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The Influence of Membrane Lipids in *Staphylococcus aureus* Gamma-Hemolysins Pore Formation

C. Potrich · H. Bastiani · D. A. Colin · S. Huck · G. Prévost · M. Dalla Serra

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Abstract The natural target of *Staphylococcus aureus* bicomponent γ-hemolysins are leucocyte cell membranes. Because a proteinaceous receptor has not been found yet, we checked for the importance of the different membrane lipid compositions by measuring the activity of the toxin on several pure lipid model membranes. We investigated the effect of membrane thickness, fluidity, and presence of nonbilayer lipids and found that the toxin pore-forming ability increased in the presence of phosphocholines with short saturated acyl chains or with unsaturated chains even though not short. An increase of activity was also evident in the presence of cone-shaped lipids like phosphatidylethanolamine or diphytanoylphosphatidylcholine, whereas cylindrical lipids, like sphingomyelin, did not favor the activity. All these results suggest that γ-hemolysins could bind to the bilayer only if the phosphatidylcholine (PC) head is freely accessible. This condition is satisfied by the concurrent presence of cholesterol and certain lipids, as highlighted by the so-called umbrella model (J. Huang and G. W. Feigenson, Biophys J 76:2142–2157, 1999). According to this model, cholesterol could help to a better exposition of PC head groups only if acyl chains are short or unsaturated. In fact, phosphatidylcholines with more than 13 carbon atoms acyl chains can cover cholesterol molecules; in this way, PC head groups pack tightly, rendering them inaccessible to the toxin, which thus shows a reduced pore-forming ability.

Keywords γ-Hemolysins · Cone-shaped lipids · Umbrella model · Phosphocholine head groups

Abbreviations

HlgA/HlgB	*Staphylococcus aureus* γ-hemolysins A and B
Cho	Cholesterol
PC	Phosphatidylcholine
DePC	DiElaidoyl PC
DMoPC	DiMyristoleoyl PC
DPoPC	DiPalmitoleoyl PC
DOPC	DiOleoyl PC
DPhPC	DiPhytanoyl PC
DPPC	Dipalmitoyl PC
PE	Egg Phosphatidyl-ethanolamine
POPC	Palmitoyl Oleoyl PC
SM	Sphingomyelin
T_m	Main transition temperature

Introduction

Bicomponent leukotoxins are pore-forming toxins active on blood cells; some can also permeabilize model membranes, especially if cholesterol is present as a lipid component (Ferreras et al. 1998). They are able to permeabilize human polymorphonuclear cells, monocytes, and macrophages (Prévost et al. 2003), thus representing an important subfamily of toxins probably developed by

C. Potrich (✉) · H. Bastiani · M. Dalla Serra
FBK-CNR Istituto di Biofisica, via alla Cascata 56/C,
38100 Povo (Trento), Italy
e-mail: cpotrich@fbk.eu

Present Address:
C. Potrich
Fondazione Bruno Kessler (FBK), Via Sommarive 18,
38100 Povo (Trento), Italy

D. A. Colin · S. Huck · G. Prévost
Institut de Bactériologie, UPRES EA-3432,
3, rue Koeberlé, 67000 Strasbourg, France

Staphylococcus aureus to refine its virulence capacities. Active toxins are couples formed by the association at the surface of membranes of one protein from the S subfamily (e.g., *Staphylococcus aureus* γ-hemolysins A [HlgA]) and one from the F subfamily (e.g., *Staphylococcus aureus* γ-hemolysins B [HlgB]) chosen among the wide family of leukotoxins. Pores are formed through several steps, including the following: (1) secretion as water-soluble monomers that diffuse from the bacteria to the target cell, (2) binding of the monomers to the cell membrane, (3) oligomerization into an insertion-competent ring-shaped structure; and (4) formation of a transmembrane β-barrel pore (Kaneko and Kamio 2004; Prévost et al. 2005; Tilley and Saibil 2006). In particular, the transition of the assembled oligomer into a functional pore probably requires a proper lipid composition (Menestrina et al. 2003). The portion of the toxin that penetrates into the membrane creates a β-barrel with hydrophilic amino acids on the inside wall constituting the pore lumen and alternate hydrophobic amino acids on the outside interacting with lipids. Each monomer participates with one β-hairpin to the assembly of the transmembrane stem (Menestrina et al. 2001). In the soluble proteins, the stem is stacked to a β-sandwich core, which forms the cap of the inserted pore. The stem domain is the key protein portion involved in the rearrangements that occur for pore opening (Viero et al. 2008).

In addition, the rim region plays a coordinating role and participates in the membrane anchoring with a region rich in aromatic residues (four tryptophans and two tyrosines per monomer). These aromatic groups form well-defined clefts where interaction with diheptanoyl- and dipropanoyl-phosphatidylcholine (PC) has been directly demonstrated in the homologous α-hemolysin heptamer (Song et al. 1996) and for the water-soluble HlgB monomer (Olson et al. 1999), respectively. Opposite to the class F components, this specific binding to phospholipids is precluded in LukS-PV, the S component of the Panton-Valentine leucocidin (Guillet et al. 2004), whereas HlgA did not have any saturation limits to target membranes (Meunier et al. 1997). Interestingly, populations of lymphocytes show variable susceptibility to the HlgA/HlgB hemolysin compared with polymorphonuclear cells, which may suggest differences in their cell membrane lipid composition.

Here, we show that the membrane lipid composition is crucial for γ-hemolysin activity. Short (fewer than 13 carbon atoms) or unsaturated PCs or cone-shaped lipids, together with cholesterol, increase the toxin pore-forming ability. These lipids may provide better PC head group availability, as will be shown here by adapting the so-called umbrella model described by Ali et al. (2008) and Huang and Feigenson (1999). According to this model, cholesterol can modulate packing and coverage characteristics of PC membranes. If the PC is long or rigid enough, cholesterol can fit below the umbrella provided by phosphocholine head group, permitting a dense membrane packing. Rather, the heads of short or unsaturated PCs cannot cover the cholesterol molecules so tightly; instead, they intercalate and separate PC heads, making them more accessible to toxin binding. Such a mechanism leads to the observed increase of the γ-hemolysins' permeabilizing ability toward liposomes.

Materials and Methods

Reagents

Calcein was obtained from Sigma and Triton X-100 from Merck. Lipids used were synthetic PCs with both saturated acyl chains from 10 to 16 carbon atoms (PC10–PC16), or with both unsaturated chains (either cis or trans) of 14, 16, or 18 carbon atoms or with only one unsaturated chain (palmitoyl oleoyl PC, POPC). We also used diphytanoyl PC (DPhPC), egg phosphatidyl-ethanolamine (PE), and brain sphingomyelin (SM), all purchased from Avanti Polar Lipids (Alabaster). These molecules were >99% pure as stated by the supplier. Cholesterol was from Fluka.

Proteins Purification

HlgA and HlgB were produced in an *Escherichia coli* expression system as described previously (Werner et al. 2002). HlgA was purified by affinity chromatography on glutathione-Sepharose 4B followed by cation-exchange fast protein liquid chromatography with a NaCl gradient from 0.36 M to 0.6 M (Werner et al. 2002), after removing the glutathione S-transferase (GST) tag with PreScission Protease (GE, Amersham Biosciences). HlgA was eluted when the NaCl concentration reaches 0.51 M. HlgB was purified by affinity chromatography on Glutathione-Sepharose 4B followed by hydrophobic interaction chromatography (Resource ISO, Amersham Biosciences) with a $(NH_4)_2SO_4$ gradient ranging from 0.96 M to 0.36 M, after removing the GST tag with PreScission Protease (Amersham Biosciences). HlgB was eluted with 0.6 M of $(NH_4)_2SO_4$. It was then dialyzed at 4°C overnight against 0.02 M HEPES, 0.5 M NaCl, pH 7.5.

Control for homogeneity was performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the proteins were then stored at −80°C.

Preparation of Lipid Vesicles

Multilamellar liposomes (MLV) were prepared in the presence of 80 mM calcein (neutralized to pH 7.0 with NaOH) and subjected to six cycles of freezing and thawing, as previously described (Tejuca et al. 1996). Large unilamellar

vesicles were prepared by extruding a suspension of MLV with a two-syringe pneumatic extruder with temperature control (LiposoFast Pneumatic LF-P, Avestin). Thirty-one passages were performed through polycarbonate filters (Millipore) with pores of an average diameter of 100 nm (MacDonald et al. 1991). The extrusion temperature was above the highest main transition temperature (T_m) of the single lipid component constituting the mixture. The initial lipid concentration was 6 mg/ml. To remove nonencapsulated calcein, the vesicles were spun through minicolumns (Strata, Phenomenex) loaded with Sephadex G50 (medium) preequilibrated with 10 mM Tris-HCl, 0.1 mM EDTA, 20 mM NaCl, pH 7.0. Large unilamellar vesicle size was between 100 and 120 nm as measured by photon correlation spectroscopy (Malvern, Z-Sizer 3).

Permeabilizing Activity

Permeabilizing activity of unilamellar vesicles was measured with a fluorescence microplate reader (Fluostar BMG). Samples were excited through a narrow-band interference filter centered at 485 nm, and the fluorescence was detected after a second filter at 538 nm. White plastic 96-well microplates (Biomat, Rovereto, Italy) were pretreated with 0.1 mg/ml Prionex (Pentapharm) to reduce unspecific binding of protein to the plastic. The final lipid concentration in the experiment was 5 μM, as determined by a Wako phospholipid kit (Wako Chemicals). The activity of variable toxin concentrations was tested at room temperature. Toxin concentrations reported in the text refer to the concentration of the single component protein and [HlgA]/[HlgB] = 1. After mixing vesicles with toxin, the release of calcein produced an increase in fluorescence. Spontaneous leakage of calcein was negligible under these conditions, even for the vesicles prepared above the transition temperature and taken to room temperature for the calcein release assay. Maximum fluorescence value was always obtained by adding 1 mM Triton X-100 (final concentration). The percentage of release, R%, was calculated as follows:

$$R\% = (F_{fin} - F_{in})/(F_{max} - F_{in}) \times 100$$

where F_{in} and F_{fin} represent the initial and the final (after 45 min) value of fluorescence before and after addition of toxin, respectively.

Fourier-Transformed Infrared Spectroscopy (FTIR) Experiments

FTIR spectroscopy was used to assess the lipid phase of several lipid compositions by the method described by Crowe et al. (1999). Multilamellar liposomes were prepared in 10 mM HEPES, 20 mM NaCl, pH 7.2, at 50 mg/ml lipid concentration. A liquid semipermanent cell with CaF_2 windows (Specac) was loaded with MLV, and transmission spectra of lipids were collected at given temperatures with a FTS 185 spectrometer (Bio-Rad) equipped with a liquid-nitrogen-cooled mercury/cadmium/telluride detector. To estimate the lipid state of different liposome mixtures, we focused on the CH_2 stretching. In fact, the phase transition induces changes in the vibrational frequency of the CH_2 bands in membrane phospholipids (Tablin et al. 1996). In particular, according to Crowe et al. (1999), we assigned absorptions below 2,851 cm^{-1} to the gel phase and above 2,853 cm^{-1} to the liquid crystalline phase. Wave numbers of between 2,851 and 2,853 cm^{-1} were defined as a transitional state, i.e., lipids did not belong to the gel phase any more but were not yet in the liquid crystalline phase (Wolkers et al. 2003). When required, a baseline subtraction was applied to correct for the water absorption centered around 3,350 cm^{-1}.

Preparation of Human Leucocyte Types and Flow Cytometry

Human polymorphonuclear neutrophil (PMNs) from healthy donors were purified from buffy coats as previously reported (Guillet et al. 2004), and suspended at 5×10^5 cells/ml in 10 mM HEPES, 140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.1 mM EGTA, pH 7.3 (EGTA buffer). During that preparation, monocytes were obtained from the ring formed by cells at the interface between plasma and the J-Prep buffer (Eurobio). To remove platelets, monocytes were washed twice by centrifugation at 600×*g* at room temperature in 1 ml of 0.9% (w/v) NaCl. Then remaining erythrocytes were removed by a 45-s incubation at room temperature after adding 36 ml of water; isotonicity was recovered with addition of 4 ml of 9% NaCl. Once again, monocytes were washed in the EGTA buffer and adjusted to 5×10^5 cells/ml.

CD4 lymphocytes were prepared from such buffy coats from healthy donors by a negative selection, which removed erythrocytes, monocytes, NK cells, B lymphocytes, gamma/delta lymphocytes, and CD8 lymphocytes by means of specific antibodies further captured by activated magnetic beads, as recommended by the manufacturer (Stemcell). HlgA and HlgB were both applied at 10 nM concentration. Flow cytometry measurements from 3000 PMNs were carried out with a FacSort flow cytometer (Becton-Dickinson) equipped with an argon laser tuned to 488 nm (Meunier et al. 1995). Pore formation and monovalent cation influx were revealed by the penetration of ethidium through the pores; cells were incubated for 30 min with 4 μM ethidium before the addition of toxins in the absence of extracellular Ca^{2+} (Joubert et al. 2007). Ethidium fluorescence was measured by Cell Quest Pro software (Becton-Dickinson). Basal level

values were obtained for each series of data from a control without addition of toxin. These were systematically subtracted from the other assays. Standard deviations values never exceeded 10% of the obtained values; only one representative among four repeated experiments are shown in Fig. 7.

Results and Discussion

Staphylococcus aureus γ-hemolysin HlgA/HlgB has a broad range of target cells, including erythrocytes, PMNs, monocytes and lymphocytes, but it is active also on model membranes. We chose to investigate the pore formation on pure lipid membranes made of PC:Cho (1:1 mol) with the γ-hemolysin couple HlgA/HlgB, which has the most potent efficacy of all known leukotoxins on synthetic bilayers (Ferreras et al. 1998). In fact, the titration of HlgA/HlgB activity on liposomes containing different ratios of PC:Cho showed that the activity increases with Cho content up to 1:2 molar ratio, being maximal at equimolar concentration. In this study, we used large unilamellar vesicles of comparable sizes, as determined by photon correlation spectroscopy.

We investigated the effect of membrane thickness, fluidity, and presence of various lipids in the mixture on the permeabilizing activity of HlgA/HlgB, and we explained the observed differences with good agreement with the umbrella model described in (Huang and Feigenson 1999) and the better accessibility of the PC head group provided by certain lipid compositions.

Effect of Membrane Thickness and Fluidity

Werner et al. (2002) demonstrated that mutations shortening the stem of some leukocidins modulated their biological activity according to target cells or lipid vesicle composition. To further investigate the role of membrane thickness, we selected PCs with saturated acyl chains ranging from 10 to 16 carbon atoms (Fig. 1).

Phospholipid membranes formed by 16 carbon length acyl chains are thick enough to accommodate the fully extended β-barrel of the well-known *S. aureus* α-toxin (Song et al. 1996) (Fig. 1a). In our case, toxin activity was conversely proportional to the acyl chain lengths; in particular, HlgA/HlgB allowed calcein release from liposomes when PC13 was present but became inactive with longer PCs (Fig. 1b). In fact, by using di14:1 or di16:1 phosphatidylcholines, i.e., unsaturated PCs with similar carbon atom length, calcein release was always detected even though it had a different efficiency (Fig. 1c). In particular, it was maximal on di18:1 and di16:1 PC liposomes (Table 1).

Besides the number of carbon atoms in acyl chains, by comparing the PCs' estimated hydrophobic thickness and calcein release (Table 1, where membrane compositions and thickness are coupled on the basis of PC species tested), no clear correlation was evidenced; liposomes containing unsaturated PCs were always better permeabilized than those with saturated PCs. Therefore, HlgA/HlgB activity could not be simply correlated to differences in bilayer thickness, but unsaturation plays also a role. Tomita et al. (1992a, 1992b) previously reported of similar results for *S. aureus* α-toxin by use of liposomes composed either of saturated PC acyl chains carrying 12–18 carbon atoms or unsaturated fatty acyl chains PC. They concluded that the assembly process of α-toxin could be promoted by the increase in membrane fluidity conferred by unsaturation on the cholesterol-containing membrane. This aspect could have physiological relevance because cell membranes are rich in unsaturated phospholipids. For example, the total unsaturated fatty acids are approximately 55% of all fatty acids present in the white blood cell membrane (Bakan et al. 2006). It is well known that the fatty acid composition and cholesterol content affect the fluidity of biological membrane. Therefore, we decided to investigate the possible correlation between membrane fluidity and HlgA/HlgB activity. We studied the fluidity of several lipid compositions by infrared spectroscopy for measuring the T_m at 25°C, i.e., the temperature used in release experiments (Fig. 2 and Table 2).

We observed changes in the vibrational frequency of the methylene symmetric stretching mode (νCH_2) at approximately 2,850 cm^{-1}, which reflects the membrane fluidity state (Tsvetkova et al. 2002). According to Crowe et al. (1999), we assigned absorptions below 2,851 cm^{-1} to the gel phase, in which the acyl chains are capable of packing tightly together. Values above 2,853 cm^{-1} were considered as liquid crystalline phase, in which the acyl chains are fluid and disordered. Values between 2,851 and 2,853 cm^{-1} were assigned to a transitional state in which lipids are in an intermediate phase. Three representatives for each phase are reported in Fig. 2. A peak shift is clearly visible that indicates the different fluidic state of the membrane. Table 2 shows the lipid compositions used in this study clustered according to their lipid state. It is immediately clear that any direct correlation does not exist between lipid phase and calcein release; in particular, membranes in the gel state can be highly damaged, moderately damaged, or undamaged by the toxin. Moreover, membranes of pure dioleoyl PC (DOPC), which should be in the fluid state, cannot be permeabilized. We concluded that the membrane fluidity measured as lipid T_m is not the main effector for the toxin activity.

This conclusion is also reinforced by experiments where the temperature varied between 5°C and 60°C (Fig. 3). The fluidity is characteristic of each lipid composition and typically increases with the temperature (Fig. 3b). Conversely, the calcein release from liposomes with a same

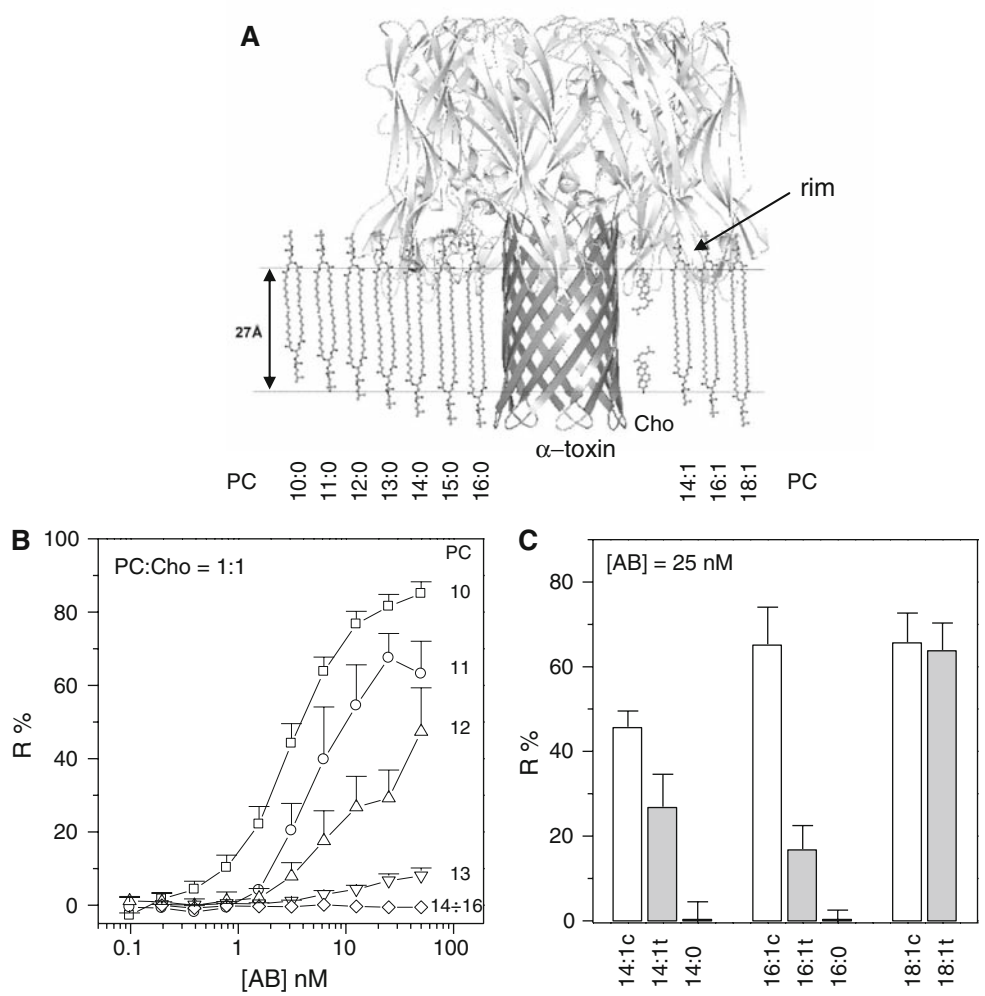

Fig. 1 Role of lipid length and chain saturation. (**a**) Schematic representation of *S. aureus* α-toxin in the membrane. *β*-Barrel spanning the double lipid layer is highlighted in dark gray; the backbone structure of the lipid molecules faced one to the other is schematized in a completely extended conformation. Phosphatidylcholine with saturated acyl chains from 10 to 16 carbon atoms (indicated as 10:0–16:0) are on the left side of α-toxin; on the right are cholesterol (Cho) and PC with monounsaturated chains from 14 to 18 carbon atoms (14:1–18:1). Two horizontal lines define the hydrophobic region corresponding to DOPC (or PC 18:1, thickness = 27 Å; Lewis and Engelman 1983). The cartoon is intended only for a visual comparison among lipids used in this study and it is based on their chemical formula. (**b**) Dose dependence of calcein release induced by HlgA/HlgB from PC:Cho (1:1 mol) large unilamellar vesicles. All PCs are saturated and their acyl chains increase from 10 to 16 carbon atoms. No release from vesicles of PC 14, 15, and 16 mixed with Cho was observed, so only one curve is shown. (**c**) Comparison of the permeabilizing activity on membranes containing cholesterol and saturated or unsaturated PC of the same length. HlgA/HlgB concentration was 25 nM, kept constant in all the reported experiments. PC and Cho are in a 1:1 molar ratio. In (**b**) and (**c**), the lipid concentration was always 5 μM. Toxin concentrations refer to the concentration of the single component protein. Buffer solution was 10 mM Tris-HCl, 0.1 mM EDTA, 20 mM NaCl, pH 7.0. Calcein release was always measured at room temperature. Points are average ± SEM of at least three experiments

lipid composition does not increase with the temperature (Fig. 3a); thus, no correlation was evidenced between changes in temperature and toxin activity. In fact, at higher temperature, i.e., when the membrane should be more fluid, the calcein release was poor both from liposomes made of DOPC:Cho (1:1 mol ratio) and dipalmitoyl PC (DPPC):Cho (1:1 mol ratio) (Fig. 3a). This correlates well with the hemolytic activity of the highly homologous *S. aureus* α-toxin, which shows the highest hemolysis at 25–30°C, while at higher temperature the activity decreased (Ikigai and Nakae 1984).

It is possible that an optimal fluid state exists for each lipid membrane giving the maximal toxin activity. Moreover, the decrease in toxin activity at high temperature could not be ascribed to toxin denaturation. In fact, toxin was still active after a 30-min preheating treatment at 50°C (data not shown). In particular, HlgA and HlgB were separately preheated at 50°C, ice cooled, and added to liposomes, and calcein release was followed at different temperatures. At both 20°C and 30°C, the toxin activity was not reduced with respect to the nontreated toxin, indicating that this toxin is stable also after the heating step.

Table 1 Comparison between PC hydrophobic thickness and HlgA/HlgB activity[a]

Lipid	Carbon atoms double bond	PC hydrophobic thickness (Å)[b]	R%[c]
DMoPC:Cho	14:1/14:1 cis[d]	20	43.0 ± 6.6
PC13:Cho	13:0/13:0	21	8.0 ± 3.8
DPoPC:Cho	16:1/16:1 cis[d]	23.5	71.2 ± 6.9
DMPC:Cho	14:0/14:0	23	0
DOPC:Cho	18:1/18:1 cis[d]	27	67.7 ± 7.7
DPPC:Cho	16:0/16:0	26	0

[a] Hydrophobic thickness is defined as the distance between two phosphate groups across the bilayer. The activity at the fixed toxin dose of 50 nM is expressed as a percentage of calcein release (last column) from vesicles whose lipid composition is reported in the first column. In the second column, the PC acyl chains' length is shown, as well as the saturation degree and unsaturated isomer used. The hydrophobic thickness values are taken from Lewis and Engelman (1983). The phospholipids are coupled by thickness similarity so that a direct comparison between saturated or unsaturated lipids is possible. DMoPC, dimyristoleoyl PC; DPoPC, dipalmitoleoyl PC; DOPC, dioleoyl PC; DPPC, dipalmitoyl PC

[b] Taken from Lewis and Engelman (1983)

[c] Calcein release average of at least three experiments ± SEM at [AB] = 50 nM

[d] Isomeric form of the double bond present in the acyl chain

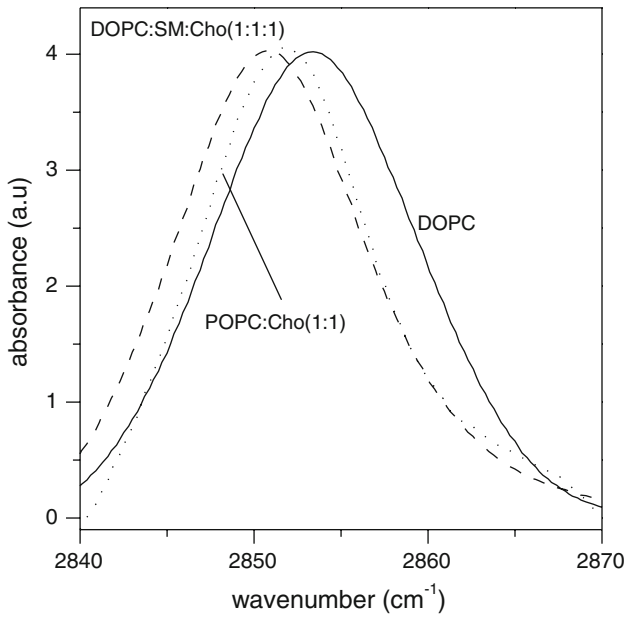

Fig. 2 Infrared spectra in the hydrocarbon stretching region of three lipid composition. Dash line: DOPC:SM:Cho (1:1:1) mixture, representative for the gel phase; dot line: POPC:Cho (1:1) vesicles, representative for the transition state; solid line: DOPC vesicles, representative for the liquid crystalline phase. Spectra were collected in transmission mode at 25°C, as described in Materials and Methods

Table 2 Comparison between lipid phase and HlgA/HlgB activity[a]

Lipid	Wave number (cm^{-1})[b]	Phase	R%[c]
PC13:Cho	2850.8	S	8.0 ± 3.8
PC14:Cho	2850.0	S	0
PC16:Cho	2850.8	S	0
DOPC:SM:Cho 1:1:1	2850.9	S	39.8 ± 4.9
POPC:DOPE:Cho 1:1:2	2849.2	S	71.3 ± 2.3
POPC:DPhPC:Cho 1:1:2	2849.6	S	64.9 ± 1.4
PC10:Cho	2851.9	T	85.0 ± 3.2
PC11:Cho	2851.1	T	63.2 ± 8.9
POPC:Cho	2851.8	T	45.3 ± 3.0
DOPC:Cho	2851.2	T	67.7 ± 7.7
DePC:Cho	2852.0	T	57.5 ± 5.6
DOPC	2853.4	L	0

[a] The lipid phase was ascribed to the different lipid compositions by measuring the Fourier-transformed infrared spectra at room temperature, as described in Materials and Methods. The wave number corresponding to the peak maximum due to the CH_2 stretching is reported, as well as the deduced lipid phase. S, gel phase; T, transitional state; L, liquid crystalline phase; DePC, dielaidoyl PC; DPhPC, diphytanoyl PC; POPC, palmitoyl oleoyl PC; SM, sphingomyelin. Calcein release (last column) shows no correlation with the membrane state

[b] Calculated standard deviation is 0.2 cm^{-1}

[c] Calcein release average of at least three experiments ± SEM at [AB] = 50 nM

Lipid Mixtures

The influence of more complex lipid mixtures on the toxin activity was investigated next; we focused on a stereotyped "lipid raft" composition (Simons and Ikonen 1997; Xu and London 2000). For this purpose, we used vesicles containing different proportions of DOPC, SM, and Cho (Lawrence et al. 2003; McIntosh et al. 2003; Pandit et al. 2004b). When SM was used in DOPC:SM:Cho liposomes either at 1:1:1 or 2:2:1 molar ratios, no significant change of activity was detected (Fig. 4a). But when we looked at the cholesterol, we noticed that the increment in HlgA/HlgB activity was directly proportional to the cholesterol content. In particular, when it increased from 0% to 50%, passing through 20% and 33% (i.e., DOPC:SM:Cho 2:2:1 and 1:1:1, respectively), there was a progressively higher release of calcein. The maximal activity was observed for DOPC:Cho (1:1). These results indicate that γ-hemolysin

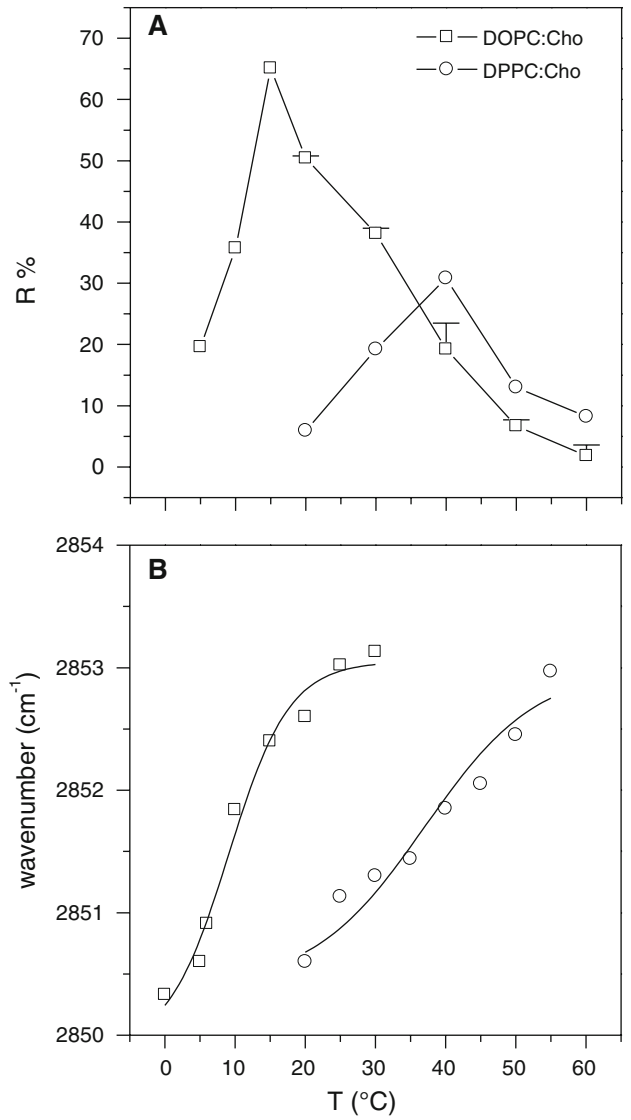

Fig. 3 Dependence of the toxin activity and the membrane fluidity on temperature. (**a**) Calcein release induced by 50 nM HlgA/HlgB from DOPC:Cho in 1:1 molar ratio (open squares) and DPPC:Cho 1:1 (open circles) liposomes at various temperatures. Experimental conditions are the same as in Fig. 1. (**b**) changing in CH_2 vibrational frequency as a function of temperature in vesicles of DOPC:Cho (open squares) and DPPC:Cho (open circles) both in a 1:1 molar ratio composition. The connecting lines were drawn manually. The wave numbers were estimated from the maximum of the peak corresponding to the symmetric CH_2 stretching vibration in the transmission spectrum collected at a fixed temperature, as described in Materials and Methods

probably does not require the presence of microdomains similar to lipid rafts, at least for the permeabilization of model membranes. This seems to be in opposition to what Nishiyama and coworkers (2006) found for the activity on human polymorphonuclear leukocytes, for other staphylococcal toxins still belonging to leukocidins class. But differences in cell binding have been reported earlier

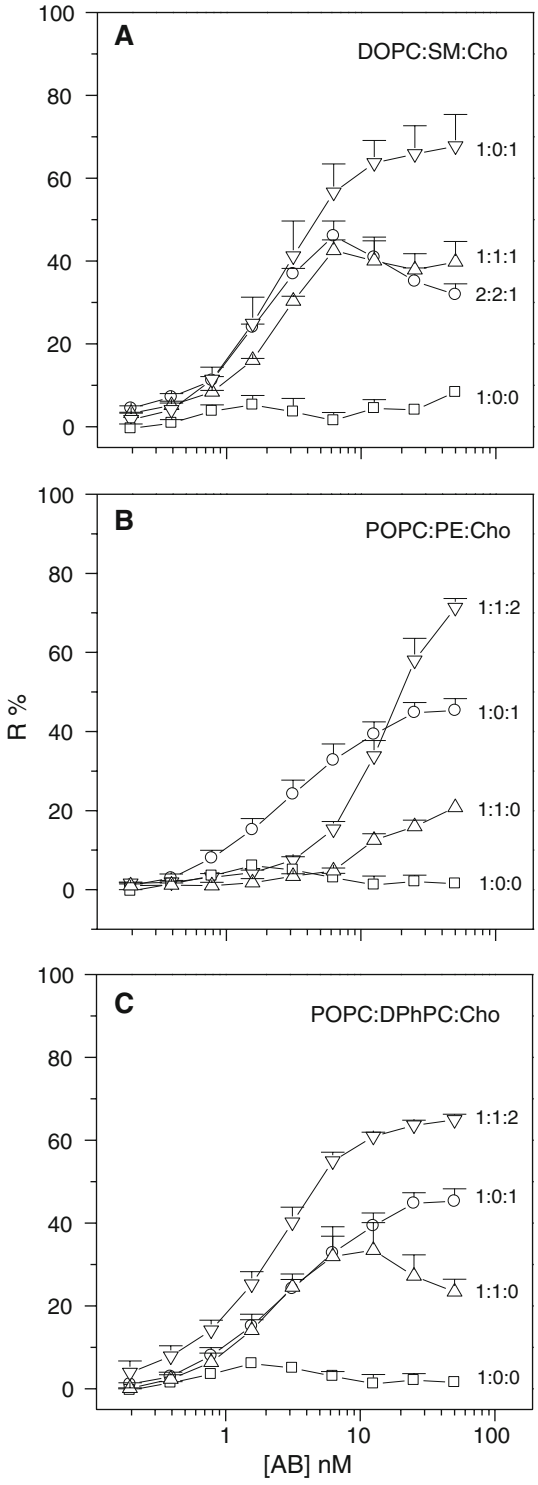

Fig. 4 Effect of different lipids. The calcein release induced by HlgA/HlgB is compared when a new lipid: SM (**a**), phosphatidylethanolamine (**b**), or diphytanoyl-PC (**c**) is introduced in PC:Cho based vesicles. The molar ratio among the different lipids is reported in each panel. All the experimental conditions are the same as in Fig. 1. Data are averaged ± SEM of at least three independent experiments

among HlgA/HlgB, HlgC/HlgB, or LukS-PV/LukF-PV (Gauduchon et al. 2001; Meunier et al. 1997), and the influence of protein receptor or minority amount of specific lipid acceptor cannot be excluded in natural membranes.

An enhancement of the activity was observed with the inclusion of conical shaped lipids like PE or DPhPC, i.e., lipids whose hydrophobic moiety occupies a cross section larger than the polar head group does (Fig. 4b and 4c, respectively) (van den Brink-van der Laan et al. 2004). The highest calcein release was observed from the 1:1:2 lipid composition, both from POPC:PE:Cho and POPC:DPhPC:Cho. On the contrary, only an approximately 20% calcein release was detectable with 50 nM HlgA/HlgB on liposomes made of POPC and a conical lipid in the absence of cholesterol. Therefore, the combination of conical lipids and cholesterol is important for the HlgA/HlgB activity.

HlgA/HlgB activity also increased when the short lipid PC10 and the lipids PC15 and cholesterol were combined together (Fig. 5a).

A 10% inclusion of PC10 in PC15:Cho liposomes (PC10:PC15:Cho in 1:4:5 molar ratio) raised the calcein release from 0 to 50%. An increase of PC10 content up to 25% of total lipid led to a further increase in toxin activity, but not as large as the previous increase. Similarly, mixing 10% of PC10 with POPC:Cho (1:4:5 molar ratio) resulted in a significant increase in activity compared with pure POPC:Cho vesicles (from 45% to 61% for 50 nM toxin, Fig. 5b). PC10 may also favor the lipid mobility within the bilayer, thus favoring the encounter of HlgA and HlgB monomers in the membrane plane, resulting in a consequent privileged formation of oligomers. This hypothesis is corroborated by Valeva et al. (2006), who reported that sufficiently dense clusters of phosphocholine head groups at the membrane surface are necessary for high-affinity binding of *S. aureus* α-toxin. Moreover, the binding of dipropanoyl-PC or 2-(N-morpholino)-ethanesulfonic acid molecules was directly demonstrated for class F leukotoxins (Guillet et al. 2004; Olson et al. 1999), as well as the binding of glycerophosphocholine or dipropanoyl-PC to the detergent-solubilized α-toxin heptamer (Galdiero and Gouaux 2004). This interaction between PC head group and toxin may therefore be privileged by the concurrent presence of short PC or conical lipids and cholesterol, resulting in an increased activity. Both effects are more evident at low PC10 content (Fig. 5).

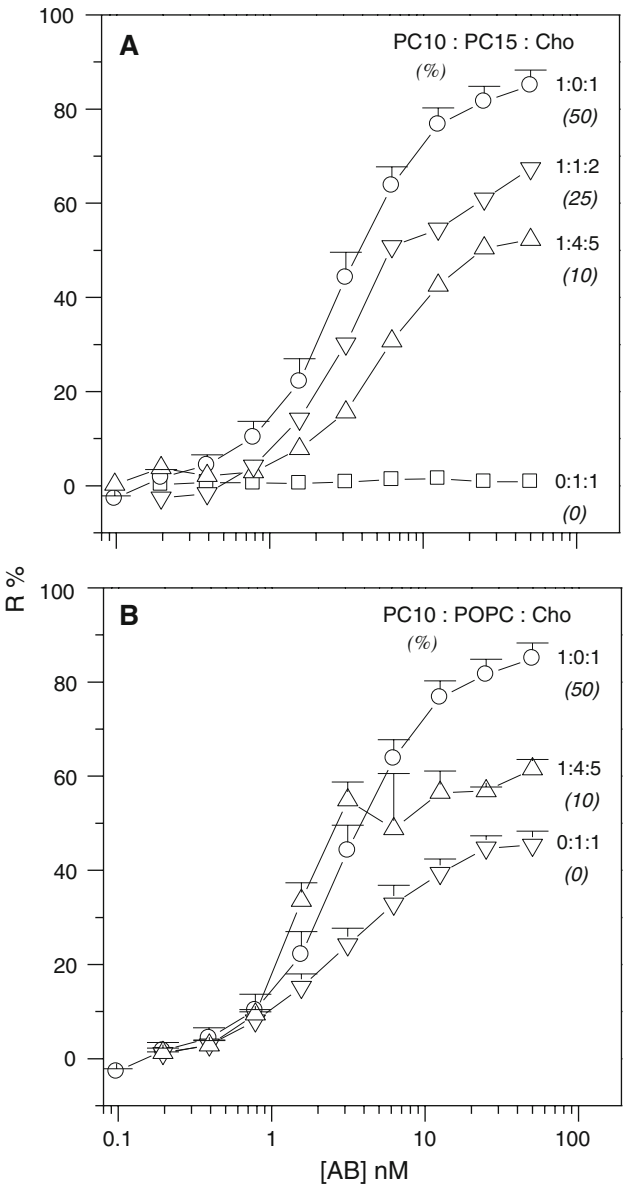

Fig. 5 HlgA/HlgB activity potentiation combining PC15:Cho (**a**) or POPC:Cho (**b**) with the short lipid PC10. The molar ratio among the different lipids is reported, as well as the PC10 content (as percentage, in italic). All the experimental conditions are the same as in Fig. 1. Points are average ± SEM of at least three experiments, except points corresponding to 1:1:2 and 1:4:5 lipid molar ratio in A, that derive from a single measurement

The Umbrella Model

All the data presented are interpretable within the umbrella model originally proposed by Huang and Feigenson (1999). This model states that Cho, which has a very small polar head group, needs to borrow adequate head group coverage from adjacent lipids to avoid the unfavorable free energy of exposure to water. In a bilayer, cholesterol stays below a polar phospholipid head group as under an umbrella. Moreover PC, a rather cylindrical lipid, makes a better umbrella for cholesterol than PE, a conical lipid, as demonstrated by the fact that when the Cho content increases in the bilayer, it tends to dissociate and precipitate more readily when combined with PE than with PC (Huang et al. 1999). To satisfy the coverage requirement, cholesterol molecules distribute in such a way that the number of cholesterol–cholesterol contacts is minimized. Consequently, a regular distribution at low cholesterol

concentration (up to 50 mol%) is seen both in simulations and in experiments (Parker et al. 2004). Molecular dynamic simulations also support the PC coverage effect on Cho molecules (Chiu et al. 2002; Pandit et al. 2004a). In these conditions, Cho can also be accessible for the binding sites of cholesterol-specific cytolysins, especially in the presence of conical lipids (Zitzer et al. 2001).

Bicomponent leukotoxins are not cholesterol dependent cytolysins, even if cholesterol inclusion in artificial membranes increases their activity, as demonstrated for γ-hemolysins (Ferreras et al. 1998). However, similar to cholesterol-dependent toxins, a boosting effect of cone-shaped lipid on γ-hemolysins activity is shown in this study.

According to the umbrella model (Fig. 6), we propose that the PC head groups may form a dense coverage hiding cholesterol molecules if the lipid acyl chains are long enough (i.e., more than 13 carbon atoms, see the simulation and the cartoon in Fig. 6a or whether they have a cylindrical shape, such as for example SM. On the contrary, this coverage is decreased by unsaturated acyl chains, saturated chains shorter than 13 carbon atoms (Fig. 6b) or cone-shaped lipids (Fig. 6c). All these three types of lipids share the possibility of increasing the distance among PC heads, thus improving the possibility for the toxin to get in contact with the better-exposed head groups.

Furthermore, cylindrical lipids may interact so strongly with cholesterol that their head groups were too close for being accessible to the toxin. This fact is also confirmed by preliminary results of cholesterol extraction from different PC:Cho membranes by methyl-beta cyclodextrins (G. Anderluh and M. Dalla Serra, personal communication).

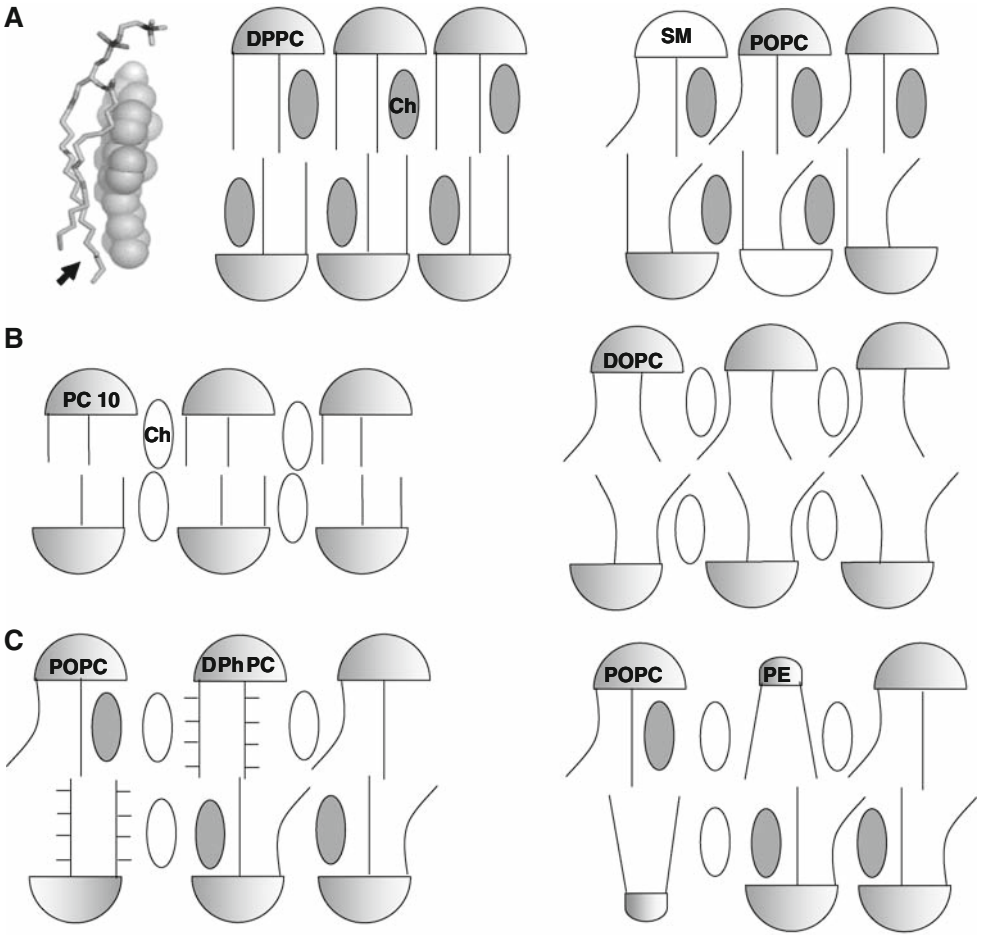

Fig. 6 The umbrella model. The phospholipid heads are represented as a dark (PC) or a white (SM) semicircle or as a small semicircle (PE), while the acylic chains are schematized as a straight (saturated) or curved (unsaturated) line. A gray oval indicates cholesterol covered by phospholipid head; the white one is used to describe free cholesterol. (**a**) At left, simulation of DPPC:Cho interaction at 1:1 ratio. Cholesterol is shown as a space-filled molecule, whereas DPPC is presented as stick molecule (adapted from Chiu et al. 2002). The arrow on a DPPC chain indicates the length corresponding to PC13. In the center and on the right, cartoons of DPPC:Cho and POPC:SM:Cho membranes, respectively, where cholesterol is completely or partially covered. (**b**) PC10:Cho and DOPC:Cho membrane cartoons, two mixtures that represent maximum cholesterol exposure and PC head accessibility. (**c**) POPC:DPhPC:Cho and POPC:PE:Cho membranes, cholesterol exposure mediated by conical lipids. In (**b**) and (**c**), the presence of exposed cholesterol favors the accessibility of the PC head groups to the toxin, by lowering the lipid lateral pressure

However, the choline exposure could be favored by the membrane curvature resulting from the presence of cone-shaped lipids or short lipids. In fact, nonbilayer lipids are expected to support membrane binding of peripheral membrane proteins and to stabilize the oligomeric structure of integral membrane proteins (van den Brink-van der Laan et al. 2004).

It is also reported that Cho can tune the lipid bilayer mobility in distinct lipid phases, but at 50 mol% of Cho content only one phase is observed in PCs:Cho mixtures (Scherfeld et al. 2003). Even though in these mixtures very few differences were observed in the lipid dynamic properties of the bilayer, it is possible that the presence of short or unsaturated PCs or conical lipids increases the diffusion coefficient of Cho, which indicates a decrease of the lipid lateral pressure. In fact, Cho provides a dynamic environment in the cell membrane that facilitates the free movement of EGF receptors and HER2 (Orr et al. 2005). It is also demonstrated for other membrane proteins and lipid probes that Cho depletion reversibly suppresses the lateral diffusion of membrane components (Vrljic et al. 2005). Similarly, Cho that is not covered by the PC umbrella could favor the free motion and thus the contact of HlgA and HlgB monomers in the membrane. This process could act through the direct binding of the phosphocholine to HlgB, which is possible as demonstrated by Olson et al. (1999).

Valeva et al. (2006) suggested that the clustering of phosphocholine head groups is required for an efficient oligomerization of *S. aureus* α-toxin. Similarly, cholesterol and nonbilayer lipids could modulate the proximity of phosphocholine head groups, so that these groups bring HlgA and HlgB in greater vicinity. The proximity of HlgA and HlgB monomers may favor the oligomerization and promote the subsequent pore opening, which globally gives an increase in toxin activity, as shown in this paper.

The membrane lipid composition could also be crucial for the in vivo activity of leukotoxins. Interestingly, we found that not all leucocytes are lysed by HlgA/HlgB, and if lysed, leucocytes are damaged to different extents. In fact, the activity on human white blood cells varies according to leucocytes and can be followed by ethidium fluorescence by flow cytometry (Fig. 7).

Not all leucocytes are permeabilized; in particular, $CD4^+$ lymphocytes are not sensitive to HlgA/HlgB because of the number of dead cells, negligible even after 1 h after treatment with the toxin (Fig. 7c). On the contrary, the pore formation raises the highest level on human PMNs both for intensity and rate (Fig. 7a), while the permeabilization of human monocytes is slower and incomplete (Fig. 7b). It is well known that HlgA/HlgB acts directly on membranes where a proteinaceous receptor has not yet been found. A possible explanation of the

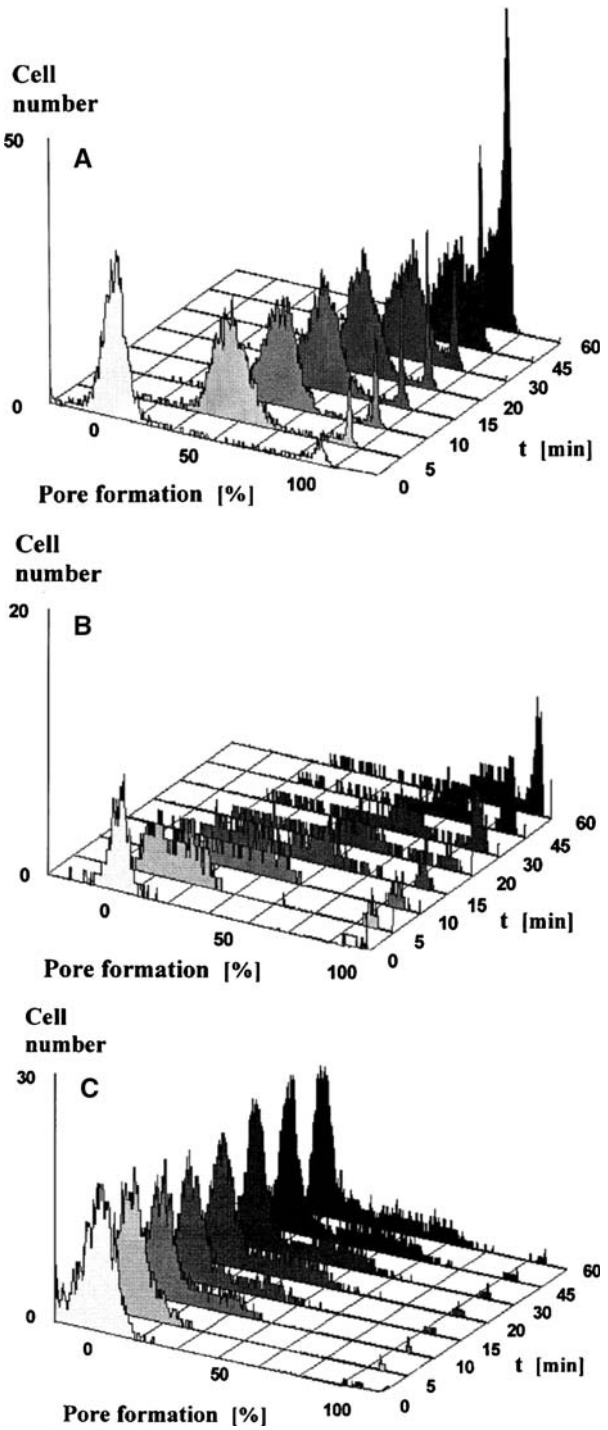

Fig. 7 By use of flow cytometry, pore-formation of blood cells by HlgA/HlgB can be followed by ethidium fluorescence after UV exposure and as a result of its combination with nucleic acids when penetrating in the cell. The maximal fluorescence was located at the region corresponding to dead cells originally tested at a density of 10^5 cells/ml. Both the two proteins HlgA and HlgB were applied at a saturating concentration of 10 nM. Diagram charts represent cell instant fluorescence recorded at 0, 5, 10, 15, 20, 30, 45, and 60 min after toxin application and for 3,000 gated cells analyzed for each assay. (**a**) Human PMNs. (**b**) Human monocytes. (**c**) Human $CD4^+$ lymphocytes. Diagrams are representative of four independent series

heterogeneity in results could rely on the different lipid composition of the cell membrane. Previous studies (Gottfried 1971; Labrouche et al. 1996) as well as more recent ones (Bakan et al. 2006; Rouzer et al. 2006) show how different could be the membrane of the leucocyte types in terms of the relative proportion among the different lipids present. In particular, the permeabilization is complete on human PMNs and PMNs membranes contain PC and Cho in an almost equimolar ratio (Leidl et al. 2008), the best ratio found also for HlgA/HlgB activity on liposomes. Furthermore, the relevance of the membrane microdomains (i.e., lipid rafts) for the $CD4^+$ functions is well known. Lipid rafts are critical to the assembly of the T-cell receptor signaling machinery and changes in their lipid composition in $CD4^+$ T cells of elderly individuals explain age-related $CD4^+$ T cell alterations (Lalazar et al. 2008; Larbi et al. 2006; Rouquette-Jazdanian et al. 2005).

Lipids have been demonstrated to be crucial for leukotoxin activity (Nishiyama et al. 2006; Valeva et al. 2006), and the present study reinforce this idea by the systematic investigation of the toxin activity on different membranes. In fact, the various extent of permeabilization on different leucocytes could be correlated to the different lipid composition of the cell membrane, which could contain different amount of unsaturated or short lipids, influencing the γ-hemolysins activity, as we demonstrate here. It is well known that dietary fat can influence the fatty acid composition of tissue lipids, including those of cell membrane and in particular the membrane composition of leukocytes (Brassard et al. 2007; Erman et al. 2006). Better accessibility to the PC head groups provided by the presence of unsaturated lipid and cholesterol could directly trigger pore formation on biological membranes.

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