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# Effect of bacterial endotoxin on the transmembrane electrical potential and plasma membrane fluidity of human monocytes

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**In order to gain insight into the physical interaction between bacterial endotoxins and the surface of human monocytes, we investigated the effects of *Salmonella typhi* endotoxin and lipid A on two functional properties of the plasma membrane of these cells: (1) the transmembrane electrical potential and (2) the fluidity of the lipid bilayer. Using the fluorescent lipophilic cationic probe 3,3′-dipropylthiodicarbocyanine (di-S-$C_3$(5)) to monitor the transmembrane electrical potential, we found that neither endotoxin nor lipid A induced depolarization of the monocyte's plasma membrane or impeded its ability to undergo depolarization in response to phorbol myristate acetate. When the resting transmembrane potential of the monocyte was analyzed by exposing di-S-$C_3$(5)-labeled cells suspended in media containing incremental concentrations of potassium ion ($K^+$) to valinomycin, no difference between the response of control cells and cells pretreated with endotoxin was noted. We next examined the effect of endotoxin and lipid A on the fluidity of the monocyte's plasma membrane by monitoring the intensity of the fluorescence of 1,6-diphenyl-1,3,5-hexatriene. By quantifying the intensity of parallel and perpendicular polarized light emitted by this membrane-embedded probe between 8 and 56°C, measurements of molecular anisotropy were used to identify temperature-dependent phase transitions within the hydrocarbon region of the plasma membrane and to estimate the relative microviscosity of the lipid bilayer before and after exposing the cells to endotoxin or lipid A. Although the temperature at which phase transitions occurred was the same in all experimental groups of cells, preincubation of monocytes with either endotoxin or lipid A appeared to increase both the apparent microviscosity of the cell membrane and the order of the lipid bilayer as reflected by a decrease in its flow-activation energy. Our data indicate that when endotoxin molecules contact the surface of the monocyte, the lipid A moiety appears to become incorporated into the plasma membrane, increasing the microviscosity of the lipid bilayer without significantly altering its ionic permeability. We therefore conclude that the metabolic activation of monocytes by endotoxin is not coupled to, or initiated by, membrane depolarization.**

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Abbreviation: di-S-$C_3$(5), 3,3′-dipropylthiodicarbocyanine.

## Introduction

Human mononuclear phagocytes undergo marked alterations in cell function following exposure to bacterial endotoxin. We and others have observed that initial stimulation of these cells by endotoxin precedes the development of a resistant state during which the monocyte is highly refractory to the stimulatory effect of these complex bacterial lipopolysaccharides [1–3]. The mechanism whereby the interaction between endotoxin and the monocyte leads to the triggering of a variety of diverse intracellular events and to a subsequent change in the ability of the cell to respond to endotoxin is poorly understood. It has been suggested that some of the effects produced by endotoxin are mediated by specific molecular reactions at the cell surface and that physical and structural properties of the plasma membrane may be important determinants of the bioactivity of endotoxin [4–8]. To investigate the physical interaction between these complex macromolecules and the cell surface, we used fluorescence spectrometry to study the fluid and electrophysical properties of the plasma membrane of monocytes before and after treatment of the cells with *Salmonella typhi* lipopolysaccharide B or lipid A isolated from this preparation of endotoxin. The temperature dependence of the intensity of the polarized fluorescence of the membrane-embedded probe 1,6-diphenyl-1,3,5-hexatriene was used to evaluate the relative fluidity of the hydrocarbon region of the membrane [9,10]. In addition, transmembrane electrical potentials were monitored by quantitating changes in the distribution of the lipophilic cation, di-S-$C_3(5)$, using techniques which have previously been applied to a number of types of cells [11–14].

## Materials and Methods

The fluorescent probe di-S-$C_3(5)$ was the kind gift of Dr. Alan Waggoner of the Carnegie-Mellon University, Pittsburgh, PA. Diphenylhexatriene and valinomycin were obtained from Sigma Chemical Co. (St. Louis, MO), phorbol myristate acetate and phorbol from P-L Biochemicals, Inc. (Milwaukee, WI), and *Salmonella typhi* lipopolysaccharide B (hereafter referred to as endotoxin) from Difco Laboratories (Detroit, MI). Endotoxin was partially purified by gel filtration using Sepharose 6B chromatography, and this preparation, which we have previously described in detail [15], served as the source of whole endotoxin for all experiments. Lipid A was isolated from endotoxin by mild acid hydrolysis using a modification [16] of a previously described technique [17].

*Preparation of monocytes.* Monocytes were separated from human venous blood by centrifugation on sodium metrizoate-Ficoll density gradients [18] followed by adherence to plastic [19]. Viability as assessed by Trypan blue exclusion [20] consistently exceeded 91%, and the average percentage of monocytes as determined by α-naphthyl-butyrate staining [21] was 91%. Monocytes ($(0.2–1.0)\cdot 10^6$/ml) were incubated in McCoy's 5A medium supplemented with 10% fetal calf serum, 50 I.U./ml penicillin G, and 50 μg/ml streptomycin for 18 h with or without endotoxin (1 μg/ml) or lipid A (5 μg/ml) at 37°C in a humid incubator containing 5% $CO_2$. Following this incubation, monocytes were washed twice with 0.15 M phosphate-buffered saline (pH 7.4) and then resuspended in the same buffer at a concentration of $(1–2)\cdot 10^6$/ml in preparation for labeling with diphenylhexatriene or treatment of the cells with di-S-$C_3(5)$.

*Labeling of monocytes with diphenylhexatriene.* A stock solution of diphenylhexatriene ($2\cdot 10^{-3}$ M) was prepared by dissolving 0.46 mg of diphenylhexatriene in 1.0 ml of tetrahydrofuran. For cell labeling, 0.1 ml of the stock solution was added rapidly to 100 ml of vigorously stirring 0.15 M phosphate-buffered saline (pH 7.4). This dispersion of diphenylhexatriene ($2\cdot 10^{-6}$ M) was stirred for 15 min at 25°C and was then added to an equal volume of the cell suspension ($(2–5)\cdot 10^6$ cells/ml). The diphenylhexatriene-monocyte mixture was incubated for 1 h at 37°C, and the cells were then washed twice in 0.15 M phosphate-buffered saline (pH 7.4).

*Measurements of fluorescence polarization of membrane-associated diphenylhexatriene.* The intensity of the polarized fluorescence of diphenylhexatriene embedded in the plasma membrane of the monocytes was measured using a Perkin-Elmer 650-10S spectrophotometer equipped with polarization filters and a compartment in which the temperature of the cell suspension could be regu-

lated. The sample was excited by light (335 nm), and the intensity of polarized fluorescence emitted parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of the excitation beam was measured at 430 nm at incremental temperatures ranging between 8 and 56°C. Molecular anisotropy ($r$) was defined by the equation:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{1}$$

An estimate of the apparent microviscosity ($\bar{\eta}$) of the lipid region of the monocyte's plasma membrane was then derived at each temperature using a modification of the Perrin equation [22] as described by Shinitzky and Barenholz [23] according to the following formula:

$$\bar{\eta} = C(r)T\tau \left(\frac{r_0}{r} - 1\right)^{-1} \tag{2}$$

Where $C(r)$ is a function of the molecular shape and the location of the transition dipole for a given fluorescent probe, $r_0$ is the molecular anisotropy of the probe at infinite rigidity, $r$ is the anisotropy of the probe measured at the experimental temperature, $T$ is the temperature expressed in degrees Kelvin, and $\tau$ is the lifetime of the excited state of the probe. Assuming $C(r)T\tau$ to be constant at 2.4 poise and $r_0$ to be 0.362 for the fluorophore diphenylhexatriene [23], the Eqn. 2 may be rewritten:

$$\bar{\eta} = 2.4 \left(\frac{0.362}{r} - 1\right)^{-1} \tag{3}$$

Since the viscosity of linear hydrocarbons in a liquid phase has been observed to diminish with temperature in an exponential manner [23], the logarithm of the apparent microviscosity ($\log \bar{\eta}$) was plotted against $1/T$ for the experiments presented in this report. The slopes of the lines derived provided an estimate of the flow-activation energy ($\Delta E$) [23] and permitted the identification of temperature-dependent phase transitions within the plasma membranes of the cells.

*Measurement of the fluorescence of di-S-$C_3$(5).* Changes in the transmembrane electrical potential were monitored by measuring the intensity of fluorescence of the cell suspension equilibrated at 37°C with $2 \cdot 10^{-6}$ M di-S-$C_3$(5). The sample was excited by light at 620 nm, and the emitted fluorescence was monitored at 670 nm. Fluorescence was measured continuously, and the change that resulted following the addition of the stimulus was reported as a relative change in fluorescence ($F/F_0$), which was calculated from the ratio of the fluorescence change measured after the addition of the membrane perturbant ($F$) to the resting fluorescence of the cell suspension measured after equilibration with di-S-$C_3$(5) but before the addition of the stimulus ($F_0$).

In experiments in which monocytes were stimulated by phorbol esters, the cells were equilibrated with di-S-$C_3$(5) in 0.15 M phosphate-buffered saline (pH 7.4). In experiments in which valinomycin was used to alter the transmembrane potential, aliquots of the cells were suspended in a series of buffers prepared by mixing 0.012 M sodium phosphate-buffered 0.15 M sodium chloride (pH 7.4) containing 1.2 mM $MgSO_4$ and 10 mM glucose with 0.012 M potassium phosphate-buffered 0.15 M potassium chloride containing 1.2 mM $MgSO_4$ and 10 mM glucose. These two solutions were mixed in varying ratios in order to obtain a series of buffers containing a wide range of $K^+$ concentrations.

## Results

*Apparent microviscosity of the monocyte's plasma membrane*

The intensities of polarized light emitted from the membrane-embedded probe diphenylhexatriene were measured parallel and perpendicular to the polarized excitation beam at multiple temperatures ranging between 8 and 56°C. These determinations were used to calculate the molecular anisotropy of diphenylhexatriene in the monocyte's plasma membrane, and the apparent microviscosity of its lipid bilayer was derived from these values at each temperature. These data were then used to identify phase transitions in the hydrocarbon region of the membrane, to estimate the flow-activation energy of the fluorophore within the membrane, and to compare the effect of endotoxin and lipid A pretreatment of the cells upon these parameters of membrane fluidity.

Shown in Fig. 1 is a representative one of three

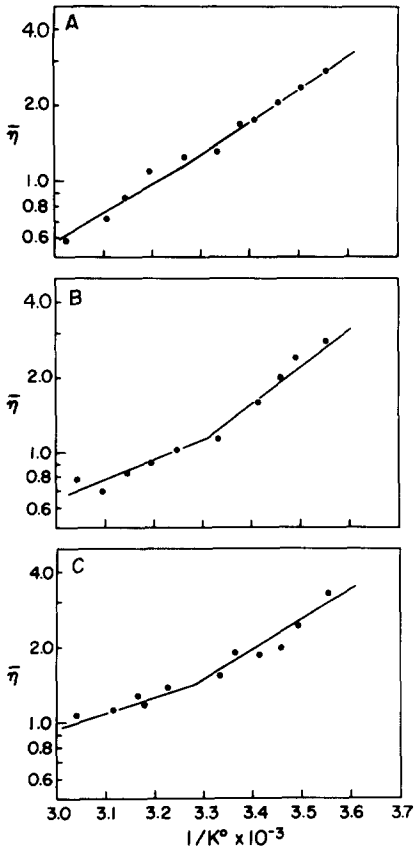


Fig. 1. Microviscosity of plasma membranes of control monocytes (A), monocytes preincubated for 18 h with endotoxin (B) and monocytes preincubated for 18 h with lipid A (C). The concentration of monocytes was $2 \cdot 10^6$ cells/ml, and microviscosity ($\bar{\eta}$) was calculated by quantitating the molecular anisotropy of membrane-embedded diphenylhexatriene at temperatures ranging between 8 and 56°C. Shown is a representative experiment repeated three times with similar results. In these experiments, the temperatures at which phase transition occurred were $31.4 \pm 6.29$ (control monocytes), $27.6 \pm 4.97$ (endotoxin) and $30.5 \pm 6.84$ (lipid A) [degrees centrigrade, mean $\pm$ standard error of the mean (M $\pm$ S.E.)].

similar experiments in which determinations of the apparent microviscosities of the plasma membranes of control, endotoxin-pretreated and lipid A-pretreated monocytes in liquid suspension were quantitated. As indicated by this graph, phase transitions occurred in all three experimental groups at approximately the same temperature. However, pretreatment of the cells with whole endotoxin (panel B) or isolated lipid A (panel C) increased the apparent microviscosity of the lipid bilayer at higher temperatures and resulted in increased order within the membrane, as reflected by a decrease in the flow-activation energy of diphenylhexatriene.

*Transmembrane electrical potentials*

The transmembrane electrical potential of the monocyte is in part a function of the transmembrane sodium ($Na^+$) and potassium ($K^+$) gradients. As illustrated in Fig. 2A, when monocytes in liquid suspension cultures are allowed to equilibrate with di-S-$C_3$(5) and subsequently are treated with the antibiotic valinomycin, which selectively discharges transmembrane potassium gradients [12], the degree of depolarization which results is to a significant extent a reflection of the transmembrane $K^+$ gradient which exists immediately prior to the addition of valinomycin and which is

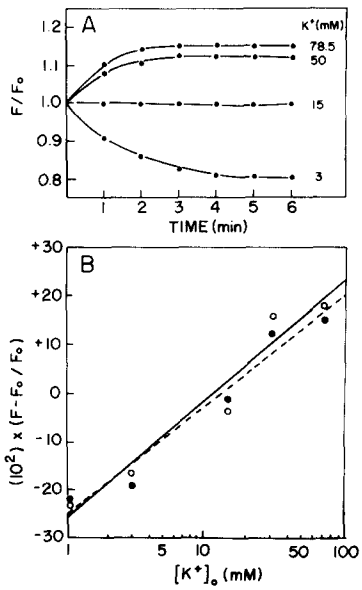


Fig. 2. (A) Changes in the relative fluorescence of di-S-$C_3$(5) ($2 \cdot 10^{-6}$ M) resulting from exposure of untreated monocytes to valinomycin ($2 \cdot 10^{-6}$ M) in the presence of increasing concentrations of $K^+$. Monocytes ($1 \cdot 10^6$/ml) were suspended in iso-osmotic buffers containing serial concentrations of $K^+$, and fluorescence was monitored upon the addition of valinomycin at time zero. (B) Goldman plot of the data shown in panel A, including a simultaneous assay using endotoxin-pretreated monocytes. The maximum change in the fluorescence of di-S-$C_3$(5) at each potassium concentration was determined for control monocytes (○——○) and for monocytes pretreated for 24 h with endotoxin (●——●).

largely determined by the extracellular $K^+$ concentration [12,14]. Alterations in the resting transmembrane potential of the monocyte, like those of the platelet [12], the erythrocyte [6,25] and the neutrophil [13], can therefore be evaluated using the fluorescent probe di-S-$C_3$(5) by verifying that the Goldman equation [26], which states that the change in observed fluorescence of the fluorophore is directly proportional to the logarithm of the external $K^+$ concentration, is obeyed. As shown in Fig. 2B, when the $K^+$ gradient was discharged by the addition of valinomycin, we found that the relative change in the fluorescence of di-S-$C_3$(5) was linearly dependent upon the logarithm of the $K^+$ concentration in the surrounding medium, an observation which justified the use of di-S-$C_3$(5) under these experimental conditions to evaluate the relative transmembrane electrical potential of isolated monocytes. No significant difference between the resting transmembrane electrical potentials of control and endotoxin-pretreated monocytes was detected, indicating that the permeability of the plasma membrane of monocytes to $Na^+$ and $K^+$ is not significantly altered by endotoxin.

In order to determine whether endotoxin or lipid A induced depolarization of the monocyte's plasma membrane upon contact with the cell surface, we studied the effects of these two compounds on monocytes which had not previously been exposed to endotoxin. For these studies, untreated monocytes were allowed to equilibrate with di-S-$C_3$(5). As shown in Fig. 3, when these cells were then treated with phorbol myristate acetate, a wave of depolarization followed which was reflected by an increase in the fluorescence of the probe. As a negative control, the cells were exposed to the biologically inert parent compound of phorbol myristate acetate, phorbol, which gave rise to no measurable change in the fluorescence of di-S-$C_3$(5). In a similar manner, when these cells were treated with endotoxin or with lipid A, neither compound appeared to induce membrane depolarization of the monocyte's plasma membrane as assessed by this technique.

In addition to these experiments, we examined the ability of phorbol myristate acetate to induce depolarization of the plasma membranes of monocytes which had been preincubated for 24 h with 1 μg/ml of *S. typhi* endotoxin. When these cells

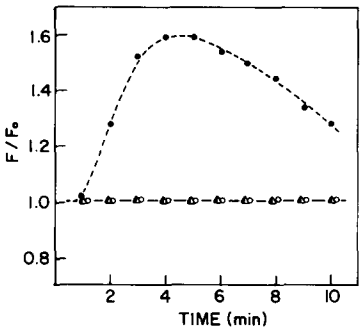


Fig. 3. Changes in fluorescence resulting from exposure of cell suspensions of monocytes ($2 \cdot 10^6$/ml) preincubated with di-S-$C_3$(5) to phorbol myristate acetate (1 μg/ml) (●), phorbol (1 μg/ml) (○), endotoxin (1 μg/ml) (△), and lipid A (1 μg/ml) (▲). Fluorescence was expressed as the ratio of observed fluorescence ($F$) to fluorescence before the addition of the stimulus ($F_0$).

were then allowed to equilibrate with di-S-$C_3$(5) and subsequently were exposed to phorbol myristate acetate, a prompt wave of depolarization followed that was identical to that observed using control cells which had been incubated for 24 h under similar conditions in media that contained no endotoxin (data not shown).

## Discussion

Bacterial endotoxins are complex lipopolysaccharide macromolecules which produce a number of pathophysiological events when introduced into the bloodstream of higher animals. In mammals, it is now apparent that a primary target cell which responds to endotoxin is the mononuclear phagocyte. In picomolar concentrations, these macromolecules have been shown to increase the rate of phagocytosis [27,28] and pinocytosis [27] by monocytes and macrophages, to induce tumor cytotoxicity mediated by these cells [29], to cause the release of their lysosomal enzymes [28,30,31], and to increase the synthesis and/or secretion of a number of proteins, including the hemopoietic growth factors erythroid burst-promoting activity [32] and granulocyte-monocyte colony-stimulating activity [1,33,34]. Of major interest to our laboratory has been the study of the kinetics of the synthesis and secretion of granulocyte-monocyte colony-stimulating activity which follow activation of the hu-

man monocyte by endotoxin. We have observed that although monocytes initially respond to bacterial endotoxins by synthesizing and releasing large quantities of this glycoprotein growth factor, within hours these cells become highly refractory to the stimulatory effects of endotoxin as assessed by the rate of its synthesis and secretion [1]. Such rapid acclimation of monocytes to endotoxin in their external environment is only one of the several well-recognized physiologic manifestations of the phenomenon of 'immediate endotoxin tolerance' which in general remains poorly understood [35].

In order to gain insight into the interaction between endotoxin and the surface of the monocyte, in earlier studies we investigated the kinetics of the binding and uptake of tritiated *S. typhi* endotoxin and $^{51}$C-labeled lipid A by viable monocytes [15] and by isolated monocyte membranes [16]. We found that the binding of the whole endotoxin molecule, but not that of isolated lipid A, to the surface of intact monocytes displayed some of the characteristics typical of receptor-ligand interaction and that pretreatment of monocytes with endotoxin appeared to reduce the number of sites on the surface of the cell available for the attachment of tritium-labeled endotoxin [15]; however, when similar binding studies were carried out using plasma membranes isolated from untreated or endotoxin-pretreated monocytes, specific, high-affinity binding could not be demonstrated [16]. Taken together, these observations suggested that specific interactions between monocyte membranes and endotoxin molecules are likely to depend on plasma membrane structures which are assembled in intact monocytes but which are disrupted when plasma membranes are isolated from the cells. To investigate the physical interaction between the endotoxin macromolecule and the lipid domains of the cell membrane of the monocyte in more detail, the current study was undertaken.

By measuring the polarization of the fluorescence of diphenylhexatriene embedded in the plasma membrane of the monocyte in order to assess the fluidity of the lipid phase, we found that incubation of the cells with either endotoxin or lipid A increased the microviscosity of the membrane and decreased the energy required to activate flow of the fluorophore within the lipid bilayer. These findings are consistent with the idea that after endotoxin becomes adsorbed onto the surface of the monocyte, presumably via an attachment mediated by the hydrophilic carbohydrate moiety of the molecule [36], the lipid component of the lipopolysaccharide molecule, lipid A, becomes directly inserted into the lipid bilayer of the cell membrane, thereby altering both its internal viscosity and its crystalline order. These observations suggest that while the initial step in the binding of endotoxin to the surface of the mononuclear phagocyte may be superficial and reversible and may display certain features characteristic of the binding of a ligand to an external surface receptor, as we [15] and others [36] have suggested, the subsequent uptake of endotoxin by the cell may not be restricted to pinocytosis of receptor-bound molecules [37], but may in addition involve direct insinuation of lipid A into the plasma membrane. Since the carbohydrate moiety of endotoxin can be hydrolytically cleaved from lipid A relatively easily under mildly acidic conditions [15–17], it is feasible that the isolated hydrophobic core of the endotoxin molecule, once separated from the polysaccharide outer shell, may become incorporated directly into the lipid bilayer of the plasma membrane of the cell.

To determine whether the insertion of lipid A into the plasma membrane of the monocyte altered the organizational state of the membrane in such a way that monovalent cation transport was affected, we indirectly assessed the transmembrane electrical potential of the monocyte's plasma membrane, both in the resting state and under conditions of perturbation, using the cyanine dye, di-S-$C_3(5)$. This lipid-soluble fluorescent cation has proven to be a useful probe for the study of transmembrane electrical potentials in many types of animal cells. When di-S-$C_3(5)$ is added to a suspension of viable cells, it inserts itself into the plasma membrane, establishing an equilibrium with di-S-$C_3(5)$ molecules in the extracellular phase. Since fluorescence of di-S-$C_3(5)$ is quenched once the dye enters the membrane, and since depolarization of the cells in physiologic buffers which contain concentrations of $Na^+$ and $K^+$ that approximate those in the extracellular environment in vivo gives rise to movement of some of the probe out of the depolarized cell membrane, de-

polarization under these conditions is associated with an increase in fluorescence (Fig. 3). The fact that we found that neither the intact endotoxin molecule nor isolated lipid A affected the resting membrane potential of monocytes or interfered with the subsequent ability of the cells to undergo depolarization in response to phorbol myristate acetate suggests that the interactions which take place between endotoxin molecules and the surface of the monocyte do not result in significant disruption of cation transport and are therefore not likely to involve extensive organizational rearrangement within the lipid phase of the membrane.

In conclusion, the data which we have summarized in this report and in earlier studies [15,16] indicate that the activation of mononuclear phagocytes by bacterial endotoxins is likely to be set into motion by the binding of these molecules onto the outer surface of the cell membrane and may in addition involve the subsequent incorporation of the lipid component of the molecule directly into the plasma membrane's lipid bilayer. Although our data do not allow us to speculate further upon the intracellular processes which are directly triggered by the attachment of endotoxin to the monocyte and which ultimately give rise to numerous metabolic events, such as the de novo synthesis and secretion of granulocyte-monocyte colony-stimulating activity [1], we hope that the studies that we have reported will provide a useful framework for such investigation.

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