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Fluorescent detection of lipopolysaccharide interactions with model membranes

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We have modeled the initial interaction of bacterial lipopolysaccharide (endotoxin) with mammalian cells as consisting of two steps. The first step, adherence, we have previously shown to be ionic in nature and contains the necessary elements to determine the observed cell specificity of lipopolysaccharide interactions. The second step, coalescence, is the hypothetical insertion of the Lipid A component of lipopolysaccharide into the cell membrane lipid bilayer. Using small, unilamellar vesicles composed of phosphatidylcholine to model the cell membrane lipid bilayer, we found that lipopolysaccharide interacted with these vesicles to change the fluidity of the lipid bilayer, as measured by an increase in the fluorescence anisotropy of diphenylhexatriene in the vesicles. Since this increase in diphenylhexatriene anisotropy could not be attributed to a transfer of diphenylhexatriene between non-interacting lipopolysaccharide aggregates and vesicles, we concluded that the lipopolysaccharide aggregate coalesced with the lipid bilayer

Introduction

Lipopolysaccharide, a major component of the outer membrane of Gram-negative bacteria, interacts with mammalian cells to activate a number of metabolic processes Although these processes have been well studied, there is little known of the molecular basis of lipopolysaccharide interaction with cells or their membranes The current model of this interaction is based on electron microscopic evidence and proposes that lipopolysaccharide bilayers 'edge associate' with cell membranes [1] However, this requires that the hydrophobic Lipid A portion of lipopolysaccharide aggregates be directly exposed to the aqueous environment which is thermodynamically untenable for aggregates of amphiphiles In addition, this proposal does not include a role for the polysaccharide portion of lipopolysaccharide in the interaction with mammalian cell membranes, although this region of lipopolysaccharide is clearly important for the solubility of lipopolysaccharide [2] and pathogenicity of Gram-negative organisms [3] in an aqueous environment

Recently, the results from studies of lipopolysaccharide binding to murine lymphocytes led Jacobs [4] to conclude that lipopolysaccharide associates with cell membranes in a two-step process The first step was reversible, could be inhibited by polyions, and was temperature independent The second step was irreversible within the time-scale of the experiment, was not inhibited by polyions, and was not seen to occur at low temperatures The two steps were modeled to be equivalent to the association of the lipopolysaccharide aggregate with the cell surface (adherence)

Abbreviations Br-PC, brominated phosphatidylcholine, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, SUV small unilamellar vesicle

and the incorporation of individual lipopolysaccharide molecules into the cell membrane (coalescence)

This two-step model takes into account the biological data, chemical structure and physicochemical constraints of lipopolysaccharide and its aggregates This is particularly important since lipopolysaccharide seems to have a preferential interaction with specific cell types [5,6] and the cellular determinants of such interactions are, as yet, unknown Further, it recognizes that the first step of this interaction depends on overcoming the electrostatic and hydration repulsions [7] of two aggregates of amphiphiles, both with charged, hydrophilic surfaces Such a process would depend on properties of lipopolysaccharide aggregates which are relatively independent of temperature but would be affected by the ion composition of the solution

The second step, hypothesized to be the incorporation of individual lipopolysaccharide molecules into the cell membrane, has commonly been termed a 'hydrophobic interaction' between the Lipid A portion of lipopolysaccharide and the hydrophobic region of the cell membrane lipid bilayer [4,8,9] Such a 'hydrophobic interaction' has not been shown directly but has been deemed a necessary step because the hydrophobic Lipid A, when made soluble in aqueous solution, can produce the same biological effects as intact lipopolysaccharide [10] and its structural integrity is necessary for the biological activity of the intact macromolecule

To test this model further it is necessary to have a model system in which the number of variables is considerably reduced from those inherent in systems using intact cells Therefore, we designed a model system that enabled us to more rigorously control the first step and to detect and measure the second step We used intact lipopolysaccharide and small, unilamęllar vesicles (SUVs) to model the interaction between lipopolysaccharide and cell membranes The interaction between SUVs, composed of phosphatidylcholine and labeled with the fluorescent probe diphenylhexatriene, and lipopolysaccharide was measured by detecting the fluorescence anisotropy of diphenylhexatriene We found that lipopolysaccharide interaction with the SUVs affected the physical properties of the lipid bilayer so that the measured anisotropy of diphenylhexatriene was increased We also found that such a change could not be attributed to transfer of the probe between lipopolysaccharide aggregates and SUVs

Materials and methods

Lipopolysaccharide Escherichia coli 055 B5 lipopolysaccharide (List Biologicals, Campbell, CA U S A) was used after extraction of free lipid by hexane/2-propanol (3 2, v/v) These preparations were also free of detectable protein or nucleic acid

Vesicles Small, unilamellar vesicles (SUVs) were composed of egg phosphatidylcholine (PC) (Avanti Polar Lipids, Birmingham, AL, U S A) and were prepared as described [11] The appropriate amount of PC solubilized in chloroform was lyophilized, and multilamellar vesicles were formed by mixing of the lipid with an aqueous buffer (150 mM NaCl/5 mM Hepes, pH 7 4) SUVs were formed by sonicating the multilamellar vesicle suspension in a bath-type sonicator at room temperature until clear Any remaining multilamellar vesicles were removed by centrifugation (100 000 $\times g$, 30 min, 4°C), and the supernatant was used as the SUV suspension Lipid quantitation was by assay of inorganic phosphate by the malachite green method [12], greater than 95% of the starting lipid was recovered in the SUV preparation

Brominated lipid Egg phosphatidylcholine was brominated by the method of Burchfield and Storrs [13] with slight modification Briefly, 1 mg of lyophilized egg PC was dissolved in 1 ml petroleum ether (30–60°C), and the tube containing the lipid was placed in a solid CO_2/ethanol slush (approx −10°C) Liquid bromine (2% in diethyl ether, v/v) was added dropwise until a stable yellow color developed, indicating complete bromination The reaction tube was removed from the slush and the petroleum ether and free bromine were evaporated by a flow of nitrogen at room temperature The brominated lipid (Br-PC) was then dissolved in chloroform and washed several times with distilled water to remove any remaining free bromine The Br-PC was then lyophilized and redissolved in fresh chloroform for storage at −20°C until used

Fluorescent labeling 1,5-Diphenyl-hexa-1,3,5-triene (Molecular Probes, Junction City, OR, U S A) was dissolved in acetonitrile, and the appropriate amount was added to SUV suspensions to obtain an appropriate labeling ratio After testing lipid probe molar ratios of between 50 1 and 1000 1, the ratio of 200 1 was selected as giving acceptable fluorescence under our experimental conditions with minimal interference by diphenylhexatriene itself The added diphenylhexatriene was incubated with the aqueous SUV suspensions for 90 min or longer at room temperature (above the phase transition temperature of egg PC) Alternatively, PC which had diphenylhexatriene covalently included in the molecule (2-[3-(diphenylhexatrienyl)propanoyl]-L-α-PC, Molecular Probes) was used with egg PC to form SUVs by the ethanol injection technique [14] with a final lipid probe molar ratio of 200 1 These vesicles were comparable in size to those formed by sonication as determined by negative-staining electron microscopy [14]

Fluorescence anisotropy measurements A Perkin-Elmer LS5 Luminescence Spectrometer equipped with excitation and emission monochromators and Polaroid film-type polarizers was used to measure diphenylhexatriene anisotropies The excitation/emission wavelengths were 357/430 nm with slit widths of 3 and 5 nm, respectively Samples were illuminated for 5 s before a signal integration of 4 s Diphenylhexatriene anisotropy (r) was calculated from the polarization ratios (P) using an instrumental correction factor [15] for diphenylhexatriene of $G = 1\ 006$, i e , $P = I_{VV}/(I_{VH}\ G)$, where I_{VV} and I_{VH} are the intensities of vertically polarized excitation with vertically and horizontally polarized emissions, respectively Anisotropy was then calculated from P as $r = (P - 1)/(P + 2)$ No attempt was made to estimate lipid bilayer microviscosities from r since this has been shown to be invalid when using diphenylhexatriene as the fluorescent probe [16]

Results

To determine the optimum conditions under which lipopolysaccharide-SUV interactions should be examined, a concentration of lipopolysaccharide was selected which had shown optimum binding to plasma membranes of intact cells (25 μg/ml) [4] The amount of lipid in the system was then titrated against this amount of lipopolysaccharide using changes in diphenylhexatriene anisotropy to indicate the extent of the interaction Increasing the amount of lipid decreased the magnitude of the change in diphenylhextriene anisotropy to a point where the lipopolysaccharide-SUV interaction was no longer detectable (Table I) Therefore, we selected a concentration of lipid (20 μM) to use in further studies that gave significant changes in diphenylhexatriene anisotropy when reacted with lipopolysaccharide and which had sufficient fluorescence intensity at the level of labeling used (lipid probe molar ratio 200 1) such that usable fluorescence intensities were obtained when multiplied by the machine by a factor of 10 or less

To determine the optimum lipopolysaccharide concentration to use in this system, we titrated lipopolysaccharide amount against the selected lipid concentration From 0 to 50 μg/ml of lipopolysaccharide, the changes in diphenylhexatriene anisotropy were indicative of a first-order interaction between lipopolysaccharide and the SUVs (Table II) At 100 μg/ml, however, the observed change in diphenylhexatriene anisotropy fell distinctly out of the pattern formed by the lower concentrations of lipopolysaccharide (Table II) We have not yet determined the reason for this deviation, but speculate that this result may have followed from a change in the phase state of lipopolysaccharide induced by its interaction with phospholipids [17]

TABLE I

CHANGES IN DIPHENYLHEXATRIENE ANISOTROPY (r) INDUCED BY LIPOPOLYSACCHARIDE INTERACTING WITH SUVs WITH DIFFERENT LIPID CONCENTRATIONS

Lipopolysaccharide 25 μg/ml, reaction volume = 2 ml T = 37°C Anisotropy values presented are the average of two separate determinations

	Amount of lipid (μM)			
	10	20	40	80
r (SUVs alone)	0 064	0 062	0 063	0 063
r (+ lipopolysaccharide)	0 091	0 071	0 065	0 064
% change in r	+42	+14	+3	+1 5

TABLE II

CHANGES IN DIPHENYLHEXATRIENE ANISOTROPY (r) INDUCED BY LIPOPOLYSACCHARIDE INTERACTING WITH SUVs WITH DIFFERENT LIPOPOLYSACCHARIDE CONCENTRATIONS

SUV lipid 20 μM reaction volume = 2 ml T = 37°C Anisotropy values presented are the average of two separate determinations

	Amount of lipopolysaccharide (μg/ml)					
	0	5	10	25	50	100
r (SUVs alone)	0 062	0 062	0 063	0 064	0 063	0 063
r (+ lipopolysaccharide)	0 062	0 065	0 070	0 073	0 074	0 089
% change in r	0	+5	+11	+14	+17	+41

Up to this point, we interpreted the results as being indicative of an interaction between lipopolysaccharide and SUVs that changes the structure of the acyl region of the SUVs and, thereby, changes the constraints on the motion of diphenylhexatriene in that region However, the observed increase in diphenylhexatriene anisotropy could also be explained by a redistribution of diphenylhexatriene between the two available hydrophobic regions In this case, the resultant diphenylhexatriene anisotropy would be the weighted sum of the anisotropies of diphenylhexatriene in the SUVs and the lipopolysaccharide aggregates [15] Two different methods were used to rule out the transfer of diphenylhexatriene as an explanation for our results

First, we used a system where diphenylhexatriene was not able to be transferred between aggregates Egg PC vesicles (20 μM lipid) containing diphenylhexatriene covalently bound to PC were reacted with lipopolysaccharide (25 μg/ml) In this system, lipopolysaccharide still produced an increase in diphenylhexatriene anisotropy (r = 0 131, SUVs alone, r = 0 143, SUV + lipopolysaccharide) although the extent of the increase (9%) was less than that observed in systems using free diphenylhexatriene (14%, Tables I and II) Since diphenylhexatriene transfer was not possible in this system, the observed increase in diphenylhexatriene anisotropy must have been the result of an interaction between the SUVs and lipopolysaccharide

The results from the experiments above also indicated that diphenylhexatriene transfer might account for a portion of the observed increase in anisotropy To determine the extent to which this mechanism affected our results, we used SUVs in which part of the diphenylhexatriene present was quenched by brominated PC (Br-PC) By using three different concentrations of Br-PC and two different concentrations of lipopolysaccharide, the fractional fluorescence intensities of diphenylhexatriene in the SUVs and lipopolysaccharide

TABLE III

FLUORESCENCE INTENSITIES OF DIPHENYLHEXATRIENE IN PRESENCE OF BROMINATED PHOSPHATIDYLCHOLINE (Br-PC) AND LIPOPOLYSACCHARIDE

Reaction volume = 2 ml T = 37°C Fluorescence intensity values presented are the average of two separate determinations

mol% Br-PC	Fluorescence intensity, amount of lipopolysaccharide (μg/ml)		
	0	25	125
0	106 1	110 2	111 3
33	62 1	60 0	64 8
66	31 1	32 6	35 4
100	1 8	2 7	4 2

TABLE IV

PERCENT OF FLUORESCENCE INTENSITY DUE TO TRANSFER OF DIPHENYLHEXATRIENE BETWEEN HYDROPHOBIC REGIONS

Calculated from data in Table III See text for details

	Amount of lipopolysaccharide (μg/ml)		
	0	25	125
Correlation coefficient	0 999	0 995	0 997
% fluorescence quenchable	96 0	93 2	92 6
% fluorescence transfer	0	2 8	3 4

TABLE V

CALCULATED ANISOTROPIES OF SUV + LIPOPOLYSACCHARIDE SYSTEM IF THEY DO NOT INTERACT

Values used for calculation $r(SUV) = 0.064$, r(lipopolysaccharide) = 0.140 ($T = 37°C$ determined separately)

	Amount of lipopolysaccharide (μg/ml)	
	25	125
f(SUV)	0.972	0.966
f(lipopolysaccharide)	0.028	0.034
$\bar{r}$	0.066	0.067
Measured r	0.073	0.089 *

* Value from 100 μg/ml lipopolysaccharide, Table II

could be estimated from the fluorescence intensities in the presence and absence of quencher (Table III)

By a linear regression calculation of the reciprocal of the fractional decrease in fluorescence versus the reciprocal of the concentration of quencher (Br-PC), i.e., a modified Stern-Volmer calculation [15], we estimated the fraction of fluorescence that was inaccessible to quenching. Thus, if diphenylhexatriene were able to transfer between SUV (quenched) and lipopolysaccharide aggregate (unquenched), a decrease in the proportion of fluorescence that was able to be quenched should have been observed. The results from such calculations are listed in Table IV. As the amount of lipopolysaccharide present increased 25 to 125 μg/ml, the percentage of fluorescence transfer increased from 2.8 to 3.4%.

If the SUVs and lipopolysaccharide aggregates were not interacting, then the average anisotropy ($\bar{r}$) of the system (SUVs + lipopolysaccharide) would be the linear sum of their individual anisotropies (r_i) multiplied by their fractional fluorescence intensities (f_i), i.e., $\bar{r} = f(SUV)\ r(SUV) + f(\text{lipopolysaccharide})\ r(\text{lipopolysaccharide})$. The data from Table IV were used to calculate the anisotropies expected if the results (Table II) were to reflect the simple transfer of diphenylhexatriene between the non-interacting aggregates (Table V). Clearly, transfer of diphenylhexatriene between aggregates could not account for the magnitude of change in diphenylhexatriene anisotropy that we observed.

Discussion

The question of how lipopolysaccharide can stimulate a cell to respond to the presence of lipopolysaccharide is difficult to answer in systems containing whole cells. We have previously shown that lipopolysaccharide associates with cells in a manner that is suggestive of a two-step process [4]. The first step is ionic in nature. However, it is not known whether such charge-mediated interactions can provide an activation signal to cells from the cell surface. The charge-mediated association of macromolecules with cell surfaces inactivates, rather than activates, cells in one system [18].

The second of these steps, proposed to be the intercalation of the hydrophobic Lipid A portion of lipopolysaccharide into the membrane lipid bilayer is not unexpected since lipopolysaccharide interacts with phospholipid monolayers [19,20]. The intrusion of such a large segment of lipid that was covalently linked together would be expected to alter the order, or 'fluidity', of the membrane lipid bilayer (or, at the very least, the outer monolayer of the bilayer). Indeed, lipid bilayers that are reconstituted with lipopolysaccharide or Lipid A are less fluid than reconstituted bilayers consisting of the lipid alone [21–23]. Therefore, we designed a test system to examine whether or not exogenous lipopolysaccharide could interact with a membrane bilayer to alter its fluidity. We concluded from our results that lipopolysaccharide interacted with the lipid bilayer and decreased the fluidity of that bilayer. Since cell activation is associated with changes in cell membrane fluidity [24,25], lipopolysaccharide-induced alterations in cell membrane fluidity may modulate cell responses. Therefore, we suggest that in our model of lipopolysaccharide-cell interactions, the first step is the association of the lipopolysaccharide aggregate with the cell surface as mediated by the charges of the lipopolysaccharide and some cell surface structure(s) (adherence). This association increases the thermodynamic probability of the intercalation of lipopolysaccharide monomers into the cell membrane lipid bilayer (coalescence the second step). This intercalation of the hydrophobic (Lipid A) portion of lipopolysaccharide decreases the fluidity of that region of the cell

membrane, and this change may play a role in cell activation We have presented evidence that the cell attempts to rid itself of this exogenous signalling substance by cytoskeleton-mediated capping of the lipopolysaccharide [26]

There are several aspects of this work that deserve further comment First, the lipopolysaccharide that we used had been delipidated by extraction Commercial (phenol-extracted) preparations of lipopolysaccharide are usually accompanied by some contamination of lipid, probably bacterial phosphatidylethanolamine, the major phospholipid of *E coli* outer membranes [27] Such lipid is most likely present in biologically occurring lipopolysaccharide aggregates [28] and contributes to the signal that lipopolysaccharide causes upon its interaction with cells (unpublished data) In this respect, the magnitude of the change in diphenylhexatriene anisotropy caused by lipopolysaccharide was larger when unextracted lipopolysaccharide was used (from 0 064 to 0 091 as compared to the values produced by extracted lipopolysaccharide, from 0 064 to 0 073, Table II) However, lipopolysaccharide alone is capable of altering the fluidity of a lipid bilayer in this model system The biological action of lipopolysaccharide and the molecular mechanism of lipopolysaccharide interaction with cell membranes is probably modified by the presence of other bacterial membrane components [17,29,30] or by heterogeneity in lipopolysaccharide structure [31] Therefore, any hypothesis concerning the interactions of lipopolysaccharide with cells should be able to accommodate the presence and role of non-lipopolysaccharide components in the lipopolysaccharide aggregate Our hypothesis of a two-step process for lipopolysaccharide aggregate interaction with, and stimulation of, cells can do so Furthermore, the model system that we have presented here should allow the elucidation of the effects of additional bacterial membrane components, e g , lipid or protein, on lipopolysaccharide interactions with lipid bilayers

Second, the use of diphenylhexatriene in a model system of unilamellar vesicles limits the changes that we observed to the hydrophobic (acyl) region of the lipid bilayer In systems using whole cells, there are multiple layers of lipid bilayers available for probes to label and for interaction with lipopolysaccharide However, lipopolysaccharide induced significant changes in diphenylhexatriene anisotropy in multilamellar vesicles only at very long incubation times, e g , 24 h at 37°C (unpublished observation) The results we observed using SUVs were apparent after a few minutes and were stable for up to 24 h Therefore, it is likely that lipopolysaccharide, when interacting with multilamellar assemblies at very long times, is involved in a redistribution of that system's components With respect to cells, this would suggest that internalization of lipopolysaccharide is likely when those cells are exposed to lipopolysaccharide for long periods of time Therefore, we found it difficult to accept the conclusion that lipopolysaccharide induced alterations only in the plasma membrane fluidity of monocytes, as reported by Larsen et al [32] In their experiments, lipopolysaccharide was incubated with monocytes for 18 h before the cells were labeled with diphenylhexatriene This is certainly enough time for internalization of lipopolysaccharide, given our results and the phagocytic capacity of activated monocytes Further, their measurements of diphenylhexatriene anisotropy were made after more than one hour's incubation of the cells with diphenylhexatriene Bouchy et al [33] reported that diphenylhexatriene is rapidly distributed throughout the cell and that after 15 minutes' incubation of the cell with diphenylhexatriene, the fluorescent signal from diphenylhexatriene arises primarily from the cell interior, not the plasma membrane Therefore, we concluded that Larsen et al [32] measured changes in diphenylhexatriene anisotropy that were characteristic of a whole cell activated by lipopolysaccharide and not just changes limited to the plasma membrane The model that we have presented will allow the systematic investigation of the factors important to lipopolysaccharide interactions with lipid bilayers, e g , lipopolysaccharide phase state or bilayer lipid composition, and, thereby, allow the elucidation of the mechanism by which lipopolysaccharide is able to activate cells through interactions with the cell membrane

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