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The Release and Detection of Endotoxin from Liposomes

Paul Harmon,[1] Donna Cabral-Lilly, Robert A. Reed,[2] Frank P. Maurio, J. Craig Franklin, and Andrew Janoff[3]
The Liposome Company, Inc., One Research Way, Princeton, New Jersey 08540

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Incorporation of lipopolysaccharide (LPS) into liposomes dramatically reduces its ability to coagulate *Limulus* amebocyte lysate (LAL). The coagulation of LAL is commonly used to signal the presence of endotoxin *in vitro.* This study demonstrates a simple method to release masked endotoxin from liposomal dispersions using moderate amounts of detergent to form mixed micelles containing lipid, detergent, and LPS. Several parameters were found to affect the degree of liposome solubilization and/or the sensitivity of the LAL assay. These included detergent type and concentration, temperature for solubilization, lipid composition, liposome morphology, and time for test incubation. The nonionic detergent polyoxyethylene 10 lauryl ether ($C_{12}E_{10}$) proved to be unique in its ability to solubilize liposomes and minimally interfere with endotoxin detection. The LAL endotoxin detection limit for samples dispersed in $C_{12}E_{10}$ varied with the phospholipid component; the sensitivity decreased in the order DSPC > DPPC = EPC ≫ DMPC. Cholesterol lowered the solubility limit of the liposomes, but did not appear to affect the LAL assay sensitivity once the liposomes were completely solubilized. The presence of negatively charged phospholipids, DSPG and Pops, also lowered the solubility limit. Pops, but not DSPG, at 10 mol% further decreased the LAL endotoxin detection limit. This detergent-solubilization method should be useful in liposomal LPS immunological studies or in other situations where accurate determination of endotoxin concentration is important.

Lipid A, the biologically active moiety of a lipopolysaccharide frequently referred to as LPS or endotoxin, is a molecule found on the outer membrane of most gram-negative bacteria. In mammalian systems, monomeric endotoxin can associate with a 60-kDa lipopolysaccharide binding protein (LBP),[4] forming a complex that recognizes CD14, a surface receptor of macrophages and other LPS-responsive cells (1). Occupation of this receptor results in the well-known endotoxin response. LPS, also through its lipid A moiety, can activate enzymes in the *Limulus* coagulation cascade that then react with a clottable protein to form a gel (2). Thus coagulation of *Limulus* amebocyte lysate (LAL) is commonly used as an *in vitro* endotoxin assay due to the ease and sensitivity of the method; endotoxin detection levels as low as several picograms per milliliter are achievable. Lipid A, when incorporated into a liposomal bilayer, is unavailable to interact with both LBP and LAL enzymes and thus both its toxic activity and detectability are masked (3).

The masking of the LAL response by liposomally incorporated endotoxin has an impact in several areas of research. The masking effect makes immunological studies comparing free and liposomal LPS-induced cellular responses more difficult to interpret, since the LAL assay measures only the "free" endotoxin concentration. Thus, the amount of LPS actually incorporated

[1,2] Present address: Merck & Co., Inc., P.O. Box 4, Mailstop WP38-3, West Point, PA 19486.
[3] To whom correspondence should be addressed. Fax: 609-520-8250. E-mail: ajanoff@lipo.com.

[4] Abbreviations used: $C_{12}E_3$, polyoxyethylene 3 lauryl ether; $C_{12}E_4$, polyoxyethylene 4 lauryl ether; $C_{12}E_6$, polyoxyethylene 6 lauryl ether; $C_{12}E_8$, polyoxyethylene 8 lauryl ether; $C_{12}E_9$, polyoxyethylene 9 lauryl ether; $C_{12}E_{10}$, polyoxyethylene 10 lauryl ether; $C_{12}E_{23}$, polyoxyethylene 23 lauryl ether (Brij 35); Chaps, 3-[3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; CHOL, cholesterol; CSE, control standard endotoxin; decyl maltoside, decyl β-D-maltopyranoside; DMPC, dimyristoylphosphatidylcholine; DOTAB, dodecyltrimethyl ammonium bromide; DPPC, dipalmitoylphosphatidylcholine; DSPC, disteroylphosphatidylcholine; DSPG, disteroylphosphatidylglycerol; EPC, egg phosphatidylcholine; LAL, *Limulus* amebocyte lysate; LBP, lipopolysaccharide binding protein; LLPS liposomal lipopolysaccharide; LPS, lipopolysaccharide; Lubrol-PX, polyoxyethylene 8 cetyl ether; MLV, multilamellar vesicle; *Mt*-LPS, mutant LPS from *Salmonella minnesota;* octyl glucoside, octyl-β-D-glucopyranoside; PC, phosphatidylcholine; Pops, 1-palmitoyl-2-oleoylphosphatidylserine; SPLV, stable plurilamellar vesicle; SWFI, sterile water for injection; T_m, temperature at which lipid changes from gel to liquid crystalline phase; TEA, triethylamine; USP, United States Pharmacopeia; W-1, polyoxyethylene ether W-1; *wt*-LPS, wild-type LPS.

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into the liposomes is never directly measured, but rather inferred from the inability of the LAL assay to detect the endotoxin at the end of the liposome incorporation procedure. The comparison is valid only if there are no other routes for endotoxin activity decrease during the incorporation procedure.

Several methods have been developed for the measurement of endotoxin concentration and all suffer from significant shortfalls (4, 5). To the best of our knowledge, no fast, simple, reliable method for the detection of endotoxin in the presence of lipid membranes exists. These considerations motivated us to investigate methods to circumvent the "masking" effect and apply the LAL assay accurately to liposomal systems. We describe here a procedure in which liposomes are initially solubilized in detergent and subsequently assayed for masked endotoxin by the LAL gel-clot method. After disruption of the bilayer structure, any endotoxin present putatively exists as part of detergent/lipid mixed micelles. We show that LAL enzymes are able to interact with the lipid A moiety under these conditions with minimal loss in LPS sensitivity. We achieved success by identification of a detergent that has excellent liposome-solubilizing properties and that also preserved the activity of LAL proteins and enzymes. The work reported here focuses on several areas: (1) the extent to which concentrated liposomal dispersions can be solubilized by detergents, (2) the effect of detergents on LAL sensitivity, (3) the demonstration of release and quantification of liposomally masked endotoxin of varying hydrophilicities, and (4) the determination of endotoxin detection limits in detergent/lipid micelles. The method described here is simple, reproducible, and sensitive enough for use in monitoring endotoxin levels in parenteral liposomal drug products, and should be useful in studies concerning the inflammatory and immunogenic responses associated with LPS and liposomal LPS (LLPS).

MATERIALS AND METHODS

DMPC, DPPC, and DSPC were purchased from Nippon Oil & Fats Co., Ltd. (Amagasaki, Japan) and Avanti Polar Lipids (Alabaster, AL). DSPG and Pops were also obtained from Avanti Polar Lipids. EPC was purchased from LIPOID KG (Ludwigshafen, Germany) and Avanti. All phospholipids were used without further purification. Cholesterol, $C_{12}E_3$, $C_{12}E_4$, $C_{12}E_6$, $C_{12}E_8$, $C_{12}E_9$, $C_{12}E_{10}$, $C_{12}E_{23}$, W-1, Chaps, Dotab, Triton X-100, and Tween 80 were purchased from Sigma (St. Louis, MO). $C_{12}E_8$, $C_{12}E_9$, Lubrol-PX (10% solution, protein grade), octyl glucoside, decyl maltoside, deoxycholate, cholate, and Tween 20 were purchased from CalBiochem (San Diego, CA). Pyrosperse (40% solution) was obtained from BioWhittaker, Inc. (Walkersville, MD). *Salmonella minnesota* lipid A and *Mt*-LPS (R595) were purchased from LIST Biologicals (Campbell, CA). *wt*-LPS was obtained from either Endosafe, Inc. (*Escherichia coli* 055:B5, Charleston, SC), United States Pharmacopeia (*E. coli* 055:B5, Rockville, MD) or LIST Biologicals (*Salmonella minnesota*, Campbell, CA), and control standard endotoxin (CSE) was from Associates of Cape Cod (Woods Hole, MA). Sterile water for injection (SWFI) and 0.9% saline for injection were purchased from Abbott Laboratories (Chicago, IL). Tubes containing lyophilized *Limulus* amebocyte lysate reagent, 0.06 EU/ml sensitivity, were purchased from Endosafe, Inc. or Associates of Cape Cod. A commercial endotoxin removal syringe tip (Pyrobind, Sepracor Inc., Marlborough, MA) was used to pretreat detergent solutions to assure the absence of endotoxin contamination. All detergent solutions were adjusted to neutral pH. All other chemicals were used as received. All glassware used for liposome preparation was depyrogenated by heating for at least 3 h at 250°C. Liposome samples were then placed in sterile, pyrogen-free polystyrene tubes for all further studies.

Liposome Preparation

MLVs of the four phosphatidylcholines were prepared by drying appropriate lipid and cholesterol from chloroform solutions on a rotaevaporator and then further drying for several hours under high vacuum. PC:Chol samples were at a 60:40 mol ratio. For MLVs with a net negative charge, DSPC:DSPG or EPC:Pops were mixed at a 90:10 mol ratio in chloroform before drying. The resulting film was hydrated with 0.9% sterile saline solution by heating the dispersion at least 10°C above the target liposome T_m for 1 h with intermittent vortexing. EPC was used for incorporation studies of LPS in MLVs shown in Table 4. EPC MLVs containing lipid A or *Mt*-LPS were made by simply premixing desired amounts of EPC and lipid A or *Mt*-LPS chloroform solutions. Chloroform was then removed by rotoevaporation and drying under vacuum for at least 3 h. The EPC/lipid A or *Mt*-LPS film was then resuspended in pyrogen-free saline. *wt*-LPS is not soluble in lipid casting solvents, and was incorporated into liposomes by hydrating dry EPC with saline containing the desired amounts of *wt*-LPS. The dispersion was then dried by rotaevaporation at 45°C and resuspended in SWFI and then dried and resuspended in SWFI one final time. Liposomes were washed by repeated pelleting by centrifugation and resuspension in fresh SWFI. SPLVs of DSPC, EPC, or EPC/Chol were made by a modification of the method of Gruner *et al.* (7). To incorporate endotoxin, CSE was added to the buffer phase before solvent removal. Large unilamellar vesicles were made by extruding the MLVs (extruder purchased from Lipex Biomembranes, Vancouver, British Columbia, Canada) 10 times through two stacked 0.1-μm polycarbonate filters (Poretics, Livermore, CA).

Detergent Solubilization of Liposomal Dispersions

Qualitative detergent solubilization was determined by taking MLVs at 20 mg/ml lipid, and adding a volume of detergent to give 14 mg/ml lipid in 1.5% detergent (v/v). The mixture was then vortexed well and heated at 65°C for 20 min and allowed to sit at room temperature for 1 h. A 1-h time frame was chosen to assure that the lipid remained solubilized throughout the LAL assay. Dispersions that appeared transparent and did not visibly scatter light at the end of this hour were considered solubilized. All dispersions that were not clear were subsequently diluted by a factor of 2 in 1.5% detergent, reheated, cooled, and then reexamined.

LAL Assay

Limulus amebocyte lysate gel-clot assays were performed, as directed by the supplier, by adding 200 μl of sample to tubes with lyophilized LAL reagent (0.06 EU/ml sensitivity) and mixing. The reagent tubes were immediately incubated at 37°C for 1 or 2 h. Formation of a clot that was stable to careful 180° inversion was considered a positive response, i.e., that there was greater than 0.06 EU/ml of endotoxin. All concentrations were calculated in EU/ml to compensate for variations in both LPS and LAL activity. The amount of endotoxin was estimated by performing successive twofold dilutions until a stable clot failed to form. The extent of inhibition due to detergent or lipid present was determined by adding known amounts of endotoxin standard to appropriate dispersions and measuring the sensitivity of the assay. Heat-inactivation of LPS was investigated by heating samples at temperatures between 37 and 65°C for various times before performing the LAL assay.

Endotoxin Detection Limits

Endotoxin detection limits for the various PC, PC/CHOL, DSPC/DSPG, and EPC/Pops samples were determined using preformed MLVs. Small amounts of CSE (1–10× the detection limit in detergent) were added to the MLVs. A minimal amount of 10% $C_{12}E_{10}$ was added to give a final detergent concentration of 0.5%. The samples were vortexed and then heated to 37°C for 30 min. If the sample was not clear to the eye, it was diluted twofold in 0.5% $C_{12}E_{10}$ and reheated and vortexed. This dilution/heat cycle was repeated until the sample was clear or until the LAL assay detection limit in 0.5% detergent was reached (0.1 EU/ml). Samples still not clear were then heated for 30–45 s at 65°C and vortexed. All samples were clear after this treatment. The concentration of lipid in the clarified dispersion was defined as the dispersion limit (i.e., solubility) of the lipid in 0.5% $C_{12}E_{10}$. LAL assays were performed for each PC and PC/CHOL sample. The endotoxin detection limit was taken to be the lowest amount of CSE giving a positive LAL gel-clot result at the dispersion limit.

RESULTS

Detergent Solubilization

Various detergents were tested to examine their ability to disrupt bilayer structures in relatively concentrated MLV lipid dispersions. As representative cases, solubilization studies of EPC, DMPC, DPPC, and DSPC MLVs, as well as their cholesterol-containing analogs (40 mol% cholesterol) were performed with the detergents listed in Tables 1 and 2. The maximum concentration of lipid that gave a transparent dispersion, at a given detergent concentration, is listed in Table 1 for cholesterol-free liposomes, and Table 2 for 40 mol% cholesterol analogs. The overall performance of a detergent's liposome-solubilizing capability was defined simply as the sum of the maximum lipid solubilities for each liposome dispersion at 1.5% detergent. The overall performances are listed in Tables 1 and 2, and serve as a qualitative gauge of the detergents' liposome-solubilizing effectiveness. The detergents investigated in this search included nonionic, anionic, cationic, and zwitterionic detergents as well as the detergent Pyrosperse which is sold commercially for use with the LAL assay. The charged detergents, Lubrol-PX and the C12EX series of polyoxyethylene lauryl ethers with $X > 6$, showed excellent liposome solubilizing properties resulting in overall performances greater than 35 for cholesterol-free liposomes. Tween 20 and Tween 80 were significantly less effective, while Triton X-100 provided acceptable liposome solubilization. It is interesting to note that Pyrosperse, the only detergent sold commercially specifically for use with the LAL endotoxin assay, was totally ineffective for solubilizing the liposomes investigated. In all cases examined, incorporation of cholesterol into the membrane lessened the detergents' effectiveness and as a result, overall performance decreased.

The morphology of the liposome also affected its rate of solubilization. EPC/CHOL large unilamellar vesicles, MLVs and SPLVs were made at 10 mg/ml total lipid, 40 mol% CHOL, and diluted into 0.5% $C_{12}E_{10}$. Although all three samples were soluble at 1.25 mg/ml in this detergent, the conditions needed were different. The unilamellar vesicles were visibly clear after vortexing at room temperature. For MLVs, heating at 37°C for 15 min followed by vortexing for 3 min was required. The SPLV sample would not completely solubilize until it was heated for 45 s at 60°C and vortexed for 1 min. Gruner *et al.* (7) noted that MLVs, but not SPLVs, are osmotically compressed. This osmotic stress may account for the less harsh conditions needed to solubilize the MLVs.

TABLE 1

Qualitative Detergent Solubilization of Non-cholesterol-Containing Liposomes (Maximum MLV Lipid Concentration Solubilized at the Indicated Detergent Levels, mg/ml)

Detergent	Liposome				Overall performance[c]
	EPC	DMPC	DPPC	DSPC	
Nonionic					
1.5% Tween 20	[a]	3.5	3.5	1.75	8.75
1.5% Tween 80	[a]	1.75	1.75	[a]	3.5
1.5% Triton X-100	3.5	14	14	[a]	31.5
1.5% octyl glucoside	7	14	14	7	42.0
1.5% decyl maltoside	7	14	14	7	42.0
1.5% Lubrol-PX	7	14	14	7	42.0
1.5% W-1	1.75	7	3.5	3.5	15.75
1.5% $C_{16}E_8$	1.75	3.5	1.75	1.75	8.75
$C_{12}E_3$[b]	—	—	—	—	
$C_{12}E_4$[b]	—	—	—	—	
1.5% $C_{12}E_6$	3.5	3.5	3.5	1.75	12.25
1.5% $C_{12}E_8$	7	14	14	7	42.0
1.5% $C_{12}E_9$	7	14	14	14	49.0
1.5% $C_{12}E_{10}$	7	14	14	14	49.0
1.5% $C_{12}E_{23}$ (Brij 35)	1.75	14	14	7	36.75
Zwitterionic					
1.5% Chaps	7	14	14	[a]	35.0
Anionic					
1.5% cholate	7	14	14	14	49.0
1.5% deoxycholate	14	14	14	7	49.0
Cationic					
1.5% DOTAB	7	14	14	14	49.0
Commercial LAL detergent					
6.6% Pyrosperse	[a]	[a]	[a]	[a]	0.00

[a] Liposome is solubilized at less than 1.75 mg/ml levels for the indicated detergent concentration.
[b] Aqueous solutions of these detergents could not be made at significant concentration (i.e., 0.5% or greater) levels.
[c] Overall performance equals the sum of columns 2 through 5.

LAL Assay Inhibition by Detergents

The degree of inhibition of the LAL assay by detergents which showed reasonable liposome solubilizing properties was determined by performing the LAL assays on detergent solutions to which known amounts of standard *wt*-LPS had been added. All detergents with an overall performance greater than about 30 were examined (Table 1). Each detergent tested inhibited the LAL assay to some degree. The charged detergents Chaps, cholate, deoxycholate, and DOTAB all dramatically decreased the LAL assay sensitivity by more than a factor of 100. Similarly, the nonionic detergents octyl glucoside, decyl maltoside, and Triton X-100 also severely inhibited the LAL assay (results not shown). The remaining detergents, all belonging to the $C_{12}E_X$ series of polyoxyethylene lauryl ethers, showed less LAL inhibition.

Table 3 gives the LAL endotoxin detection limits for 0.5% levels of the $C_{12}E_X$ detergents in the presence of known amounts of added endotoxin. $C_{12}E_{10}$ and $C_{12}E_{23}$ inhibited the LAL assay by a factor of only about 2 compared to detergent-free solution. Curiously, while $C_{12}E_{10}$ and $C_{12}E_{23}$ showed the same twofold inhibition, progressively shortening the ether repeat number (X) from 10 to 6 led to systematic increases in LAL inhibition. $C_{12}E_6$ inhibited the LAL assay by a factor of 42 at the 0.5% detergent level. Figure 1 shows the LAL endotoxin detection limit for $C_{12}E_{10}$ at increasing detergent concentrations. The detection limit is better at lower detergent concentrations, but the amount of lipid dissolved is also lower. At the 1.5 or 3.0% detergent levels required for solubilization of 10–20 mg/ml lipid dispersions, $C_{12}E_{10}$ inhibited the LAL assay by an additional factor of 2 or 4. In contrast, $C_{12}E_{23}$ inhibited the LAL assay by an additional factor of at least 64 at these higher levels (data not shown). Therefore, we identified $C_{12}E_{10}$ as a candidate for release and quantification of liposomal endotoxin since it inhibited the LAL assay the least and provided excellent liposome solubilization. In fact, the detergent inhibition could be partially overcome by increasing the incubation time for the LAL test from 60 to 120 min at 37°C. All negative controls (SWFI or detergent alone) were still negative after 120 min, and standard dilution curves for CSE gave similar results at 60 and 120 min.

Heating was required to solubilize some of the lipid samples prior to performing the endotoxin assay. No inhibition of LAL sensitivity was found for any sample

TABLE 2

Qualitative Detergent Solubilization of Cholesterol-Containing Liposomes (Maximum MLV Lipid Concentration Solubilized at the Indicated Detergent Levels, mg/ml)

Detergent	Liposome				Overall performance[c]
	EPC/CHOL	DMPC/CHOL	DPPC/CHOL	DSPC/CHOL	
Nonionic					
1.5% Tween 20	[a]	[a]	[a]	[a]	0.00
1.5% Tween 80	[a]	[a]	[a]	[a]	0.00
1.5% Triton X-100	1.75	[a]	[a]	[a]	1.75
1.5% octyl glucoside	7	3.5	3.5	1.75	15.75
1.5% decyl maltoside	1.75	1.75	1.75	1.75	7.0
1.5% Lubrol-PX	3.5	3.5	3.5	3.5	14.0
1.5% W-1	1.75	[a]	[a]	[a]	1.75
1.5% $C_{16}E_8$	[a]	[a]	[a]	3.5	3.5
$C_{12}E_3$[b]	—	—	—	—	
$C_{12}E_4$[b]	—	—	—	—	
1.5% $C_{12}E_6$	3.5	3.5	[a]	[a]	7.0
1.5% $C_{12}E_8$	3.5	3.5	1.75	1.75	10.5
1.5% $C_{12}E_9$	3.5	1.75	1.75	1.75	8.75
1.5% $C_{12}E_{10}$	7	3.5	1.75	1.75	14.0
1.5% $C_{12}E_{23}$ (Brij 35)	[a]	[a]	[a]	[a]	0.00
Zwitterionic					
1.5% Chaps	[a]	[a]	[a]	[a]	0.00
Anionic					
1.5% cholate	[a]	[a]	[a]	[a]	0.00
1.5% deoxycholate	[a]	1.75	[a]	[a]	1.75
Cationic					
1.5% DOTAB	[a]	3.5	[a]	[a]	3.5
Commercial LAL detergent					
6.6% Pyrosperse	[a]	[a]	[a]	[a]	0.00

[a] Liposome is solubilized at less than 1.75 mg/ml levels for the indicated detergent concentration.
[b] Aqueous solutions of these detergents could not be made at significant concentration (i.e., 0.5% or greater) levels.
[c] Overall performance equals the sum of columns 2 through 5.

when heated to ⩽37°C. Incubating samples at temperatures greater than 37°C for sustained periods of time resulted in loss of LAL sensitivity (e.g., see Table 3) and inconsistent detection. It was possible, however, to apply as many as two short bursts of high heat (⩽45 s at 65°C) to solubilize liposomal formulations not dissolved at 37°C and not interfere with the LAL assay.

TABLE 3

Detergent Inhibition of LAL Gel-Clot Assay[a]

Detergent type (0.5% by vol)	Endotoxin detection limit (EU/ml, *wt*-LPS)
None	0.06
$C_{12}E_{10}$	0.10
$C_{12}E_{23}$	0.12
Lubrol-PX	0.33
$C_{12}E_9$	0.68
$C_{12}E_8$	1.3
$C_{12}E_6$	2.5
$C_{12}E_{10}$[b]	0.20

[a] All other detergents inhibited LAL greater than 100-fold or had dispersion indices less than 30.
[b] Sample heated to 65°C for 4 min.

Masking and Release of Endotoxin from Liposomes

The following set of experiments were designed to determine the degree to which liposomes mask endotoxin detection. Lipid A was incorporated into EPC MLVs to give 48,000 EU/ml lipid A and 10 mg/ml EPC. An aliquot of these liposomes was serially diluted in pyrogen-free saline and LAL tested. The liposomes tested LAL positive until a dilution factor of about 20. $C_{12}E_{10}$ was then added to each dilution to give a concentration of 1.5%, converting the liposomes to micelles. Each dilution was then LAL tested. Even with the fourfold loss in LAL sensitivity expected from the presence 1.5% $C_{12}E_{10}$, LAL activity was observed until a total dilution factor of 160,000. The use of the detergent increased the sensitivity by a factor greater than 8000. This demonstrates endotoxin release and detection upon bilayer disruption. As a control, an identical quantity of lipid A in chloroform was added to an empty flask, dried, and rehydrated in 10 ml of a saline solution with 0.5% TEA added to solubilize the lipid A. For this lipid A control (no liposomes or detergent) the LAL sensitivity is the standard 0.06 EU/ml, and LAL activity was found until a dilution factor of 800,000.

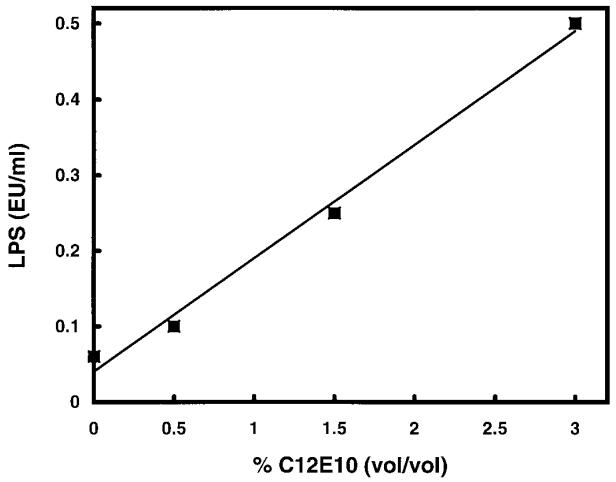

FIG. 1. Inhibition of the LAL gel-clot assay by increasing concentrations of $C_{12}E_{10}$ detergent. Known amounts of endotoxin standard were added to the solutions, and the minimum required for a positive gel result is plotted for each detergent concentration. The data were fitted to a line using least-squares linear regression analysis, $r^2 = 0.992$.

Table 4 shows the lipid A results, as well as data from similar studies of somewhat less hydrophobic *Mt*-LPS and hydrophilic *wt*-LPS. Data are also shown for incorporation experiments starting with 4800, 480, 48, and 4.8 EU/ml levels of lipid A. All LAL results are expressed in terms of EU/ml as described above. Column I shows the result of the *wt*-LPS, *Mt*-LPS, and lipid A in the absence of liposomes or detergent. Column II shows the LAL activity in the presence of intact liposomes. Column III gives the LAL results after repeated washing of the liposomes with pyrogen-free saline. Column IV shows the release of masked endotoxin from the washed liposomes by $C_{12}E_{10}$ solubilization. The large increases in the levels of detected endotoxin in column IV compared to column III demonstrate the success of the unmasking protocol. In column V, we have estimated the approximate percentage of the LPS which was initially incorporated into the EPC liposomes. The values in column V are calculated as:

$$\%\ \text{masked endotoxin} = \frac{\text{total added} - \text{detected in intact liposome}}{\text{total added}} \times 100 = \frac{\text{column 1} - \text{column 2}}{\text{column 1}} \times 100.$$

Clearly, the percentage of the initial endotoxin that actually becomes inserted into the EPC bilayers is very different for *wt*-LPS versus *Mt*-LPS or lipid A. The ease of liposomal incorporation is related to the relative hydrophobicity of the endotoxins, which is diminished as the length of the polysaccharide chain increases. For *Mt*-LPS and lipid A incorporated at 48,000 EU/ml or greater, 90% or more of the endotoxin is undetectable by the LAL assay. The incorporation efficiency of lipid A into EPC MLVs drops to ~70% if less than ~5 EU of lipid A is initially added. *wt*-LPS shows much less incorporation using the procedures described here for MLVs and demonstrates the hydrophilicity of *wt*-LPS.

Masked Endotoxin Detection Limits

Once all bilayer structures are completely disrupted by $C_{12}E_{10}$, the endotoxin detection limit is determined only by the LAL inhibition from the detergent and the micellar lipid. Because 0.5% $C_{12}E_{10}$ gave the least inhibition of the LAL assay (Table 3), we determined the endotoxin detection limits for the PC, PC/CHOL, and negatively charged MLVs at this detergent concentration. The maximum amount of lipid which could be added to 0.5% $C_{12}E_{10}$ and produce a transparent dispersion (dispersion limit) was found by successive heating and dilution as described under Materials and Methods for endotoxin limits. These limits are shown in Table 5. Heating to 37°C followed by vortexing was sufficient to solubilize all but the DSPC-containing MLVs. A 30-s incubation at 65°C was needed for the DSPC samples. EPC liposomes did not form a transparent solution until the concentration was lowered to 2.4 mg/ml compared with 4 mg/ml for the other PC liposomes. In the

TABLE 4

Summary of Endotoxin Incorporation into EPC MLVs

	I Initial LAL activity of endotoxin added to lipid (EU/ml)	II LAL activity measured in liposomal preparation (EU/ml)	III LAL activity after washing liposomes with pyrogen-free saline (EU/ml)	IV LAL activity of washed liposomes solubilized in 1.5% $C_{12}E_{10}$ (EU/ml)	V Approximate percentage of endotoxin initially masked by liposome
wt-LPS	>1,200,000	1,200,000	1,200	480,000	—
Mt-LPS	480,000	6,000	6,000	400,000	>90%
Lipid A	48,000	1	0–0.6	40,000	>90%
	4,800	1	0–0.6	4,000	>90%
	480	1	0	400	>90%
	48	1	0	40	>90%
	4.8	1	0	4	>70%

TABLE 5

Combined $C_{12}E_{10}$/Lipid Inhibition of LAL Gel—Clot Assay

Lipid	Dispersion limit of lipid (mg/ml)[a]	Endotoxin detection limit (EU/ml, CSE)[b]
EPC	2.4	0.3
DMPC	4.0	6.0
DPPC	4.0	0.3
DSPC	4.0	0.1
EPC/Chol	1.25[c]	0.3
DMPC/Chol	1.25[c]	1.2
DPPC/Chol	1.25[c]	0.1
DSPC/Chol	1.25[c]	0.1
EPC/Pops	2.0[d]	1.2
DSPC/DSPG	3.0[d]	0.1

[a] Solubility of lipid in 0.5% $C_{12}E_{10}$.
[b] Detection limit determined at the dispersion limit in 0.5% $C_{12}E_{10}$.
[c] Total lipid concentration (PC + CHOL) at 40 mol% CHOL.
[d] Total lipid concentration at 10 mol% Pops or DSPG.

presence of 40 mol% cholesterol, the dispersion limit for all samples was 1.25 mg/ml. Incorporating 10% negative charge, as either PG or PS, also lowered the dispersion limit but to a lesser extent than cholesterol (3 mg/ml for DSPC/DSPG and 2 mg/ml for EPC/Pops).

Table 5 also shows the endotoxin detection limits, defined as the minimum amount of CSE needed in the final diluted, clear sample to give a positive LAL test. Note that CSE was added before the heating/dilution cycle to mimic conditions needed for practical testing for LPS contamination during liposomal preparation. The detection limit for DSPC was not significantly different from that of 0.5% $C_{12}E_{10}$ alone (see Table 3). The presence of EPC or DPPC increased the limit ~3-fold. Remarkably, DMPC at 4 mg/ml had a dramatic inhibitory affect on the LAL assay with a 600-fold decrease in sensitivity. The presence of CHOL had little effect on the detection limit once the sample was solubilized. The improved detection limit for DMPC/CHOL may reflect the much smaller amount of DMPC in this sample (0.96 mg/ml). The effect of a liposome net negative charge on the LAL endotoxin detection limit depended on the lipid component providing the charge. DSPG, at 10 mol% in DSPC liposomes, had no significant effect on LAL sensitivity (0.1 EU/ml). However, when 10 mol% Pops was incorporated into EPC MLVs, the endotoxin detection limit increased from 0.3 EU/ml for EPC alone to 1.2 EU/ml for the EPC/Pops liposomes.

LPS was also incorporated into EPC and DSPC SPLVs, by adding CSE to the buffer/solvent mixture before the solvent was removed. Complete masking of the LPS occurred in both SPLV preparations. After solubilization of the SPLVs, however, inhibition of the LAL assay was found to be independent of the preparation process, i.e., MLVs vs SPLVs gave identical endotoxin detection limits for these two lipids.

DISCUSSION

Several studies have shown that the immunological effects of endotoxin can be altered by incorporating LPS into liposomes. These include changes in cytokine production and monocyte activation as well as a reduction in the ability to coagulate *Limulus* amebocyte lysate (8–10). This last effect, interference with the LAL assay, makes it difficult to quantitate the biological properties of endotoxin and to rule out LPS contamination in liposomal formulations. Our studies were designed to examine the extent of endotoxin masking and to determine a method of unmasking using detergents to allow accurate quantitation using the LAL gel clot test.

The results indicate that the detergent $C_{12}E_{10}$ is unique in its capacity to solubilize liposomes while preserving the LAL sensitivity. Using this detergent with the LAL allowed us to recover endotoxin which was originally masked in liposomes. The use of detergent to form mixed micelles with the lipid provides a simpler method for LPS unmasking than the extraction methods used by others (6, 12) and does not require incorporation of fluorescently labeled LPS (11). Moreover, the detergent solubilization described here unmasked 70 to >90% of the endotoxin originally added to the liposome preparation. This recovery is significantly higher than the 25 to 50% obtained by Schmidtgen and Brandl using ultrafiltration and resuspension of ethanol/water-extracted liposomal endotoxin (12). Although it may not be necessary in all cases to convert the liposomes into mixed micelles, solubilization to a clear solution provides an easy, reliable endpoint for a standardized assay. The conditions used can be varied to ensure complete lipid solubilization at a sample dilution that will be within the endotoxin detection limit of the LAL assay. It was possible to detect picogram quantities of LPS in the samples tested. Our findings and conclusions about the use of detergents with the LAL assay for the detection of endotoxins differ from those of Schmidtgen and Brandl (12). Our results clearly indicate that there are large differences among detergents in their interference with the LAL assay. The conclusions of Schmidtgen and Brandl about detergents may be based on a detergent which does not easily solubilize liposomes or significantly interferes with LAL clotting. Further, our results demonstrate that the endotoxin detection limit is dependent on several parameters, including detergent type and concentration, temperature needed for solubilization, and lipid composition. Liposome morphology affected the ease of solubilization, but did not appear to change the final assay detection limit.

The dramatically different tendencies of lipid A, *Mt*-LPS, and *wt*-LPS to become incorporated into PC liposomes as shown in Table 4, column V have been de-

scribed (13, 14). *wt*-LPS is relatively difficult to completely mask in unilamellar vesicles and MLVs, even with repeated drying procedures. However, LPS incorporation procedures have been developed for immunological studies which appear to give rise to complete wild-type endotoxin masking (6). Our data show that complete masking of endotoxin is possible in SPLVs as well.

The solubilization and LAL assay methods described here obviate the need for concerns about masked endotoxins since the method is sensitive enough to be applied to liposomal end-product testing for most of the liposomal systems studied. The LPS detection limits shown in Table 5 are less than 0.5 EU/ml for all liposomes tested except DMPC and EPC/Pops. The detection limit for lipid dispersions which have larger concentrations of lipid than the dispersion limits shown in Table 5 can be calculated by accounting for the dilution factors necessary to reach the dispersion limits (mg/ml) shown in the table. Samples which initially have less lipid than these dispersion limits will have masked endotoxin limits between the values in Table 5 and the detection limit measured in the presence of detergent alone. In any case, detection of pharmaceutically relevant levels of endotoxin is achievable in a variety of liposomal systems, even if all the endotoxin was initially masked.

This method may be useful in improving our understanding of the immunological response to LLPS. $C_{12}E_{10}$ bilayer disruption followed by LAL detection would provide a direct measurement of liposomal LPS, and would reflect any losses in endotoxin activity not derived from liposomal masking. Utilization of the method described here would ensure that cellular responses from identical quantities of liposomal LPS and free LPS are being compared.

In conclusion, we have demonstrated a simple method to release masked endotoxin from liposomal dispersions. Liposomes are solubilized by moderate levels of the detergent $C_{12}E_{10}$ to form micellar structures containing detergent, LPS, and lipid. LAL proteins can interact with the lipid A moiety under these conditions and retain enough activity to allow for the detection of picogram levels of endotoxin. This method should be useful in liposomal LPS immunological studies as well as in other areas where accurate detection of endotoxin is required.

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