Biochimica et Biophysica Acta 1848 (2015) 775–780

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

Zoltan Beck [a,b], Gary R. 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 12 December 2014

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.

Published by Elsevier B.V.

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/Published by Elsevier B.V.

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. Purified QS-21 was purchased from Desert King International (San Diego, CA, USA). Saponin stock solutions (1 mg/ml) were made in PBS (pH 7.0).

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, 2×10^7 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, $\Delta OD = 0.2$, number of reads = 40. The results were presented in EU/ml units.