated for DPPC-ricin, DPPC-cer-ricin and a non-lyophilised suspension of DPPC.

Perturbations induced by cerebrosides and ricin on the polar and interfacial regions of DPPC bilayers

As a first comment, we note that the signals detected in the 1,400–1,000 cm^{-1} and 1,800–1,600 cm^{-1} spectral regions are only relevant to the DPPC molecules: that is, no C=O and PO_2^- groups are present in the cerebroside molecules.

According to Fig. 3 b, addition of cerebrosides and ricin only perturbs the antisymmetrical stretching mode of the PO_2^- groups. In both cases a more homogeneous environment of PO_2^- is detected as probed by the decrease in the halfwidth of the peak. Considering the location of the maxima, it appears that cerebrosides and ricin induce different perturbations in the polar region of the membrane. With DPPC-cer samples (Fig. 3 b) the feature at 1,248 cm^{-1} is dominant. According to Green et al. (1987), these data suggest that in the presence of cerebrosides fewer water molecules would interact with the PO_2^- groups of DPPC. It seems that galactose groups screen the interaction of water with PO_2^- groups. However, in lipid-ricin recombinants the major signal is centered at 1,242 cm^{-1}. This indicates that the interaction with ricin increases the accessibility of PO_2^- groups to the aqueous solvent. Quite similar signals are obtained with DPPC-ricin and DPPC-cer-ricin which suggests that the effects induced by ricin dominate those of the cerebrosides. This agrees with the existence of a ricin–cerebroside interaction via the galactosyl residues.

In the C=O ester stretching region the same perturbation is observed after addition of cerebrosides and/or ricin. The decrease of the bandwidth is associated with the decrease in intensity of the 1,723 cm^{-1} feature related to the sn-2 C=O ester groups of DPPC. Both ester groups appear now to be in the same environment corresponding to the sn-1 C=O group i.e. the C=O group is located deeper within the membrane. These IR data clearly demonstrate that ricin interacts with the surface of the membranes. This conclusion is supported by the observation that addition of increasing amounts of protein does not amplify the perturbations.

Perturbations induced by cerebrosides and ricin on the hydrophobic core of the membrane

Taking into account that both DPPC and cerebrosides contain long acyl chains, the information obtained by Raman spectroscopy cannot be specifically attributed to either one of these two membrane constituents. As compared to what we observe in the polar and interfacial regions, cerebrosides only slightly perturb the hydrophobic region of the membrane, although we demonstrate that lyophilisation enables their complete incorporation in DPPC multilayers.

Analysis of the data obtained in the presence of ricin indicates that the lipid composition of the membrane significantly modulates the interaction process. Although only slight perturbations of the hydrophobic regions of DPPC are detected after addition of ricin, a large ordering of the DPPC-cer lipid acyl chains is observed in DPPC-cer-ricin mixtures. Such an effect would arise from both the ability of ricin to bind the galactosyl residues of the cerebrosides and the specific organisation of the DPPC-cer-membranes. Based on the data published by Levin on amphotericin-lipid mixtures (1988), the observed ordering effect induced by ricin could result from a partial interdigitation of the two lipid layers involving Van der Waals interactions (Maulik et al. 1986). Since such an ordering effect is not observed when mixing DPPC and cerebrosides, the ricin–lipid interaction would be the driving force of this interdigitation.

Conclusion

By combining Raman and FTIR spectroscopy, we have detailed, for the first time, the membrane perturbations that ricin may induce. We show that the protein mainly interacts with the interfacial domains of the membrane. On the other hand we demonstrate that ricin induces different perturbations depending on the lipid composition of the membrane, as observed by several groups using other lipid-protein mixtures (Surolia and Bachhawat 1978; Marsh and Watts 1982; Chicken and Sharon 1984). It is clear from Fig. 3 that if ricin interacts with both DPPC and DPPC-cer, the induced effects significantly differ. With DPPC, the membrane perturbation is only detected at low temperatures; although with DPPC-cer mixtures, the ef-

fect is observed below and above T_m. In the first case, the protein decreases the lipid packing order. In contrast, with DPPC-cer membranes large stabilizing effects are detected. Such a difference between systems certainly arises from the lectin property of ricin to bind the galactosyl residues present in the cerebrosides and from the specific organisation of the DPPC-cer membranes.

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