Molecular Interactions between Bacterial Endotoxins and Mammalian Cells. A Spin-label Study

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Endotoxin molecules from *E. coli* 0111:B4, J5 and lipid A have been spin-labelled with 2,2,6,6-tetramethylpiperidin-*N*-oxyl(TEMPO) free radicals in their sugar residues. Measurement of the rotational correlation times indicates that these saccharide residues do not bind to cell membrane surface structures. A lipid-labelled derivative of the lipid A component of LPS has also been synthesized with the spin-label group positioned on the myristic acid chain. When this is incubated with macrophage cells, an EPR signal characteristic of a spin-label motionally restricted in a lipid bilayer is obtained. This indicates that this LPS derivative binds to the cell membrane through its lipid component. The majority of this 'binding' (66%) is shown to be due to non-specific insertion in the bilayer.

The membrane-intercalated LPS appears to aggregate into patches of high endotoxin concentration, which are stabilised by their strong interaction with membrane phospholipids (phosphatidylethanolamine). Uptake of these spin-labelled derivatives into cells is found to be dependent on a chemical energy source (ATP) and an intact cytoskeleton. We speculatively suggest that the aminophospholipid translocase enzyme present in cell membranes might be responsible for cellular uptake of endotoxin.

Endotoxin, the lipopolysaccharide (LPS) constituent present in the outer membrane of Gram-negative bacteria, causes a variety of physiological effects in man and experimental animals, including fever, haemodynamic disturbances, shock and death.[1] It is recognised that the mononuclear phagocytes are the primary target cells, and that their interaction with LPS leads to secretion of cytokines which are responsible for many of its biological effects. However, the precise biochemical and molecular mechanisms of endotoxin action are unknown.

We have previously reported that LPS induces an increase in rigidity in cell membranes;[2] such a change in the lipid environment might have important consequences on the activity of membrane-bound enzymes[3,4] and this may play a significant role in the development of the pathophysiology of endotoxic shock at the cellular level.

Many investigators believe that in order for LPS to influence cellular functions, it must first bind to specific cell-surface receptors. For example, receptors have been identified which recognise particular regions of the polysaccharide (PS) component of LPS.[5] Release of LPS from the bacterium also allows the hydrophobic lipid A component, initially immersed in the bacterial outer membrane, to become available for interaction with host cell receptors.[6] It is possible that this interaction of LPS with cells is non-specific and that binding of the lipid region does not require the mediation of cell surface receptors.

In an attempt to answer this controversy, we synthesized novel 'sugar'- and 'lipid'-labelled derivatives of LPS[7,8] and monitored their interaction with macrophage cells. This provided valuable information about the substructures that are important for LPS–cell interaction, and also on the fate of LPS once bound to the membrane.

Experimental

Spin-labelled derivatives of endotoxin (from native *E. coli* 0111:B4, its J5 mutant, which lacks the ability to incorporate galactose, or lipid A, the smallest toxic component) were prepared such that the nitroxide spin-labels were covalently linked to specific sugar residues (Fig. 1).

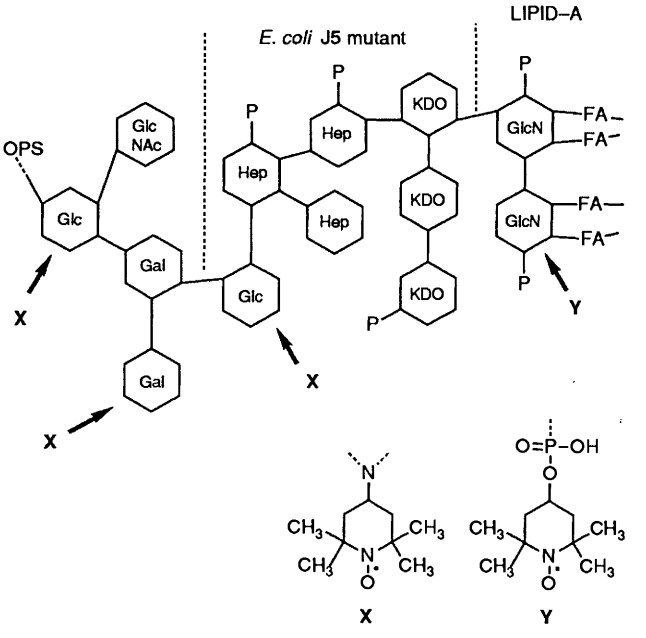
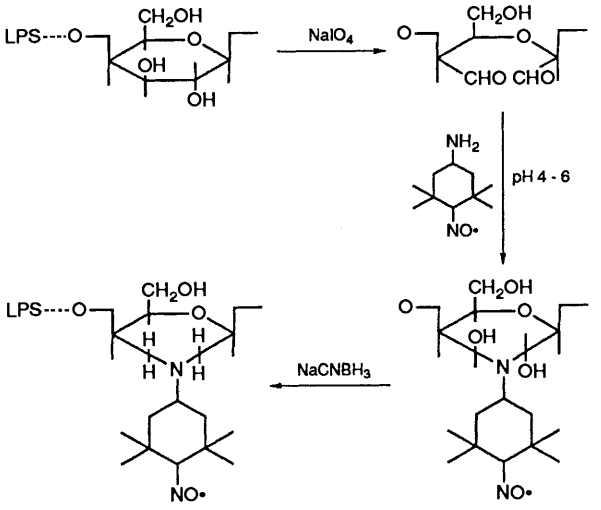


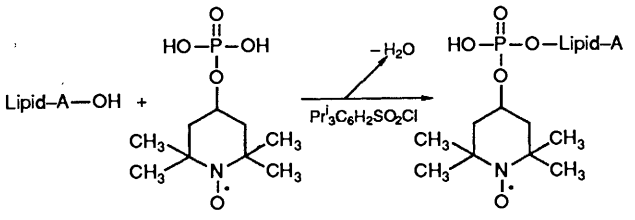
Fig. 1 Schematic diagram to show the possible sites of attachment of amino-TEMPO spin-label (arrowed X) to lipopolysaccharide from *E. coli* 0111.B4 and its rough mutant J5, and attachment of phospho-TEMPO (arrowed Y) to lipid A

All syntheses are described in greater detail elsewhere.[7,8] Briefly, the method for spin-label attachment to B4 or J5 endotoxins is outlined in Scheme 1, and is basically similar to that used for coupling proteins to insoluble polysaccharides. The method for addition of a spin-label group onto a glucosamine residue on lipid A is outlined in Scheme 2, and was carried out using the condensing agent triisopropylbenzenesulfonyl chloride (TBSC).[9] Scheme 3 outlines the preparation of a spin-labelled lipid analogue of lipid A and was a modification of Schwartzmann's synthesis.[10]

Comparison of the endotoxicity of these spin-labelled



Scheme 1 Addition of amino-TEMPO spin-label to sugar residues on endotoxin that have di-hydroxyl groups



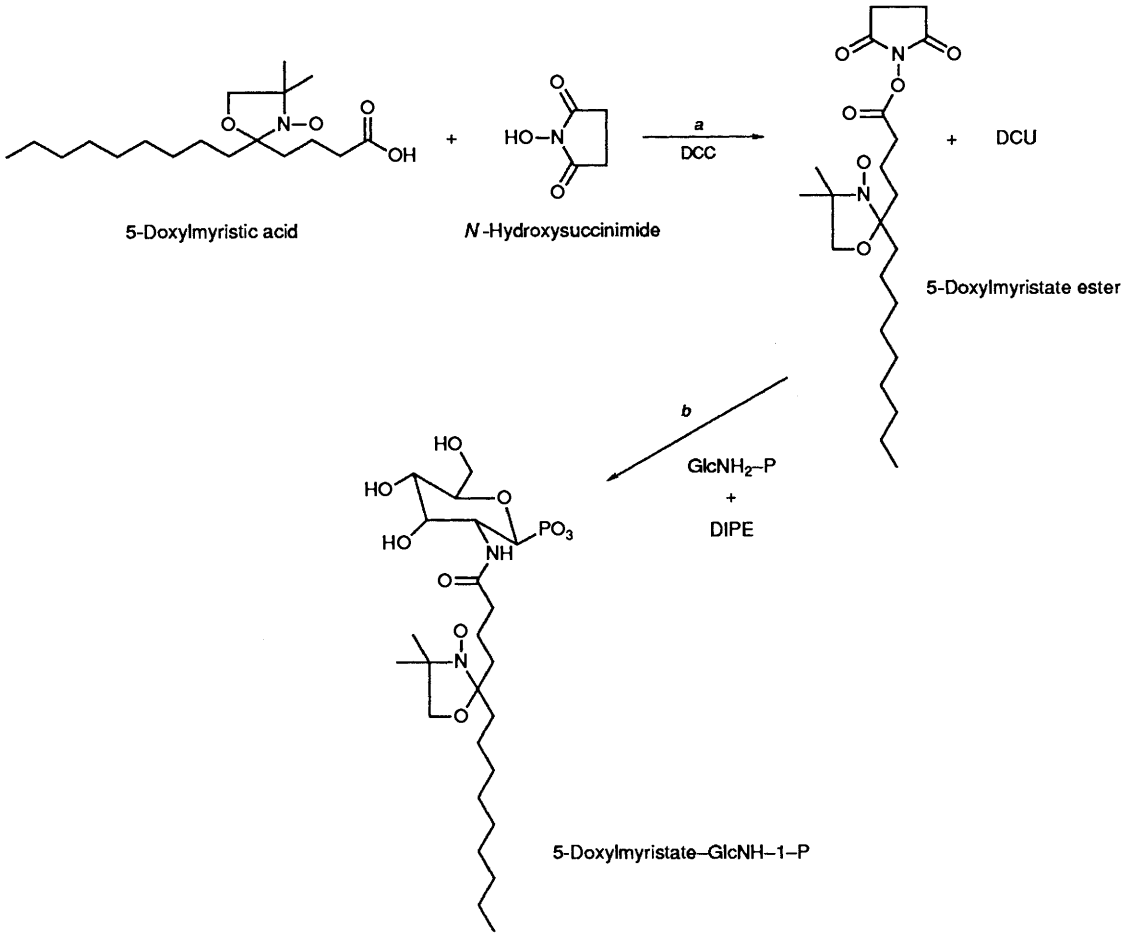
Scheme 2 Addition of phospho-TEMPO spin-label to the primary alcohol group on a glucosamine residue on lipid A

analogues was achieved by: (i) Limulus Amoebocyte Lysate (LAL) activity, which is a specific assay for endotoxin.[11] (ii) Their ability to induce Tumour Necrosis Factor (TNF) production in human monocytes.[12] (iii) Lethality in mice. Each compound was found to compare closely with its native (unlabelled) counterpart. This was important as it ensured that introducing a nitroxide spin-label onto the molecule of interest did not significantly alter its chemical or biological characteristics.

Critical Micellar Concentration.—The concentration at which each spin-labelled compound aggregated was measured by gradually increasing its concentration in PBS (1 cm^3) and taking spectra of the solutions at each increment. The central peak height was plotted against the concentration of compound used, and the CMC was calculated from the point on the graph where non-linear increase in signal intensity with concentration began (due to spin–spin broadening). The lipid-labelled monosaccharide, 5-DMGlcN-P, aggregated above 2×10^{-5} mol dm^{-3}, whereas TEMPO-labelled endotoxin (B4 and lipid A) had a CMC of 6.6×10^{-5} mol dm^{-3}.

Cell Lines.—The murine macrophage-like cell line P388D was cultured in Dulbeccos modified minimal essential medium (DMEM; Sigma) supplemented with 10% foetal calf serum (Gibco) which contained antibacterials and antifungals.

Preparation of Liposomes.—Fresh liposomes were prepared by drying the appropriate phospholipid (100 mm^3, 10 mg cm^{-3} in chloroform) under a stream of nitrogen in a glass test tube.



Scheme 3 Chemical synthesis of a spin-labelled monosaccharide analogue of lipid A. DCC, dicyclohexylcarbodiimide; DIPE, diisopropylethylamine; DCU, dicyclohexylurea.

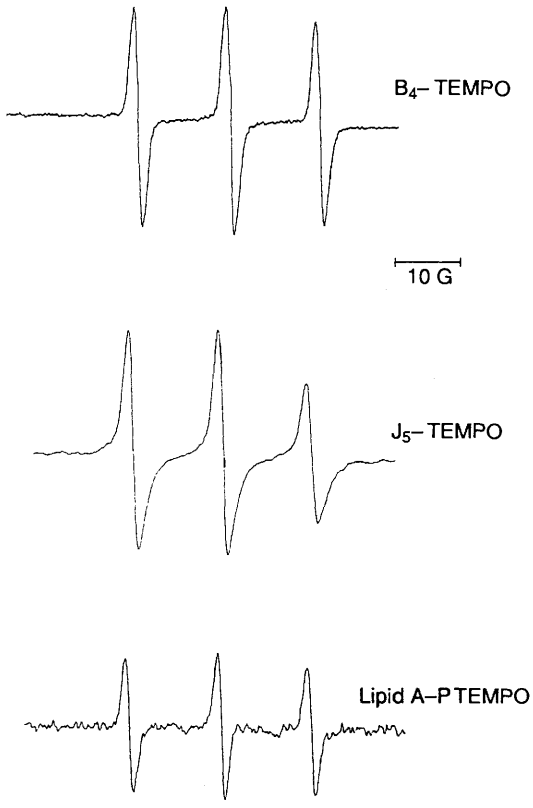


Fig. 2 EPR spectra of the spin-labelled LPS from *E. coli* 0111.B4, J5 and lipid A (25 μg cm^{-3}) in medium at 22 °C. Spectrometer conditions; power 5 mW, modulation amplitude 0.4 G, the gain was adjusted to give spectra of similar size (2.5×10^3–1×10^4).

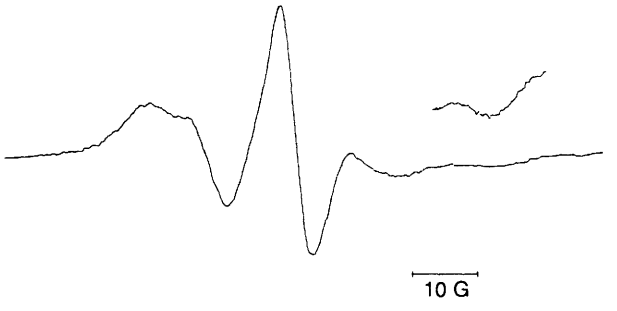


Fig. 3 Typical spectrum of the lipid-labelled monosaccharide, 5-DMGlcN-P inserted into macrophage membranes. The cell pellet was washed three times in order to remove any free, unbound label. Spectrometer settings as in Fig. 2.

The dried lipid mixture was resuspended in phosphate buffered saline (PBS; 200 mm^3) and dispersed by sonication. Liposomes were collected after centrifugation at 13 000 r.p.m. for 10 min and re-suspended in PBS (200 mm^3) for each experiment.

Phospholipid Extraction.—Approximately 2×10^7 cells were suspended in distilled water (1 cm^3) and sonicated (on ice) for 30 min. To this was added 3.75 cm^3 of chloroform–methanol (1:2 v/v) which was then allowed to stand at room temperature for a further 30 min. 1.25 cm^3 of chloroform was added, and then 1.25 cm^3 of Garbus wash (2 mol dm^{-3} KCl in 0.5 mol dm^{-3} K_3PO_4 buffer, pH 7.4). This was shaken well and the two phases allowed to separate. The lower (lipid-containing) layer was extracted three times with light petroleum. These were then reconstituted into liposomes or cell ghosts by the same method as liposome preparation (above).

Spin-labelling Experiments.—In a typical experiment, 2×10^7 cells were incubated with 1.25 μg cm^{-3} of labelled LPS in DMEM at 37 °C. After 10 min, the cells were centrifuged free of the supernatant and washed three times in ice cold PBS. Samples for EPR analysis were made by drawing a small volume into a 1 mm i.d. capillary tube sealed at one end.

The influence of surface protein on this binding was assessed by pre-treating the macrophages in culture with proteinases [trypsin, 0.25% for 5 min, or proteinase K, 0.02% for 5 min, made up in culture medium (DMEM)] and incubated at 37 °C and 95% O_2. The cells were removed from the flask by gentle aggitation and the spin-labelled compound added as above.

Kinetic studies were performed in order to measure the rate of reduction of the labelled LPS compounds by cells. In this case, the sample tube (containing labelled cells) was placed in the spectrometer cavity and repeat scans (2, 4 or 8 min) were taken. Signal reduction was measured from the mid-peak height in each spectrum, and plotted as the percentage of the original signal that remained at that time.

The influence of metabolic inhibitors on this rate of uptake was assessed by pre-treating the cells with sodium azide (a respiration inhibitor; 0.5% for 1 h); colchicine (inhibits microtubule formation; 75 mmol dm^{-3} for 1 h); 2-deoxyglucose (depletes ATP levels; 12 mmol dm^{-3} for 1 h) or *N*-ethylmaleimide (a thiol group reagent; 10 mmol dm^{-3} for 5 min), and the rate of signal reduction measured as above.

External ascorbate reduction was measured by finally suspending the labelled cells in 50 mm^3 of 25 mmol dm^{-3} sodium ascorbate in PBS and then into the glass capillary for measurement.

EPR Spectroscopy.—EPR measurements were made on a JEOL JES RE 2X ESR spectrometer, operating at 9.5 GHz. Spectrometer settings used are given in the figure legends.

Results

Lipopolysaccharide from E. coli *B4, J5 and Lipid A, Spin-labelled at Specific Sugar Residues.*—Typical spectra of each of the LPS molecules spin-labelled in their saccharide residues in aqueous buffer (Hanks' balanced salt solution; HBSS) are shown in Fig. 2. The spectra show three sharp lines characteristic of a nitroxide radical in solution, and indicate that the TEMPO spin-labels are freely rotating. On incubation with macrophage cells, any restriction in the motion of these labelled sugar residues, such as that induced by binding to a receptor, would result in characteristic changes in the EPR spectra, typified by broadening and the appearance of outer peaks and troughs. However, the spectrum of cell-associated LPS is very similar to that for free spin-labelled LPS (Fig. 2) and consists of three sharp lines of almost equal intensity which is typical for a spin-label undergoing rapid molecular motion. This suggests that these saccharides were not directly involved in the binding of LPS to the cell.

5-Doxyl Myristate Glucosamine Phosphate. A Spin-labelled Monosaccharide Analogue of Lipid A.—On incubation of this lipid-labelled compound (5-DMGlcN-P) with macrophage cells, the isotropic signal obtained with the compound in aqueous solution became broadened to give an anisotropic signal (Fig. 3). This spectrum was similar to those obtained from spin-labelled fatty acids intercalated into cell membranes and suggests that the hydrophobic lipid component of LPS intercalates directly into the cell membrane.

If the concentration of compound added to the cells was increased, this anisotropic signal became further broadened with some loss of spectral detail (Fig. 4). At the highest concentration shown (1×10^{-5} mol dm^{-3}) an extensively broadened EPR signal developed which was similar to the

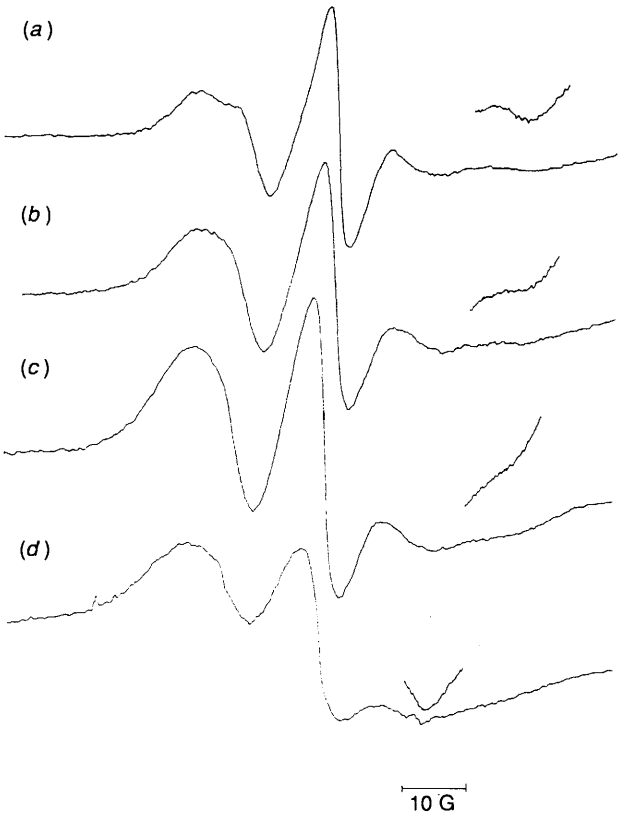


Fig. 4 Spectra showing the formation of regions of high concentration of 5-DMGlcN-P within the membrane. The broadening of the spectrum was due to spin–spin interaction of the nitroxide labels. The concentration of compound added to the cells in each case are shown for each spectrum. (*a*) 0.2×10^{-5}; (*b*) 0.4×10^{-5}; (*c*) 0.6×10^{-5}; (*d*) 1×10^{-5} mol dm^{-3}. Spectrometer settings were as for Fig. 2 except the gain was 5×10^{-5}.

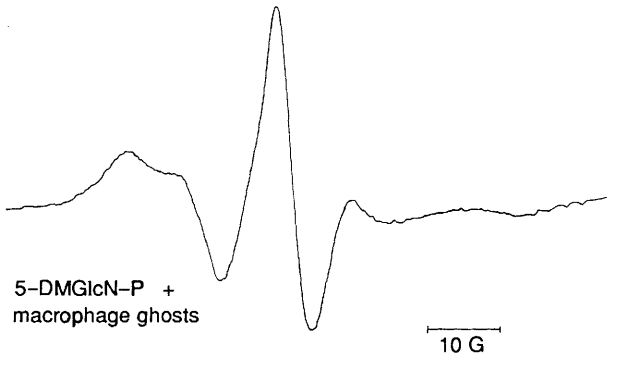


Fig. 5 5-DMGlcN-P incubated with liposomes or membrane ghosts made up of the reconstituted phospholipids from macrophages. Spectrometer settings as for Fig. 4.

single, large 20 G peak obtained when a high degree of nitroxide–nitroxide interaction occurs. This broadened signal must have been due to the close proximity of the spin-labelled compounds within the membrane and not due to spin-broadening of the compound added in the aqueous medium, as the concentration ranges used were below the critical micellar concentration (2×10^{-5} mol dm^{-3}) and any free label that was not cell-associated was removed and subsequently recovered in the washes.

Effect of Proteinases.—Cells that had been pre-incubated in either trypsin or proteinase K in order to cleave any surface proteins, showed a marked decrease in the amount of lipid-labelled compound they could incorporate when compared

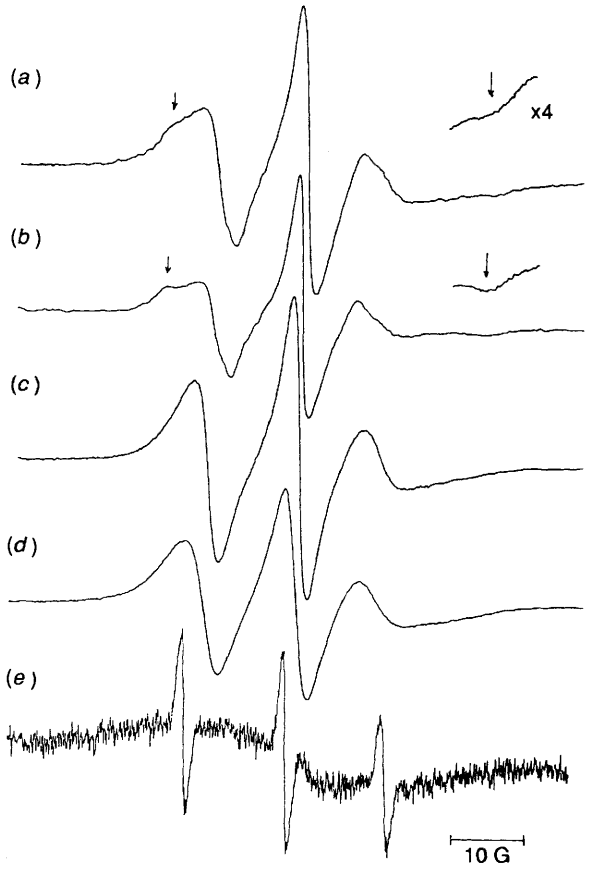


Fig. 6 Interaction of 5-DMGlcN-P with liposomes made up with each phospholipid; (*a*) phosphatidylcholine (PC); (*b*) phosphatidylethanolamine (PE); (*c*) phosphatidylglycerol (PG) and (*d*) phosphatidylinositol (PI). Arrows show the additional component from spin-labels in a more motionally-restricted environment with liposomes of PC or PE. Spectrum (*e*) shows that there is no such association between this compound and vesicles made up from lipids such as arachidonic acid. This spectrum shows spin–spin broadening due to a high nitroxide concentration (large 20 G signal) superimposed onto the signal from the free, unrestricted spin-label.

with control macrophages. This decrease was monitored by measuring the height of the mid-field peak in the EPR spectra obtained from 2×10^7 cells labelled with 25 μg of compound. Pre-incubation with trypsin resulted in 29.5% and with proteinase K 41% less compound being incorporated into the membrane.

Vesicles or liposomes were made up from the extracted phospholipids of macrophage membranes in an attempt to mimic a cell membrane without proteins. The spectrum of 5-DMGlcN-P incorporated into these membrane 'ghosts' clearly shows a broad signal similar to that in Fig. 3, and is indicative of the compound being able to intercalate into the phospholipid bilayer to the same extent as when whole cells were used (Fig. 5).

The interaction of 5-DMGlcN-P with liposomes made up of each phospholipid alone was also monitored (Fig. 6). All spectra (*a*)–(*d*) gave an EPR signal typical of labels undergoing fast molecular motion. This signal must have been from labels loosely associated with the phospholipid liposomes, since continued washing did not reduce the signal amplitude. The spectra from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were two-component in nature, containing the signal seen in all the spectra [phosphatidylglycerol (PG) or phosphatidylinositol (PI)] along with an additional component (shown by arrows in Fig. 6). This appears to be from spin-labels in a more motionality restricted environment.

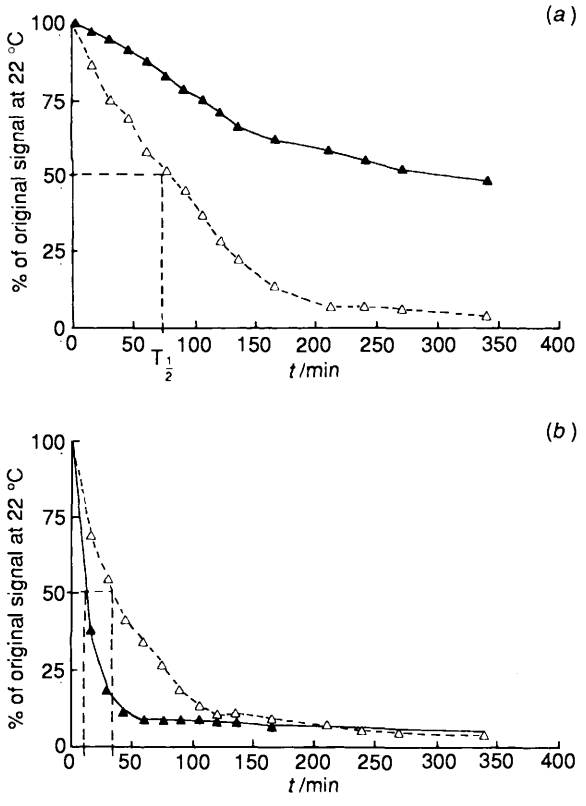


Fig. 7 The rate of reduction of the lipid-labelled compound 5-DMGlcN-P compared to the fatty acid spin-label, 5-doxyl stearate (5-DS). This is expressed as the percentage of the original mid-field peak height remaining at any one time. (*a*) By macrophages (△, 5-DMGlcN-P + cells; ▲, 5-DS + cells) and (*b*) by macrophages which were bathed in medium containing 25 mmol dm^{-3} sodium ascorbate (△, 5-DMGlcN-P + cells + ascorbate; ▲, 5-DS + cells + ascorbate).

When similar experiments were performed using vesicles of stearic or arachidonic acid [Fig. 6(*e*)] the signal obtained from the washed vesicle pellet was that of the free spin-label, superimposed onto a spin–spin broadened signal. This signal was probably due to the formation of spin-label–arachidonic acid vesicles, and the spin-exchanged spectrum from the high concentration of unrestricted nitroxide spin-labels.

These results indicate that the lipid component of LPS inserts into particular regions of a cell membrane to form patches of high endotoxin concentration. Phospholipids such as PE and PC bind the inserted LPS and probably stabilise these domains after intercalation.

In the presence of cells, nitroxide labels are taken up and reduced intracellularly to the corresponding hydroxylamines, with the result that the EPR signal disappears. This metabolism of nitroxide labels was used in this study to monitor the uptake of spin-labelled LPS to the cytosolic side of the membrane.

Fig. 7(*a*) shows the decay in EPR signal intensity (measured from the mid-field line height) with time observed for 5-doxyl stearate-labelled cells compared with 5-DMGlcN-P intercalated into cells. Signal reduction was slow for the fatty acid probe 5-DS ($t_{\frac{1}{2}} > 4$ h) whereas the lipid-labelled molecule was reduced relatively quickly ($t_{\frac{1}{2}} = 75$ min).

When similar experiments were performed in the presence of 25 mmol dm^{-3} ascorbate [Fig. 7(*b*)] the results were reversed; the signal from 5-DS was reduced immediately whereas that from the lipid-labelled compound was not reduced as quickly. Ascorbate can reduce nitroxide labels, but is unable to cross the membrane bilayer. The stearate spin-probe remained in the outer region of the membrane and was therefore reduced by the ascorbate in the surrounding solution. The lipid-labelled molecule on the other hand was protected from the external ascorbate and was probably either translocated to the cytosolic side of the membrane or taken up by endocytosis into membrane bound vesicles where it could still give an EPR signal until reduced intracellularly.

To show that signal reduction could not have occurred extracellularly, medium was taken from macrophages treated by LPS. This was unable to reduce the spin-labelled monosaccharide over a time period of up to 6 h.

Further evidence in support of 'active translocation' of the spin-labelled LPS came from kinetic experiments performed by pre-treating the macrophages in culture. On adding 5-DMGlcN-P to cells treated with sodium azide (a metabolic inhibitor), colchicine (inhibits microtubule formation), 2-deoxyglucose (depletes ATP levels) or *N*-ethylmaleimide (a thiol group reagent), the EPR signal was stable and did not decrease with time. This suggests that uptake and/or reduction of the nitroxide radical on LPS by macrophages was dependent on (*a*) a chemical energy source, (*b*) presence of cytosolic reductants, and (*c*) an intact cytoskeleton. Cells treated by these metabolic inhibitors could not maintain the cellular functions required for active uptake of LPS and consequently showed a much slower rate of reduction.

Discussion

In order for LPS to influence cellular function, it must first interact with the plasma membrane. A better understanding of this initial interaction might help to elucidate the precise biochemical and molecular mechanism of LPS action and ultimately result in more effective treatments.

This study has produced several interesting results from the interaction of spin-labelled analogues of bacterial LPS with cells, and in particular their interaction with cell membranes. Different LPS molecules (0111:B4, J5 or lipid A) which vary according to the amount of polysaccharide, were spin-labelled in their saccharide residues. The results showed that there was no immobilisation of these saccharide regions of LPS upon interaction of these molecules with macrophages, and favour incorporation of LPS into the membrane in a way that does not directly involve the PS region. This is in agreement with similar studies that looked at the interaction of radiolabelled LPS with cells—the PS region of this LPS did not bind to the cell surface but binding was observed with a serum albumin–lipid A conjugate.[13]

Further evidence to suggest that the PS region on spin-labelled B4 LPS did not bind to the cell surface and was anchored to the membrane by lipid A, was obtained on finding that subsequent addition of polyclonal antisera to these LPS-TEMPO-treated cells could immobilise the freely rotating spin-labelled sugar residues.[7]

When 5-DMGlcN-P was incubated with macrophages, the spectrum obtained was that of a motionally restricted spin-label, with the appearance of outer hyperfine maxima. From this it was concluded that the myristic acid chain on 5-DMGlcN-P intercalated into the lipid phase of the macrophage membrane. Once inserted into this outer leaflet, it can move laterally in the plane of the membrane and at a critical concentration (1×10^{-5} mol dm^{-3}), it appears that aggregates or regions of high concentration of nitroxide (and hence LPS) occur within the membrane.

The LPS-binding capacity of these macrophages could be reduced by degrading cell surface proteins. However, when compared with control cells, 66% of the compound was still able to 'bind' to these membranes stripped of protein. Similar results were obtained on incubating the lipid-labelled compound with membrane ghosts (made up from the extracted phospholipids of macrophages). The compound could inter-

calate into these ghosts to the same extent as when the whole cells were used. This indicates that LPS might interact with cells *via* protein-dependent or protein-independent mechanisms.

It is possible that LPS recognises specific sites of the membrane, and in particular, its phospholipid composition; thus, variations in phospholipid composition among cell types might be responsible for the specificity of the biological reactions elicited by LPS. Indeed, binding of the lipid-labelled analogue to liposomes made up of a single type of phospholipid showed a strong interaction between the labelled myristic acid residues of 5-DMGlcN-P with PE and PC.

LPS and PE are the two major lipid constituents of the outer membrane of Gram-negative bacteria. Studies on mixed bilayers of LPS and spin-labelled PE[14] have previously shown that patches of either pure LPS or PE are formed, and this spontaneous formation of domains of LPS within the membrane, stabilised by their interaction with the headgroups of particular phospholipids, such as PE, may have important implications on the mode of action of LPS.

In cultured mammalian cells, reduction of lipophilic nitroxides, such as those used in this study, principally occurs at the level of ubiquinone in the respiratory chain in mitochondria.[15] The rate at which the EPR signal disappeared from cells incubated with the labelled LPS derivatives therefore represented the rate at which the nitroxide compound was taken up and metabolised intracellularly.

5-DMGlcN-P was taken up by macrophages at a rate much faster than that for the fatty acid probe, 5-doxyl stearate. This uptake was shown to be dependent on (i) a chemical energy source (such as ATP), (ii) intracellular reductants (thiol groups), and (iii) an intact cytoskeleton. Experiments performed in the presence of external ascorbate indicated that this spin-labelled monosaccharide was taken up from the outer leaflet of the bilayer and protected from reduction near the outside of the membrane.

These results can be explained in terms of active translocation of the compound to the inner half of the membrane leaflet. A transport system, dependent on cytoplasmic ATP and involving selective interactions between lipids and cytoskeleton proteins, has been identified.[16] This so called 'translocase' enzyme is responsible for the selective translocation of aminophospholipids across the bilayer, and results in the asymmetrical distribution of these molecules between both halves of the lipid bilayer of eukaryotic cells[18]—the outer leaflet generally rich in the cholinephospholipids whereas the aminophospholipids (PE and phosphatidylserine) preferentially occupy the inner leaflet.[17]

This study has demonstrated a high degree of association between the spin-labelled LPS and PE. We suggest speculatively that this enzyme might translocate LPS along with the PE, or otherwise recognise LPS directly. This asymmetrical distribution of phospholipids might also be involved in the early stages of endocytosis,[18] inducing an expansion of the cytoplasmic side of the membrane relative to the outer part of the bilayer, and might explain how the LPS derivatives were taken up into the cytoplasm and reduced.

Conclusions

Taken together, these findings suggest that LPS binds macrophages *via* hydrophobic interactions of the fatty acyl chains of the lipid A component, the PS region is left on the cell surface only anchored to the cell by lipid A. The majority of LPS interaction with cells can be attributed to its binding to membrane hydrophobic regions by non-specific insertion, and it is probably this hydrophobic interaction which is responsible for much of its diverse biological action. It is possible that the strong association found between LPS and PE is important to its stability once inserted into the membrane, and that the aminophospholipid translocase present in eukaryotic membranes might be responsible for its uptake into host cells.

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References

1 D. C. Morrison and R. J. Ulevitch, *Am. J. Path.*, 1978, **93**, 527.
2 S. K. Jackson, P. E. James, C. C. Rowlands and J. C. Evans, *Free Rad. Res. Comms.*, 1989, **8**, 47.
3 C. D. Linden, K. L. Wright, H. M. McConnell and C. F. Fox, *Proc. Natl. Acad. Sci. USA*, 1973, **70**(8), 2271.
4 M-S. Liu, S. Ghosh and Y. Yang, *Life Sciences*, 1983, **33**, 1995.
5 S. Gordon, V. H. Perry, S. Rabinowitz, L. P. Chung and H. Rosen, *J. Cell. Sci. Suppl. 9*, 1988, 1.
6 R. Y. Hampton, D. T. Golenbock and C. R. H. Raetz, *J. Biol. Chem.*, 1988, **263**, 14802.
7 S. K. Jackson, P. E. James, C. C. Rowlands and B. Mille, *Biochim. Biophys. Acta*, 1992, in the press.
8 S. K. Jackson, P. E. James, C. C. Rowlands and B. Mille, manuscript in preparation.
9 F. G. Sharom and C. W. M. Grant, *Biochem, Biophys. Res. Commun.*, 1977, **74**, 1039.
10 G. Schwarzmann, P. H. Bleihauer, J. Schubert, K. Sandhoff and D. Marsh, *Biochemistry*, 1983, **22**, 5041.
11 P. Feiberger, M. Knos and L. Mellstam, *Endotoxins and their detection with the Limulus amoebocyte Lysate*, eds. S. W. Watson, J. Levin and T. Novitsky, Alan R. Liss, New York, 1982.
12 N. Matthews and M. L. Neale, in *Lymphokines and Interferons*, eds. M. J. Clemens, A. G. Morris and A. J. N. Gearing, IRC Press, Oxford 1986, p. 221–225.
13 M. Tahri-Jouti and R. Chaby, *Mol. Immunol.*, 1990, **27**, 751.
14 Y. Takeuchi and H. Nikaido, *Rev. Infect. Diseases*, 1984, **6**(4), 488.
15 K. Chen, P. D. Morse and H. M. Swartz, *Biochim. Biophys. Acta*, 1988, **943**, 477.
16 A. Sune, P. Bette-Bobillo, A. Bienvennue, P. Fellman and P. F. Devaux, *Biochemistry*, 1987, **26**, 2972.
17 R. F. A. Zwaal, B. Roelofsen, P. Cumfurius and L. L. M. Van Deenen, *Biochim. Biophys. Acta*, 1975, **406**, 83.
18 A. Zackowski, H. Herrmann, A. Paraf and P. F. Devaux, *Biochim. Biophys. Acta*, 1987, **897**, 197.

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