Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# Detection of liposomal cholesterol and monophosphoryl lipid A by QS-21 saponin and *Limulus polyphemus* amebocyte lysate

Q2 Q1 Zoltan Beck [a,b], Gary Matyas [b], Carl R. Alving [b,*]

[a] U.S. Military HIV Research Program, Henry M. Jackson Foundation for the Advancement of Military Medicine, 6720A Rockledge Drive, Bethesda, MD 20817 USA
[b] Laboratory of Adjuvant and Antigen Research, US Military HIV Research Program, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910, USA

ARTICLE INFO

*Article history:*
Received 2 October 2014
Received in revised form 26 November 2014
Accepted 5 December 2014
Available online xxxx

*Keywords:*
Liposomal model membranes
Lipid bilayer heterogeneity
Cholesterol
QS-21 saponin
Monophosphoryl lipid A
Limulus amebocyte lysate

ABSTRACT

Liposomes containing cholesterol (Chol) have long been used as an important membrane system for modeling the complex interactions of Chol with adjacent phospholipids or other lipids in a membrane environment. In this study we utilize a probe composed of QS-21, a saponin molecule that recognizes liposomal Chol and causes hemolysis of erythrocytes. The interaction of QS-21 with liposomal Chol results in a stable formulation which, after injection into the tissues of an animal, lacks toxic effects of QS-21 on neighboring cells that contain Chol, such as erythrocytes. Here we have used liposomes containing different saturated phospholipid fatty acyl groups and Chol, with or without monophosphoryl lipid A (MPLA), as model membranes. QS-21 is then employed as a probe to study the interactions of liposomal lipids on the visibility of membrane Chol. We demonstrate that changes either in the mole fraction of Chol in liposomes, or with different chain lengths of phospholipid fatty acyl groups, can have a substantial impact on the detection of Chol by the QS-21. We further show that liposomal MPLA can partially inhibit detection of the liposomal Chol by QS-21. The Limulus amebocyte lysate assay is used for binding to and detection of MPLA. Previous work has demonstrated that sequestration of MPLA into the liposomal lipid bilayer can block detection by the Limulus assay, but the binding site on the MPLA to which the Limulus protein binds is unknown. Changes in liposomal Chol concentration and phospholipid fatty acyl chain length influenced the detection of the liposome-embedded MPLA.

© 2014 Published by Elsevier B.V.

UNCORRECTED PROOF

## 1. Introduction

Molecular heterogeneity of mixtures of phospholipids, cholesterol (Chol), and other lipids in liposomal model membranes can lead to complex spatial patterns, different thermotropic phase distributions, lipid polymorphisms, and structural changes of different classes, types, and groups of individual species of the membrane lipids [1–6]. It is believed that the liposomal lipid bilayer can exist simultaneously and dynamically in a “liquid-disordered (fluid)” and “liquid ordered” phase in which the coexisting liquid phases differ in the different degrees of acyl chain order [7,8]. An intermediate phase has been further postulated that is ordered in the conformational structure of the lipid chains but is disordered based on the lateral positions of the molecules [7,8]. Intermediate phases of membrane lipids may be manifested in the form of lipid “rafts” that can be microscopic or even nanoscopic domains [9,10].

In the elucidation of membrane organization, Chol has played a key role and exhibits great versatility from a functional standpoint because of the many types of chemical and physical interactions both with other membrane lipids [11–13], and even with membrane-associated proteins [14,15]. The complexities of self-association of Chol molecules, with resultant superlattice and other types of geometric formations [16–18], result in liposomal surface areas or patches that can be probed at the membrane–water interface with a variety of water-soluble chemicals, such as cholesterol oxidase [6,19], cytolytic toxins [20,21], or even monoclonal antibodies [22].

In the present study, in order to model the surface characteristics of complex lipid bilayers we utilized variations of lipids related to those of a unique liposomal formulation known as AS01, a liposomal membrane system that serves as an adjuvant constituent in commercial vaccines to malaria and other diseases [23]. As originally described in a 1996 patent application publication [24], and also taught in subsequent disclosures [25,26], a preferred form of AS01 comprises liposomes containing dioleoyl phosphatidylcholine (DOPC), Chol and monophosphoryl lipid A (MPLA) (the lipid moiety of Gram-negative bacterial lipopolysaccharide) [27], together with QS-21, a member of the saponin family. QS-21 is extracted from the bark of *Quillaja saponaria* tree in Chile [28], and consists of two hydrophilic head groups with several sugar residues, and a hydrophobic region comprised of a triterpene group

* Corresponding author at: Laboratory of Adjuvant and Antigen Research, U.S. Military HIV Research Program, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910, USA. Tel.: +1 301 319 7449; fax: +1 301 319 7518.
*E-mail address:* calving@hivresearch.org (C.R. Alving).

http://dx.doi.org/10.1016/j.bbamem.2014.12.005
0005-2736/© 2014 Published by Elsevier B.V.

ARTICLE IN PRESS



with structural similarity to a sterol, and an alkyl ester [29]. Saponins bind to Chol in lipid bilayers of erythrocytes or liposomes, resulting in irreversible pore formation that is associated with hemolysis of erythrocytes and permeability of liposomes [30,31]. Binding of QS-21 to liposomal Chol results in reduced toxicity to neighboring erythrocytes, but this detoxification process still allows retention of adjuvant activity [24–26]. Here we utilized QS-21 as a probe to examine the roles of liposomal phospholipids and other lipids, and the effects of varying mole fractions of liposomal Chol, on the surface accessibility of liposomal Chol.

AS01 liposomes are prepared by hydration of a dried lipid film [24–26], and this is thus a preparation that theoretically might contain some demixed free cholesterol [32]. In addition, the physical structure of the liposomes may be dramatically altered by the interaction of QS-21 with membrane Chol [33]. Thus, AS01 is an interesting, complex, and stable suspension of liposomal lipids in which the manufacture is sufficiently reproducible to have been employed as an adjuvant structure in numerous government-regulated experimental vaccines [23].

Lipid A is a set of acylated and amidated diglucosamine diphosphate molecular congeners, and MPLA represents one or more congeners lacking the glucosamine C1 phosphate [27,34]. In this study we used the Limulus amebocyte lysate (LAL) assay as a probe to examine the roles of phospholipid chain length and mole fraction of Chol on the liposomal surface expression of MPLA. A lysate of amebocytes from the blood of *Limulus polyphemus* (Atlantic horseshoe crab) containing a clotting protein is widely used as a surrogate probe for detecting the endotoxic activity of LPS or lipid A [35]. Although the exact molecular epitope or structure of lipid A (or MPLA) to which the Limulus protein binds is still not completely clear, incorporation of lipid A into the liposomal bilayer greatly masks both the endotoxic and the LAL activities [36–38]. It is believed that masking of the LAL activity is due to sequestration of the "Limulus-reactive" group of lipid A in the liposomal lipid bilayer resulting in inhibition of binding of the Limulus protein to the lipid A. However, "Limulus-positive" (i.e., reactive) and "Limulus-negative" (non-reactive) liposomes can be created by varying the concentration of liposomal lipid A to higher or lower amounts, respectively [39]. As with other liposomal lipids, lipid A can self-associate to form lipid A-enriched domains [40], and these may be lamellar or non-lamellar [41]. High concentrations of liposomal lipid A presumably lead to self-association or phase separation, with increased surface visibility of the Limulus-reactive group of lipid A.

In the work described here we have found that changes of the mole fractions of liposomal Chol in liposomes having different phospholipid compositions can have substantial impacts on the detection of Chol by the QS-21. Similarly the changes in liposomal Chol concentration can influence the detection of the liposomal MPLA by the Limulus assay. Likewise, the presence of liposomal MPLA itself can also influence the detection of the liposomal Chol by the QS-21.

## 2. Materials and methods

### 2.1. Lipids and saponins

Dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphocholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), cholesterol (Chol), and synthetic monophosphoryl lipid A (MPLA) (PHAD™) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DMPC, DPPC, DSPC, and Chol were dissolved in chloroform and DMPG was dissolved in chloroform: methanol (9:1). Each lipid stock solution was prepared fresh using distilled chloroform. Saponin mixture (cat#: S7900) was purchased from Sigma (St. Louis, MO, USA) and purified QS-21 was purchased from Desert King International (San Diego, CA, USA). Saponin stock solutions were made in PBS.

### 2.2. Preparation of liposomes

Liposomes were prepared as previously described [42]. Lipids were mixed, dried under vacuum, and then liposomes were formed in PBS, pH:7.4, in a final concentration of either 50 mM or 1.272 mM of total phospholipids, as noted. Liposomal phosphatidylcholine and phosphatidylglycerol were in a molar ratio of 9:1. Liposomal Chol varied as indicated. When MPLA was used the molar ratio of total phospholipid:MPLA was 45:1, or 5.6:1 where indicated. The mole percent concentrations of liposomal Chol indicated in each figure are based on the ratio of Chol:total phospholipid (i.e., phosphatidylcholine and phosphatidylglycerol) originally used in the preparation of the liposomes.

### 2.3. Cholesterol analysis

Cholesterol analysis was routinely used to confirm the cholesterol content in the liposome preparation [43]. One to 100 μl of liposomes was diluted in water in a final volume of 100 μl and then was added to 3 ml of glacial acetic acid. Two milliliters of 0.1% ferric chloride/glacial sulfuric acid was slowly layered on the samples. After mixing and then equilibrating the samples to room temperature, the absorbance was read at 560 nm. Standard cholesterol concentration curve with linear regression was used to determine the cholesterol concentration in each preparation of liposomes.

### 2.4. Hemolytic assay

Hemolysis of red blood cells was used as a measure both of the relative amount of free QS-21, and of the toxicity of QS-21 under the indicated experimental conditions. Human red blood cells were purchased from the Research Blood Components LLC (Boston, MA, USA) under a Walter Reed Army Institute of Research protocol reviewed by the independent Institutional Review Board, Division of Human Subjects. Erythrocytes were washed with PBS and were quantified by a Beckman Coulter counter model ACT10 (Indianapolis IN, USA). In each assay of this study, hemolytic activity of QS-21 incubated with or without liposomes was determined in 220 μl volume and each step of the assay was performed at room temperature (22 °C). One hundred microliters of QS-21 dilution was incubated with 100 μl of liposomes, or PBS only, on a Daigger Rocker (Vernon Hills, IL, USA) for 15 minutes. After mixing the liposomes, $2 \times 10^7$ erythrocytes in 20 μl of PBS were added to the mixture and incubated on a Daigger Rocker for an additional 30 minutes. Plates were centrifuged at 800 $\times g$ for 6 min. Supernatant was transferred to a polystyrene 96-well plate, and absorbance was read at 541 nm. Hemolysis by QS-21 binding to liposomal Chol was expressed as % of maximum hemolysis by free QS-21.

All of the experiments were highly reproducible. In each figure, all of the data are shown as the mean of at least two independent experiments. The curves are closely representative of the conclusions drawn with each of the independent experiments. To illustrate this, the QS-21 dose curves shown in Fig. 2 were repeated in numerous independent experiments, each with a separately manufactured liposome (L) or L (MPLA) preparation containing 50 mol% Chol.

### 2.5. Limulus amebocyte lysate assay

Limulus amebocyte lysate (LAL) Kinetic-QCL assay was purchased from Lonza (Allendale, NJ, USA). The assay was performed on the Spectramax M5 (Molecular Devices) platform using the SoftMax Pro Chromo-LAL protocol at 37 °C, using the following parameters: $\Delta t =$ 150 seconds, measurement filter = 405 nm, $\Delta$mOD = 200, number of reads = 40. The results were presented in EU/ml units.

Please cite this article as: Z. Beck, et al., Detection of liposomal cholesterol and monophosphoryl lipid A by QS-21 saponin and *Limulus polyphemus* amebocyte lysate, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamem.2014.12.005

ARTICLE IN PRESS



## 3. Results

### 3.1. Effect of liposomal Chol on hemolytic activity of co-incubated QS-21

The degree of erythrocyte damage caused by QS-21 was inversely related to the mol% of liposomal Chol that had been pre-incubated with QS-21 (Fig. 1). Free QS-21 caused maximum hemolysis at approximately 2 μg, but when QS-21 was pre-incubated with liposomes containing either 33.7 or 43 mol% Chol maximum hemolysis occurred at approximately 4 μg of QS-21. Liposomes having 55 mol% Chol completely blocked the hemolytic effect of QS-21 up to 200 μg (Fig. 1).

### 3.2. Inhibition of binding of QS-21 to liposomal Chol by liposomal MPLA

Binding of QS-21 to DMPC/DMPG/Chol liposomes containing 0.127 μmol total phospholipids and 50 mol% Chol was compared to the same liposomes that also contained 0.025 μmol of MPLA (DMPC/DMPG/Chol/MPLA). As shown in Fig. 2, 50 mol% Chol liposomes without MPLA (L) reached 100% hemolysis only at the concentration of 25 μg of QS-21. With liposomes having MPLA [L(MPLA)], hemolysis reached a maximum level at 5 μg of QS-21, which was similar to the hemolytic curve of free QS-21. It is thus clear that 50% Chol was less visible to QS-21 in liposomes containing MPLA when compared to those lacking MPLA.

### 3.3. Fatty acyl chain length of the liposomal saturated phospholipids changes the accessibility of liposomal Chol for binding of QS-21

Three liposome compositions containing 0.114 μmol of phosphatidylcholine, consisting of DMPC, DPPC, and DSPC, respectively, together with 0.0127 μmol of DMPG, and containing either 33.7% or 55% Chol with or without 2.85 nmol MPLA, were pre-incubated with QS-21 (Fig. 3). As shown in Fig. 3A, with up to 10 μg of QS-21, little or none of the QS-21 bound to DMPC, DPPC, or DSPC liposomes having 33.7 mol% Chol. However, increasing the liposomal Chol to 55% (Fig. 3B) increased the binding of QS-21 to Chol in DSPC liposomes, and especially in DMPC liposomes. In contrast, DPPC liposomes with 55% Chol bound much less QS-21 than the DMPC or DSPC liposomes (Fig. 3B). The presence of MPLA inhibited the binding of QS-21 to DMPC liposomes with 55% Chol, but did not inhibit the binding to either DPPC or DSPC liposomes (Fig. 3C).

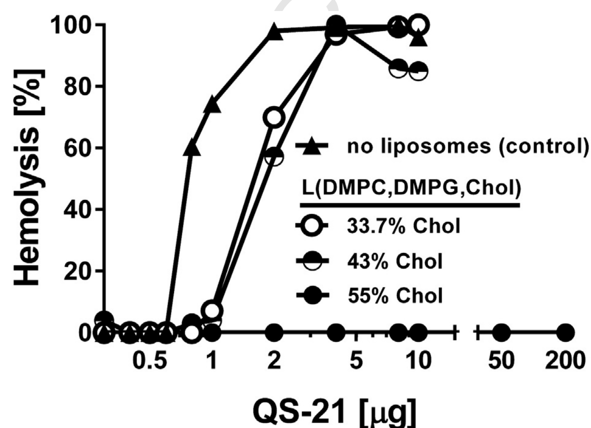


**Fig. 1.** Hemolysis of erythrocytes by QS-21 pre-incubated with liposomes. QS-21 was mixed with DMPC/DMPG liposomes containing a total of 5 μmol of phospholipids at 22 °C. The liposomes also contained either 33.7, or 43, or 55 mol% Chol, respectively.

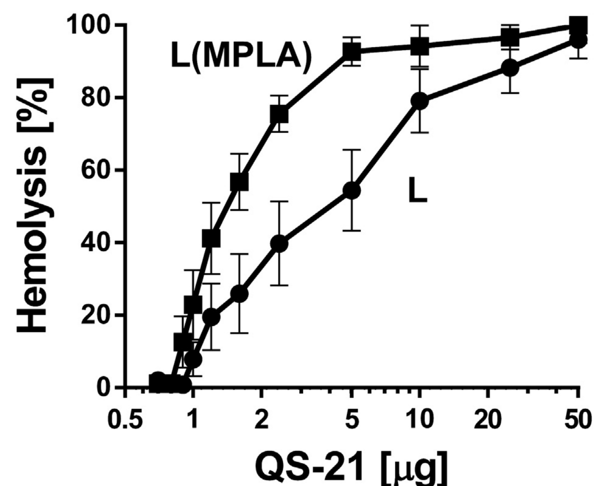


**Fig. 2.** Inhibition of binding of QS-21 to liposomal Chol by liposomal MPLA. QS-21 was pre-incubated with liposomes consisting of a total of 0.127 μmol of phospholipid (DMPC/DMPG, 9/1), 50 mol% Chol, and either lacking or containing MPLA, (L) or L(MPLA) (MPLA:phospholipid = 1:5.6), as indicated. The mean ± S.D. is shown with 14 independent liposome batches of L, and with 4 independent batches of L(MPLA).

### 3.4. Cholesterol transfer between liposomes

As shown in Fig. 4, Chol transfer from liposomes containing 66% Chol to liposomes lacking Chol was detected by using QS-21 as a probe. Preincubation of QS-21 with [L(0% Chol) + L(66% Chol)] resulted in hemolytic activity of QS-21 that was intermediate between that observed after preincubation of QS-21 separately with either L (0% Chol) or L(66% Chol) (Fig. 4A). Preincubation of QS-21 with an equal mixture of liposomes containing MPLA, and containing or lacking Chol [L(0% Chol + MPLA) + L(66% Chol + MPLA)] also resulted in an intermediate level of hemolysis due to QS-21 (Fig. 4B). However, the curve of [L(0% Chol + MPLA) + L(66% Chol + MPLA)] shown in Fig. 4B was shifted toward greater hemolytic activity when compared with liposomes lacking MPLA [L(0%Chol) + L(66%Chol)] (Fig. 4A). It is thus clear that the presence of MPLA in liposomes either inhibited the Chol transfer between liposomes or shielded the visibility of the Chol in the mixture of liposomes.

### 3.5. Limulus amebocyte lysate recognition of DMPC/DMPG/Chol/MPLA liposomes can be suppressed by increasing the amount of liposomal Chol

Fig. 5A shows that the MPLA in DMPC/DMPC/Chol/MPLA, DPPC/DMPC/Chol/MPLA, or DSPC/DMPC/Chol/MPLA liposomes containing 33.7 mol % Chol was detected by the LAL assay. The recognition of MPLA by LAL in liposomes containing 33.7% Chol was proportional to the length of saturated fatty acyl chains of phosphatidylcholine, with LAL binding in the order DSPC > DPPC > DMPC. However, at 55 mol% Chol the ability of LAL to detect MPLA in each of the liposomes was dramatically reduced (Fig. 5B).

## 4. Discussion

Strong interactions of QS-21 with Chol make this an interesting reagent to examine as a probe of liposomal Chol and adjacent bilayer lipids. Here we utilized QS-21 as a unique probe for studying the accessibility of Chol in complex liposome formulations that contained DMPC, DPPC, or DSPC, together with DMPG, and that contained or lacked MPLA. These lipid combinations were employed because of extensive previous work in which combinations of these and related lipids, including other

Please cite this article as: Z. Beck, et al., Detection of liposomal cholesterol and monophosphoryl lipid A by QS-21 saponin and *Limulus polyphemus* amebocyte lysate, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamem.2014.12.005

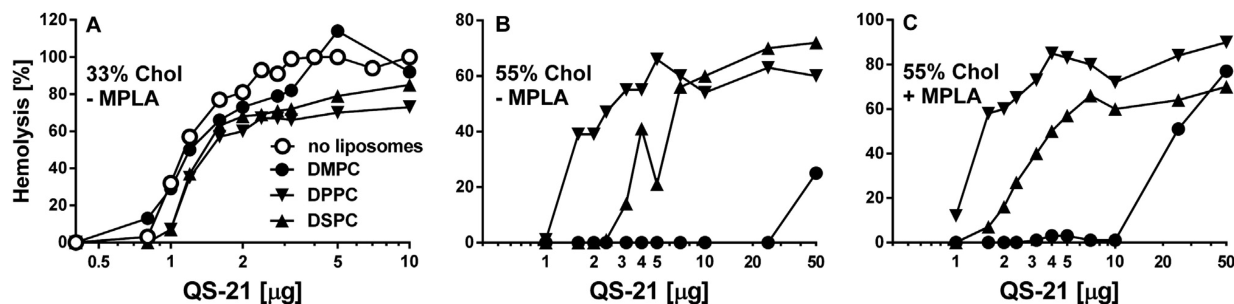


**Fig. 3.** Effect of phospholipid fatty acyl chain length on accessibility of liposomal Chol for binding of QS-21. The liposomes contained DMPC, DPPC, or DSPC, as indicated. (A) Liposomes with 33.7 mol% Chol and no MPLA. (B) Liposomes with 55 mol% Chol, and no MPLA. (C) Liposomes with 55 mol% Chol and MPLA.

saponins, have been used for biophysical and immunological studies of liposomal model membranes hydrated from dried lipid films [20,33, 44–50].

The major goals of this study were to utilize QS-21 as a probe to determine the relative effects of saturated phospholipid fatty acyl chain length and the presence of MPLA on the visibility of Chol in a geometrically complex membrane. Due to its six 14-carbon fatty acids that serve as a hydrophobic anchor, MPLA is strongly embedded in the liposomal bilayer. LAL served as an important measure of the relative visibility of the liposomal MPLA. It is well known that the ratio of Chol:phospholipid solubility in liposomes is not a linear function, with major structural differences occurring at 1:2, 1:1, and at 2:1 which is the theoretical upper limit of association. Some release of free Chol due to de-mixing has been reported to occur during hydration of liposomes from dried films [32]. A mean of 51 $\pm$ 6 mol% Chol uptake was found with different batches of Chol:DPPC liposomes containing initial ratios of 2:1 [32]. However, as indicated in Fig. 2 variation caused by different amounts of free Chol present in different liposome batches, if it occurred, was relatively small when compared to the interactions of liposomal Chol and MPLA that were detected by QS-21.

The complex nano-architecture of the lipid bilayer is governed by the size, shape, and orientation of the linear display of individual neighboring lipid molecules and groupings of the molecules. In the present work we have found that the lengths of the saturated fatty acyl chain lengths of neighboring phospholipids, and the self-association of Chol into membrane surface patches, each played an important role in the ability of QS-21 to bind to Chol. The physical characteristics of liposomal Chol are determined by the three main structural elements, in which a small polar group (3β-hydroxyl) is attached to a rigid asymmetrical steroid structure, and a short hydrocarbon chain attached to the steroid ring structure at position 17 [16]. When compared to surrounding bulk phospholipids it is estimated that Chol has a hydrophobic length approximately equivalent to a glycerol phospholipid having 17 carbon saturated fatty acyl chains [51]. It was proposed that functional mismatches can occur between the hydrophobic length of the Chol molecule and the hydrophobic thickness of the surrounding phospholipid bilayer, based on phospholipid fatty acyl chain length [12,51].

As evidence for a functional mismatch of hydrophobic length of Chol and adjacent phospholipids as proposed above, when Chol-dependent cytolytic toxins (a class of molecules known as cytolysins) are used as probes of the lipid bilayer, the ability of the cytolysin to interact with liposomal Chol is governed by the self-association properties of the Chol in the context of the phospholipid composition of the bilayer, such that tetanolysin cytolysin reached a maximum of binding at 50% Chol [20]. Binding of the θ-toxin cytolysin of *Clostridium perfringens* [52], and the perfingolysin O cytolysin [21], to liposomes containing Chol was in the order DMPC < DPPC < DSPC. It was proposed that phospholipids with >17 carbon saturated fatty acyl chains (such as DSPC) pushed Chol molecules out from under the headgroup, thus making them more exposed for binding to the cytolysin [21]. Interestingly, in the present study we found that DPPC was more effective than DMPC or DSPC in shielding liposomal Chol from binding of QS-21 (resulting in hemolysis by QS-21) in the order DPPC < DSPC < DMPC (see Fig. 3B). Although the reason for this differential recognition of Chol by QS-21 is unclear, we presume that it could indicate a close physical association of Chol with DPPC because of similarities in the hydrophobic lengths of the two compounds, and it might be related to increased points of contact between DPPC and Chol in the context of the surrounding bilayer.

To add to the complexity, liposomal Chol can also transfer between co-incubated liposomes having different mole fractions of Chol resulting in an intermediate concentration of liposomal Chol between the donor and recipient liposomes [53–55]. The transfer process between liposomes apparently involves an aqueous-soluble form of Chol rather than exchange through collision of liposomes [56–59]. In the present study we found that QS-21 can be useful as a probe for detecting transfer of Chol from liposomes containing 66% Chol to those lacking Chol. In addition, we also found that the presence of MPLA in DMPC liposomes interfered not only with the detection of liposomal Chol by

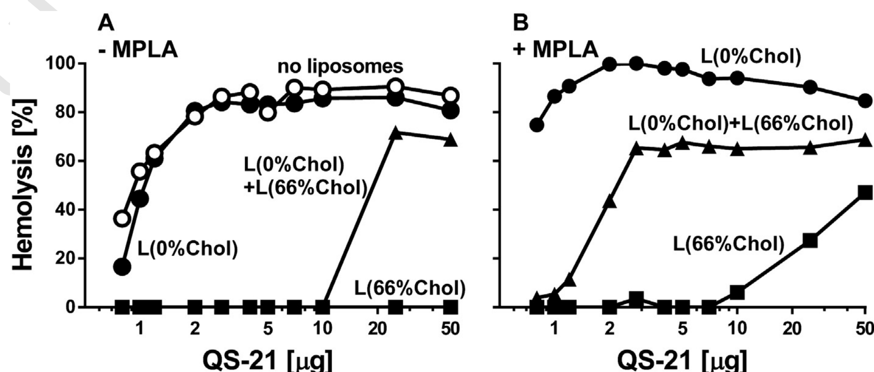


**Fig. 4.** Inhibition by MPLA of Chol transfer between DMPC/DMPG liposomes. Chol transfer was detected by QS-21 binding to liposomes containing either: (A) no MPLA, or (B) MPLA.

Please cite this article as: Z. Beck, et al., Detection of liposomal cholesterol and monophosphoryl lipid A by QS-21 saponin and *Limulus polyphemus* amebocyte lysate, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamem.2014.12.005

ARTICLE IN PRESS



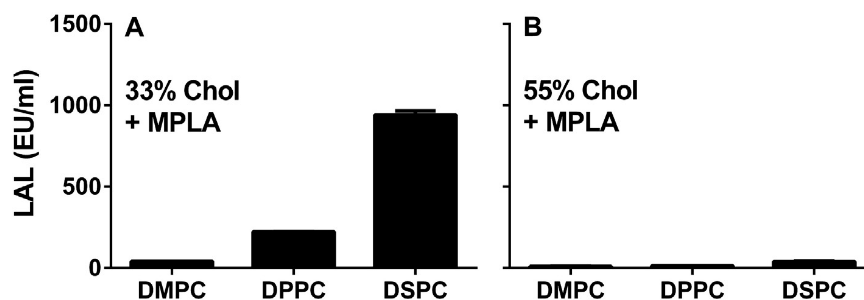


**Fig. 5.** Increasing amounts of liposomal Chol suppress LAL positivity of DMPC/DMPG/Chol/MPLA liposomes. (A) LAL binding to liposomes containing 33.7 mol% Chol. (B) LAL binding to liposomes containing 55 mol% Chol.

QS-21, but also with the transfer of Chol from 66% Chol liposomes to liposomes lacking Chol. It should be noted that uptake of Chol by liposomes lacking Chol (Fig. 4A) theoretically could have been partly due to pre-existing free Chol in the donor liposome preparation [32]. However, the increased (ca. 10-fold) by liposomal MPLA of Chol transfer from donor to recipient liposomes (Fig. 4B vs. Fig. 4A) was an independent function of the MPLA that was present in the donor and recipient liposomes. Interestingly, the ability of ≥50% liposomal Chol to transfer between adjacent lipid bilayer membranes does not seem to have a substantial effect on the properties of liposomes *in vivo*. As evidence of this, we previously showed that after injection of DMPC liposomes containing both lipid A and 71% Chol into mice, the liposomes were sufficiently stable *in vivo* to induce highly specific antibodies to Chol that could bind to lipid A-free liposomes having ≥50% Chol, but not to those containing 43% Chol [22].

As noted in Introduction, detection of liposomal MPLA by the LAL assay is strongly dependent on the molar concentration of MPLA in the liposomes. Upon examining liposomes containing 33.7% Chol and MPLA, in which the liposomes had different phospholipid fatty acyl groups, the detection of MPLA by LAL was directly related to the phospholipid fatty acyl chain length, with DMPC < DPPC < DSPC (Fig. 5A). The MPLA that we employed is a synthetic congener of MPLA that contains six acylated or amidated myristoyl fatty acids; and after inclusion of this MPLA in liposomes the mismatch of surrounding phosphatidylcholines having longer fatty acyl groups (i.e., DPPC or DSPC, compared to DMPC) was associated with, and perhaps somehow promoted, greater visibility of the MPLA for detection by the LAL on the surface of the liposomes. It is not yet clear which local site on MPLA is shielded from LAL binding by DMPC,

In this study we have focused on QS-21 and LAL as representing novel probes of the surfaces of liposomes containing Chol and MPLA. Saponins such as QS-21 are lipid glycans that have profound physical effects on the liposomes after binding to Chol in that they cause complex and stable trans-membrane holes (pores) in the liposomes [31], and interactions of liposomes with Quil A, a mixture of Quillaja saponins, can result in highly complicated lipid structures [33]. The exact geometric structure of the holes or other structures in the liposomes caused by QS-21-Chol interaction, and their orientation with respect to adjacent liposomal MPLA, is not clear. However, it is possible that that after binding of QS-21 to liposomal Chol hydrophobic regions of the liposomes could be exposed under some conditions to allow binding of LAL to liposomal MPLA. It thus appears that the LAL assay represents yet another unique probe that could be useful for examining the influence of complex or mismatched lipid mixtures that display reactive groups on the surface of liposomal lipid bilayer membranes.

## Acknowledgements

This work was supported through a Cooperative Agreement (W81XWH-11-2-0174) between the Henry M. Jackson Foundation for the Advancement of Military Medicine and the U.S. Army Medical Research and Materiel Command (MRMC). The authors thank Mr. Christopher Spiridon for technical assistance. The views expressed in this article are those of the authors and do not necessarily reflect the official policy of the Department of the Army, Department of Defense, or the U.S. Government.

## References

[1] J.L. Rubenstein, B.A. Smith, H.M. McConnell, Lateral diffusion in binary mixtures of cholesterol and phosphatidylcholines, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 15–18.
[2] P.R. Cullis, B. de Kruijff, Lipid polymorphism and the functional roles of lipids in biological membranes, Biochim. Biophys. Acta 559 (1979) 399–420.
[3] B. Snyder, E. Freire, Compositional domain structure in phosphatidylcholine–cholesterol and sphingomyelin–cholesterol bilayers, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 4055–4059.
[4] D. Bach, E. Wachtel, Phospholipid/cholesterol model membranes: formation of cholesterol crystallites, Biochim. Biophys. Acta 1610 (2003) 187–197.
[5] S.J. Marrink, A.H. de Vries, D.P. Tieleman, Lipids on the move: simulations of membrane pores, domains, stalks and curves, Biochim. Biophys. Acta 1788 (2009) 149–168.
[6] Y. Lange, S.M. Tabei, J. Ye, T.L. Steck, Stability and stoichiometry of bilayer phospholipid-cholesterol complexes: relationship to cellular sterol distribution and homeostasis, Biochemistry 52 (2013) 6950–6959.
[7] O.G. Mouritsen, The liquid-ordered state comes of age, Biochim. Biophys. Acta 1798 (2010) 1286–1288.
[8] O.G. Mouritsen, Model answers to lipid membrane questions, Cold Spring Harb. Perspect. Biol. 3 (2011) a004622.
[9] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[10] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, Science 327 (2010) 46–50.
[11] D. Tang, B. Wieb van der Meer, S.-Y. Chen, Evidence for a regular distribution of cholesterol in phospholipid bilayers from diphenylhexatriene fluorescence, Biophys. J. 68 (1995) 1944–1951.
[12] H. Ohvo-Rekilä, B. Ramstedt, P. Leppimäki, J.P. Slotte, Cholesterol interactions with phospholipids in membranes, Prog. Lipid Res. 41 (2002) 66–97.
[13] M.L. Berkowitz, Detailed molecular dynamics simulations of model biological membranes containing cholesterol, Biochim. Biophys. Acta 1788 (2009) 86–96.
[14] R.M. Epand, Do proteins facilitate the formation of cholesterol-rich domains? Biochim. Biophys. Acta 1666 (2004) 227–238.
[15] R.M. Epand, Proteins and cholesterol-rich domains, Biochim. Biophys. Acta 1778 (2008) 1576–1582.
[16] T. Róg, M. Pasenkiewicz-Gierula, I. Vattulainen, M. Karttunen, Ordering effects of cholesterol and its analogues, Biochim. Biophys. Acta 1788 (2009) 97–121.
[17] P.L. Chong, W. Zhu, B. Venegas, On the lateral structure of model membranes containing cholesterol, Biochim. Biophys. Acta 1788 (2009) 2–11.
[18] I.P. Sugár, P.L. Chong, A statistical mechanical model of cholesterol/phospholipid mixtures: linking condensed complexes, superlattices, and the phase diagram, J. Am. Chem. Soc. 134 (2012) 1164–1171.
[19] M.R. Ali, K.H. Cheng, J. Huang, Assess the nature of cholesterol-lipid interactions through the chemical potential of cholesterol in phosphatidylcholine bilayers, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 5372–5377.
[20] C.R. Alving, W.H. Habig, K.A. Urban, M.C. Hardegree, Cholesterol-dependent tetanolysin damage to liposomes, Biochim. Biophys. Acta 551 (1979) 224–228.
[21] J.J. Flanagan, R.K. Tweten, A.E. Johnson, A.P. Heuck, Cholesterol exposure at the membrane surface is necessary and sufficient to trigger perfringolysin O binding, Biochemistry 48 (2009) 3977–3987.
[22] G.M. Swartz Jr., M.K. Gentry, L.M. Amende, E.J. Blanchette-Mackie, C.R. Alving, Antibodies to cholesterol, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 1902–1906.
[23] N. Garçon, M. Van Mechelen, Recent clinical experience with vaccines using MPL- and QS-21-containing adjuvant systems, Expert Rev. Vaccines 10 (2011) 471–486.
[24] N.M. Garcon, M. Friede. Vaccines containing a saponin and a sterol. European patent specification No. EP 0 822 831, Publication No. WO 96/33739 (1996). Also filed as

Please cite this article as: Z. Beck, et al., Detection of liposomal cholesterol and monophosphoryl lipid A by QS-21 saponin and *Limulus polyphemus* amebocyte lysate, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamem.2014.12.005

ARTICLE IN PRESS



U.S. patent application No. 10/967,395, Publication No. 2005/0214322 (Sep 29, 2005).
[25] P. Vandepapeliere. Vaccine compositions comprising a saponin adjuvant. US patent application No. 12/096,838, Publication No. US2008/0279926 (Nov. 13, 2008).
[26] P. Vandepapeliere. Vaccine compositions comprising a saponin adjuvant. US patent application No. 13/020,045, Publication No. US2011/0206758 (Aug. 25, 2011).
[27] C.R. Alving, M. Rao, N.J. Steers, G.R. Matyas, A.V. Mayorov, Liposomes containing lipid A: an effective, safe, generic adjuvant system for synthetic vaccines, Expert Rev. Vaccines 11 (2012) 733–744.
[28] C.R. Kensil, U. Patel, M. Lennick, D. Marciani, Separation and characterization of saponins with adjuvant activity from Quillaja saponaria Molina cortex, J. Immunol. 146 (1991) 431–743.
[29] J.L. Cleland, C.R. Kensil, A. Lim, N.E. Jacobsen, L. Basa, M. Spellman, D.A. Wheeler, J.Y. Wu, M.F. Powell, Isomerization and formulation stability of the vaccine adjuvant QS-21, J. Pharm. Sci. 85 (1996) 22–28.
[30] P. Seeman, Ultrastructure of membrane lesions in immune lysis, osmotic lysis and drug-induced lysis, Fed. Proc. 33 (1974) 2116–2124.
[31] A.D. Bangham, Physical structure and behavior of lipids and lipid enzymes, Adv. Lipid Res. 1 (1963) 65–104.
[32] J. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta 1417 (1999) 89–100.
[33] T. Paepenmüller, C.C. Müller-Goymann, Influence of Quil A on liposomal membranes, Int. J. Pharm. 475 (2014) 138–146.
[34] N. Qureshi, K. Takayama, E. Ribi, Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of Salmonella typhimurium, J. Biol. Chem. 257 (1982) 11808–11815.
[35] K. Brandenburg, J. Howe, T. Gutsman, P. Garidel, The expression of endotoxic activity in the Limulus test as compared to cytokine production in immune cell, Curr. Med. Chem. 16 (2009) 2653–2660.
[36] C.R. Alving, R.L. Richards, Liposomes containing lipid A: a potent nontoxic adjuvant for a human malaria sporozoite vaccine, Immunol. Lett. 25 (1990) 275–279.
[37] C.R. Alving, Lipopolysaccharide, lipid A, and liposomes containing lipid A as immunologic adjuvants, Immunobiology 187 (1993) 430–446.
[38] P. Harmon, D. Cabral-Lilly, R.A. Reed, F.P. Maurio, J.C. Franklin, A. Janoff, The release and detection of endotoxin from liposomes, Anal. Biochem. 250 (1997) 139–146.
[39] E.C. Richardson, B. Banerji, R.C. Seid Jr., J. Levin, C.R. Alving, Interactions of lipid A and liposome-associated lipid A with Limulus polyphemus amoebocytes, Infect. Immun. 39 (1983) 1385–1391.
[40] J. Kubiak, J. Brewer, S. Hansen, L.A. Bagatolli, Lipid lateral organization on giant unilamellar vesicles containing lipopolysaccharides, Biophys. J. 100 (2011) 978–986.
[41] K. Brandenburg, W. Richter, M.H. Koch, H.W. Meyer, U. Seydel, Characterization of the nonlamellar cubic and HII structures of lipid A from *Salmonella enterica* serovar Minnesota by X-ray diffraction and freeze-fracture electron microscopy, Chem. Phys. Lipids 91 (1998) 53–69.
[42] G.R. Matyas, J.M. Muderhwa, C.R. Alving, Oil-in-water liposomal emulsions for vaccine delivery, Methods Enzymol. 373 (2003) 34–50.
[43] A. Zlatkis, B. Zak, A.J. Boyle, A new method for the direct determination of serum cholesterol, J. Lab. Clin. Med. 41 (1953) 486–492.
[44] C.R. Alving, K.A. Urban, R.L. Richards, Influence of temperature on complement-dependent immune damage to liposomes, Biochim. Biophys. Acta 600 (1980) 117–125.
[45] B. Banerji, J.A. Lyon, C.R. Alving, Membrane lipid composition modulates the binding specificity of a monoclonal antibody against liposomes, Biochim. Biophys. Acta 689 (1982) 319–326.
[46] N.M. Wassef, C.R. Alving, R.L. Richards, Liposomes as carriers for vaccines, Immunomethods 4 (1994) 217–222.
[47] P. Vandepapelière, Y. Horsmans, P. Moris, M. Van Mechelen, M. Janssens, M. Koutsoukos, P. Van Belle, F. Clement, E. Hanon, M. Wettendorff, N. Garçon, G. Leroux-Roels, Vaccine adjuvant systems containing monophosphoryl lipid A and QS21 induce strong and persistent humoral and T cell responses against hepatitis B surface antigen in healthy adult volunteers, Vaccine 26 (10) (Mar 4 2008) 1375–1386.
[48] J. Lorent, C.S. Le Duff, J. Quetin-Leclercq, M.P. Mingeot-Leclercq, Induction of highly curved structures in relation to membrane permeabilization and budding by the triterpenoid saponins, α- and δ-Hederin, J. Biol. Chem. 288 (20) (May 17 2013) 14000–14017.
[49] F1. Ciesielski, D.C. Griffin, M. Rittig, I. Moriyón, B.B. Bonev, Interactions of lipopolysaccharide with lipid membranes, raft models - a solid state NMR study, Biochim. Biophys. Acta 1828 (8) (Aug 2013) 1731–1742.
[50] V.J. Venditto, L. Wieczorek, S. Molnar, F. Teque, G. Landucci, D.S. Watson, D. Forthal, V.R. Polonis, J.A. Levy, F.C. Szoka Jr., Chemically modified peptides based on the membrane-proximal external region of the HIV-1 envelope induce high-titer, epitope-specific nonneutralizing antibodies in rabbits, Clin. Vaccine Immunol. 21 (8) (2014 Aug) 1086–1093.
[51] T.P.W. McMullen, R.N.A.H. Lewis, R.N. McElhaney, Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated phosphatidylcholines, Biochemistry 32 (1993) 516–522.
[52] Y. Ohno-Iwashita, M. Iwamoto, K. Mitsui, S. Ando, S. Iwashita, A cytolysin, θ-toxin, preferentially binds to membrane cholesterol surrounded by phospholipids with 18-carbon hydrocarbon chains in cholesterol-rich region, J. Biochem. 110 (1991) 369–375.
[53] B. Bloj, D.B. Zilversmit, Complete exchangeability of cholesterol in phosphatidylcholine/cholesterol vesicles of different degrees of unsaturation, Biochemistry 16 (1977) 3943–3948.
[54] M.J. Poznansky, Y. Lange, Transbilayer movement of cholesterol in phospholipid vesicles under equilibrium and non-equilibrium conditions, Biochim. Biophys. Acta 506 (1978) 256–264.
[55] Y. Nakagawa, K. Inoue, S. Nojima, Transfer of cholesterol between liposomal membranes, Biochim. Biophys. Acta 553 (1979) 307–319.
[56] J.M. Backer, E.A. Dawidowicz, Mechanism of cholesterol exchange between phospholipid vesicles, Biochemistry 20 (1981) 3805–3810.
[57] L.R. McLean, M.C. Phillips, Mechanism of cholesterol and phosphatidylcholine exchange or transfer between unilamellar vesicles, Biochemistry 20 (1981) 2893–2900.
[58] L.R. McLean, M.C. Phillips, Cholesterol desorption from clusters of phosphatidylcholine and cholesterol in unilamellar vesicle bilayers during lipid transfer or exchange, Biochemistry 21 (1982) 4053–4059.
[59] K.R. Bruckdorfer, J. Crowe, M.K. Sherry, Evidence for a water-soluble intermediate in exchange of cholesterol between membranes, Biochim. Biophys. Acta 778 (1984) 489–496.

UNCORRECTED PROOF

Please cite this article as: Z. Beck, et al., Detection of liposomal cholesterol and monophosphoryl lipid A by QS-21 saponin and *Limulus polyphemus* amebocyte lysate, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamem.2014.12.005