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EFFECTS OF *E. COLI* 0111.B4 LIPOPOLYSACCHARIDE ON SPIN-LABELLED MURINE MACROPHAGE AND HEPATOCYTE MEMBRANES

S.K. JACKSON*, P.E. JAMES

Dept Medical Microbiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN.

C.C. ROWLANDS and J.C. EVANS

Dept of Chemistry, University of Wales College of Cardiff, P.O. Box 78, Cardiff CF1 1XL.

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Macrophages and hepatocytes from normal and BCG-primed mice have been spin-labelled in their membranes with 5- and 16-doxyl stearic acid. Incubation of spin-labelled cells from BCG-primed animals with lipopolysaccharide from *E. coli* 0111.B4 produced a detectable and transient disturbance in the cell membranes as reflected by an increase in the order parameter measured from the electron spin resonance spectra of 5-doxyl-stearate.

This membrane disturbance was maximal at 3–4 hours of incubation and was only detected with cells from mice primed with BCG. Spectra obtained from the 16-doxyl-stearate-labelled cells showed no change in order parameter on incubation with lipopolysaccharide.

KEY WORDS: Spin label, electron spin resonance, lipopolysaccharide, macrophage, hepatocyte.

INTRODUCTION

Bacterial lipopolysaccharides (endotoxins) are known to have multiple biological activities in mammalian host systems. Among these are the induction of haematological events such as complement activation[1] and immunological events such as B-lymphocyte proliferation[2] and the activation of macrophages.[3]

Central to many of the host responses to endotoxin is the blood monocyte or tissue macrophage.[4] Injection of BCG into mice can 'prime' macrophages for subsequent activation by lipopolysaccharide.[5] This primed state may render the animal several thousand times more sensitive to the lethal effects of lipopolysaccharide.[6] Lipopolysaccharide is known to induce the production of many immunoregulatory and inflammatory molecules from these cells including tumour necrosis factor[7], interleukin-1,[8] prostaglandins and leukotrienes.[9] Therefore, studies of the mechanism of lipopolysaccharide-macrophage interactions are essential to the understanding of the host response to lipopolysaccharide. A first step in the analysis of this interaction is to

*Author for correspondence.

detect the biochemical or biophysical changes induced by lipopolysaccharide prior to the final expression of activation.

This report describes the electron spin resonance-detected membrane changes induced by lipopolysaccharide in spin-labelled macrophages and hepatocytes from mice made sensitive to the toxic effects of lipopolysaccharide by prior injection with Bacille-Calmette-Guerin (BCG).

MATERIALS AND METHODS

Mice

Adult strain TO mice were injected intravenously with BCG (Glaxo, ca 10^7 live organisms per mouse) 14–18 days before the animal cells were used for experiments. Mice not receiving BCG were used as controls.

Macrophages were obtained by peritoneal lavage with 5 mls sterile phosphate buffered saline (PBS; 0.15 M). Cells were incubated for 2 hours at 37° C in plastic petri dishes (Sterilin). After this time, adherent cells were removed by gentle scraping, washed × 3 in sterile PBS, counted and resuspended at 6×10^6 cells per ml in Hanks Balanced Salt Solution (HBSS). Cells obtained in this way were > 95 % macrophages after Giemsa staining and microscopic examination.

Heptocytes

Livers were removed and 0.7 mm thick slices were obtained with a tissue chopper (McIlwain). Slices containing 95 % hepatocytes, were washed in sterile PBS and resuspended in HBSS. Cell viability was measured by exclusion of the dye trypan blue.

Spin-labelling

To the cell suspensions in 3 ml HBSS was added 100 μl of 0.5 mg/ml 5- or 16-doxyl stearic acid (Sigma) in ethanol. The mixtures were then incubated at 37° C for one hour. The spin-labelled cells were then washed three times with PBS to remove unbound label.

Endotoxin exposure

Lipopolysaccharide from *E. coli* 0111.B4 at 1 μg/ml was added to the spin-labelled cells and incubated at 37° C for between 1 and 8 hours. ESR spectra were recorded during these times. Each sample exposed to endotoxin was compared to a control sample of spin-labelled cells without endotoxin.

ESR measurements

Macrophage cells were pelleted and resuspended in 200 μl of HBSS and then carefully pipetted into a 2 mm i.d. sealed glass capillary tube. Liver slices were placed into an ESR tissue cell (Wilmad, USA). All spectra were recorded at either room temperature or 37° C on a Varian E109 ESR spectrometer operating at 9.3 GHz at 3300 G field and 10 mW microwave power.

Order parameters

The empirical order parameter (S) – a measure of membrane fluidity – was derived from the following equation:

$$S = \frac{A\| - A\perp}{Azz - Axx} \quad \text{(i)}$$

Where A‖ is half the distance between the outer hyperfine lines and A⊥ is half the distance of the inner hyperfine splittings (see Figure 1). Azz – Axx was taken as 25 Gauss[10].

The 2A‖ value is used to estimate the freedom of motion of the spin-label and therefore to determine the rigidity of the membrane in which it is located[11] From equation (i) it can be seen that an increase in 2A‖ will result in an increase in order parameter (S) and thus reflect an increased rigidity in the membrane.

RESULTS

The ESR spectra obtained from peritoneal macrophages spin-labelled with 5- and 16-doxyl stearate are shown in Figure 1. The spectra show that the 5-doxyl stearate is more motionally restricted than the 16-doxyl label as evidenced by the broadening of the peaks and the appearance of the outer peaks and troughs (Figure 1). This is consistent with the labelled stearic acids being incorporated into the cell membrane; if they were simply associated with the cell surface then both labels should give identical spectra.

Incubation of 5-doxyl stearate-labelled BCG-primed macrophages with *E. coli* 0111.B4 lipopolysaccharide (1 μg/ml) for 3 hours produced the spectrum showed in Figure 2. Similar spectra were recorded from hepatocytes incubated with lipopolysaccharide. From Figure 2 it is clear that the lipopolysaccharide has disturbed the membrane to produce a change in the stearic acid mobility. Incubation of cells from animals not primed with BCG did not produce the spectral changes observed with cells from primed animals. Comparison of the high-field peak maxima shows that addition of lipopolysaccharide has caused an increase in the value of 2A‖.

In contrast, incubation of 16-doxyl stearate-labelled cells with *E. coli* 0111.B4 lipopolysaccharide did not change the values of 2A‖.

The calculated order parameters, for both spin-labels are presented in Table 1.

Incubation of any of the spin-labelled cells with either lipopolysaccharide at concentrations above 10 μg/ml or below 0.1 μg/ml did not produce measurable changes in the resultant ESR spectra (Figure 3). Similarly incubations of less than 3 hours or greater than $4\frac{1}{2}$ hours also produced no change in ESR measurable parameters (Figure 4). From this it appears that the membrane changes induced by lipopolysaccharide are both time and concentration dependent.

DISCUSSION

This study has produced several interesting results from the interaction of bacterial lipopolysaccharide with macrophage and hepatocyte cells. First, incubation of these cells with lipopolysaccharide produces a change in the membrane fluidity which is

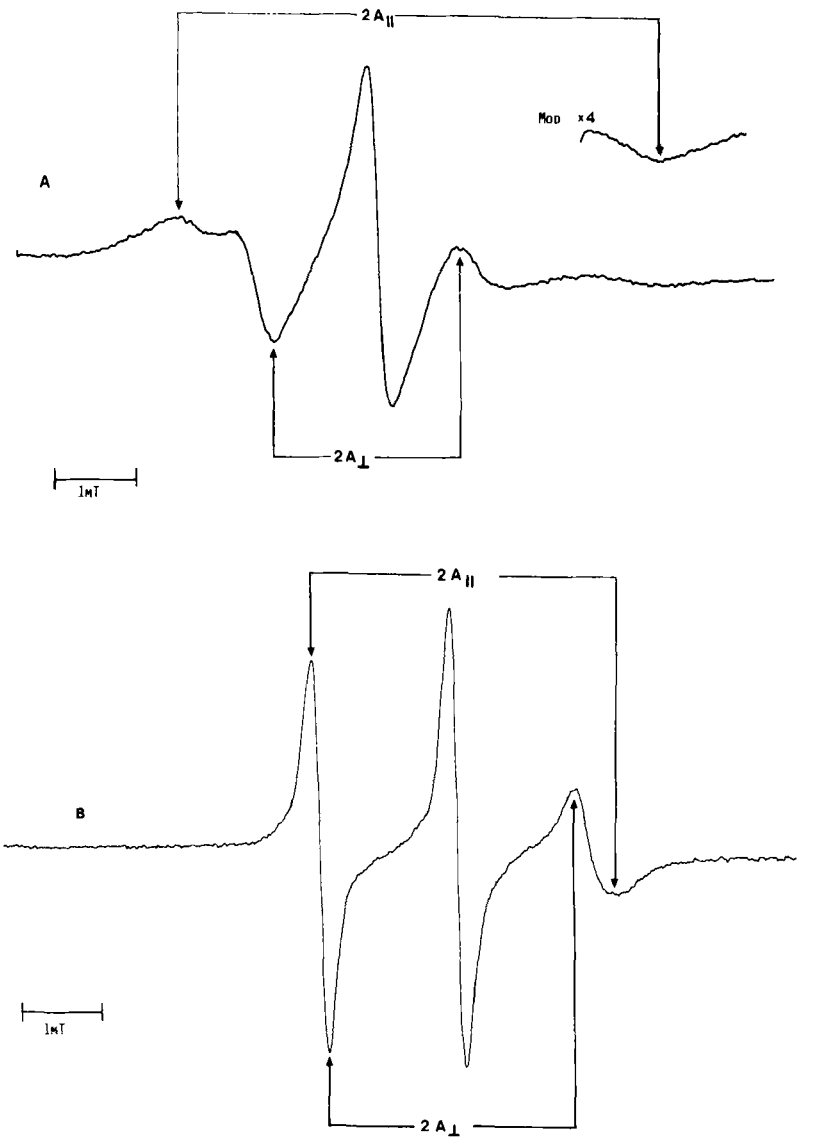

FIGURE 1 A. ESR spectrum of murine peritoneal macrophages labelled with 5-doxyl stearate. B. ESR spectrum of murine peritoneal macrophages labelled with 16-doxyl stearate. Spectrometer settings were: Gain 1×10^4, modulation (Mod) 1 gauss, sweep time 8 minutes, time constant 0.3 seconds.

both time and concentration dependent. Secondly, only cells from animals primed with BCG showed this disturbance in membrane order. Thirdly, the interaction of lipopolysaccharide with cell membranes results in a decrease in membrane fluidity as measured near the surface of the lipid bi-layer; such a disturbance could not be detected deeper in the hydrophobic regions of the bi-layer.

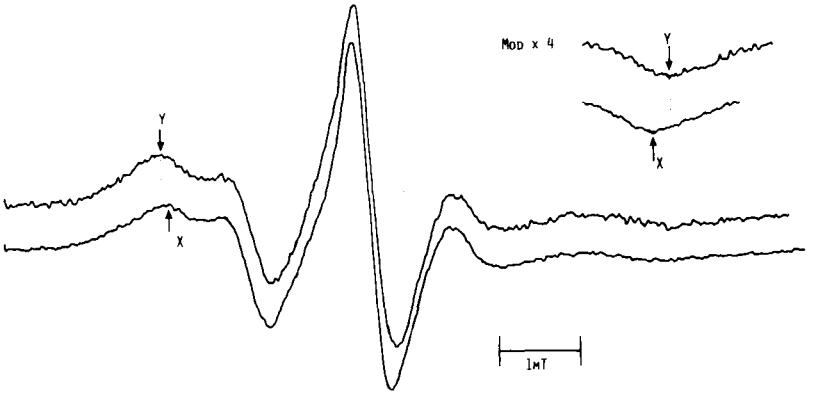

FIGURE 2 ESR spectra from murine peritoneal macrophages labelled with 5-doxyl stearate before (x) and 3 hours after (y) incubation with 1 μg *E. coli 0111* lipopolysaccharide. Spectrometer settings were as Figure 1, except gain = 5×10^4.

TABLE 1
Calculated order parameters from macrophages and liver cells labelled with 5 or 16-doxyl stearate in the absence (control) or presence of 1 μg lipopolysaccharide (LPS). (Mean ± S.D.)

	Macrophage			Liver	
	Control	1 μg LPS		Control	1 μg LPS
5-DS	0.70 ± 0.015	0.74 ± 0.013	5-DS	0.61 ± 0.014	0.66 ± 0.015
16-DS	0.17 ± 0.011	0.17 ± 0.011	16-DS	0.13 ± 0.010	0.13 ± 0.010

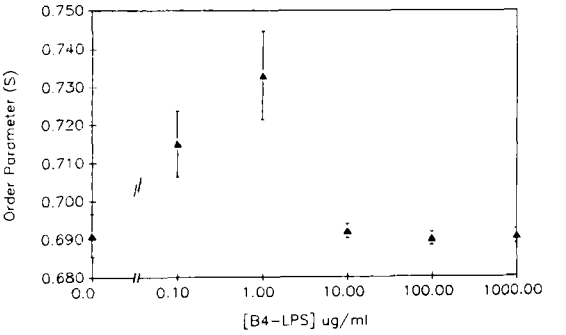

FIGURE 3 Typical concentration dependent change in order parameter(s) measured from 5-doxyl stearate labelled cells incubated with lipopolysaccharide from *E. coli* 0111.B4.

The changes in order parameter, calculated from the measured A values in the ESR spectra, in cells treated with lipopolysaccharide are summarised in Table 1. The increase in order parameter measured with cells labelled with 5-doxyl stearate (6 % and 8 % increase for macrophages and hepatocytes respectively) represent significant changes in membrane composition.[12]

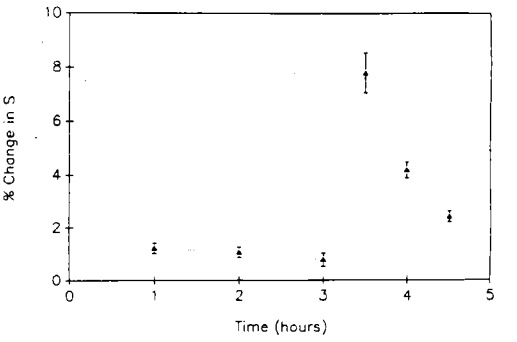

FIGURE 4 Typical time-dependent change in order parameter(s) measured from 5-doxyl stearate cells in the presence of 1 μg/ml *E. coli* 0111.B4 lipopolysaccharide.

The measured increase in membrane rigidity must represent the initial change induced in these cells by bacterial lipopolysaccharide. The ESR spectra from 16-doxyl stearate did not change following incubation of the cells with lipopolysaccharide showing that the disturbance in membrane fluidity was not detected deep in the lipid bi-layer. Thus translocation of lipopolysaccharide through the membrane is unlikely. The disturbance of the membrane is probably an initial event which triggers a 'second message', such as the production of inositol phosphates through which the cell responds.[13]

The decrease in membrane fluidity described here is in contrast to the work of Esser[14] who, in an earlier study using a macrophage tumour cell line found a transient increase in the membrane fluidity. In this case peritoneal macrophages elicited with thioglycollate showed no change.

The present study suggests that other cell types can respond to lipopolysaccharide with detectable changes in membrane fluidity. A decrease in fluidity was measured in both hepatocytes and macrophages. Both these cells are important targets for bacterial lipopolysaccharides[15]. However, only peritoneal macrophages from BCG-infected animals showed significant membrane perturbation following lipopolysaccharide exposure. BCG is known to prime macrophages for triggering to full 'activation' and tumouricidal capacity[16]. This priming is accompanied by the expression of both physical and chemical markers by these cells[17]. Furthermore, mice injected with BCG become up to several thousand times more susceptible to the biological and toxic effects of lipopolysaccharide[5].

A recent study has suggested[18] that BCG increases the ratio of polyunsaturated fatty acids in the plasma membranes of cells. In particular, an increase in macrophage arachidonic acid was reported following infection with BCG. Additionally, after incubation with lipopolysaccharide, the unsaturated/saturated fatty acid ratio decreased by a third in these cells[19]. An increase in membrane fluidity which may occur as a consequence of increased unsaturation might facilitate the binding of lipopolysaccharide by allowing the expression of surface receptor proteins.

Lipopolysaccharide is known to stimulate the production of arachidonic acid-derived prostaglandins and leukotrienes[20], which would lead to a decrease in membrane unsaturation and fluidity. The membrane perturbation reported here might represent the actual binding of lipopolysaccharide, by as yet unknown mechanisms, and the subsequent decrease in unsaturated fatty acid content.

The membrane disturbances reported here were dependent on both the concentration of lipopolysaccharide and the time of exposure. At concentrations above 5 μg/ml lipopolysaccharide may form complex aggregates which could inhibit the binding process. An incubation time of several hours was required to detect the membrane perturbations, perhaps this time was required for protein synthesis and fatty acid turnover. At incubation times longer than four hours the ESR-detectable changes had disappeared, suggesting that the membrane disturbance was transient and reversible.

The results of this study are interesting because they show that two different cell types, both thought to be intimately involved in the numerous effects of lipopolysaccharide *in vivo*, can respond *in vitro* by a measurable decrease in membrane fluidity. Furthermore, factors which enhance the susceptibility of some host cells to lipopolysaccharide, such as BCG, might prime those cells to interact with this ligand.

Whether the membrane perturbation described here reflects true ligand-receptor binding, or binding of the lipopolysaccharide by non-specific means has not been determined. Studies are now in progress to attempt to elucidate the mechanism of binding of lipopolysaccharide to mammalian cells.

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