Role for L-selectin in lipopolysaccharide-induced activation of neutrophils

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The activation of leucocytes by bacterial cell wall lipopolysaccharide (LPS) contributes to the pathogenesis of septic shock. LPS is known to interact with several cell-surface proteins, including CD14, when presented as a complex with serum LPS-binding protein. However, the identity of the receptor responsible for LPS signalling and leucocyte activation is unknown. Interestingly, mice deficient in cell-surface L-selectin were dramatically resistant to the lethal effects of high doses of LPS in a model of septic shock. Recently we reported that L-selectin binds to cardiolipin and other charged phospholipids at a site distinct from the carbohydrate-binding site. Structural similarities between charged phospholipids and the lipid A moiety of LPS prompted us to investigate interactions between L-selectin and LPS. Herein we show that L-selectin is a neutrophil surface receptor for LPS and lipotechoic acid. The binding of LPS to L-selectin is independent of serum and Ca^{2+}, and is blocked by antibodies to L-selectin and fucoidan. Furthermore, the interaction of LPS with cell-surface L-selectin results in superoxide production, indicating that L-selectin can mediate both binding and activation of human neutrophils. These findings suggest novel therapeutic approaches for the treatment of septic shock.

INTRODUCTION

Bacterial lipopolysaccharides (LPSs) are major constituents of the cell walls of Gram-negative bacteria [1]. The release of LPS from bacterial cell walls into the host circulation results in activation of leucocytes and endothelial cells, resulting in the release of cytokines, nitrous oxide and tissue factors. These responses to LPS or lipotechoic acid cause a clinical syndrome in humans known as septic shock, which has a 40–60 % mortality rate [1]. The CD14 molecule, a glycosylphosphatidylinositol-anchored membrane protein, has been characterized on monocytes, and to a lesser extent on neutrophils, as a receptor for LPS and the serum LPS-binding protein (LBP) complex. However, several studies indicate that CD14 does not mediate intracellular signalling directly, and evidence exists for an independent pathway for the LPS-induced activation of cells [2–7]. It is possible that additional membrane components may be involved in the direct recognition of LPS and may in turn may co-operate with CD14.

Interestingly, L-selectin-deficient mice were shown to be dramatically resistant to the lethal effects of high doses of LPS in a model for septic shock [8]. Selectins are cell-surface integral membrane glycoproteins that are involved in leucocyte trafficking, thrombosis and inflammation. The three known selectins (E-, P- and L-selectins) are characterized by an N-terminal carbohydrate recognition domain followed by an epidermal growth factor domain, between two and nine complement control protein domains, a single membrane-spanning region and a cytoplasmic C-terminal cytoplasmic domain.

Recently we reported that L-selectin binds to cardiolipin and other charged phospholipids via a site distinct from the proposed carbohydrate-binding site [9]. Structural similarities between charged phospholipids and the lipid A moiety of LPS prompted us to investigate the possibility of a direct interaction between L-selectin and LPS. In this paper we show that LPS binds to L-selectin and induces superoxide production in neutrophils in the absence of serum.

MATERIALS AND METHODS

Materials

A plasmid containing the human L-selectin gene was kindly provided by Professor B. Seed (Harvard Medical School, Boston, MA, U.S.A.). Recombinant human selectins, lacking the transmembrane and cytosolic domains, were produced by Bernard Allet (Glaxo Institute of Molecular Biology, Geneva, Switzerland) as C-terminal chimeras with the ZZ-domain of Protein A. The ZZ-domain binds tightly to human IgG. A baculovirus/insect-cell expression system was used, and the IgG-purified proteins were partially characterized in cell adhesion assays [9,10].

Four anti-L-selectin monoclonal antibodies were used: LAM1-3 and TQ1 (subtype IgG1; Coulter Immunology, Coulter Corp., Hialeah, FL, U.S.A.), MCA 1076 (subtype IgG2; Serotec, Oxford, U.K.) and Dreg 56 (subtype IgG1; ams Biotechnology, Witney, Oxon, U.K.). The anti-E-selectin monoclonal antibodies used were clones BBA (subtype IgG1; cat. no. MON 6011; Bradsure Biological Ltd., Loughborough, Leics., U.K.) and MCA 883 (subtype IgG1; Serotec). Other antibodies were the isotype control antibody MCA 928 (subtype IgG1; Serotec), the anti-CD31 antibody MCA 793 (subtype IgG1; Serotec) and the anti-CD14 antibody MCA 660 (subtype IgG1; Serotec).

Horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (cat. no p217; lot no. 111) was from Dako-Immunoglobulin. Sialyl Lewis X was from Dextra Laboratories, Reading, U.K.

Solid-phase binding assays

Microtitre plate wells (Maxisorb; Nunc) were coated with LPS (Sigma) or lipotechoic acid (Sigma) in 50 mM sodium bicar-

Abbreviations used: LPS, lipopolysaccharide; LBP, LPS-binding protein; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate.

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bonate buffer (pH 9.6) at ambient temperature for 16 h. Non-specific binding sites were blocked with ovalbumin (3 mg/ml) in PBS for 2 h at ambient temperature. Selectin-ZZ (1.4 μg) in the presence or absence of competitors was added to coated wells in PBS containing ovalbumin (1 mg/ml) and HRP-conjugated IgG (1:500 dilution). The plates were incubated for 2 h at ambient temperature. After extensive washing, the amount of bound selectin-ZZ–IgG complex was determined by adding the HRP substrate *o*-phenylenediamine dihydrochloride (Fast enzyme system, Sigma; cat. no. P-9187). The reaction was stopped after 5 min with 3 M HCl, and the A_{490} was measured.

LPS binding to neutrophils

Freshly drawn human blood was layered slowly on to Hypaque/dextran (1:1.6, v/v) at a 1:1 (v/v) ratio of blood to Hypaque/dextran. After 50 min, the clear plasma layer was removed and centrifuged (1000 ***g***; 20 min) in a solution of Ficoll. The lymphocyte layer was removed and the supernatant was treated with a hypotonic lysis buffer to remove red blood cells. The neutrophils were pelleted and then resuspended in ice-cold PBS. Neutrophils (1×10^6 cells/ml) were treated with fluorescein isothiocyanate (FITC)-labelled LPS or antibodies as described above. The tubes were stored on ice and the A_{488} was measured using a Becton–Dickinson FACScan Analyser.

LPS-induced superoxide production

Isolated neutrophils were pretreated with 1,2,3-dihydrorhodamine (1 μg/ml) in PBS for 10 min at 37 °C. The subsequent incubation with LPS (*Escherichia coli* serotype 055:B5) was for 15 min at 37 °C. The cells were then fixed with 0.03 % paraformaldehyde. Tubes were stored on ice and the A_{488} was measured using a Becton–Dickinson FACScan.

RESULTS

Binding of L-selectin to LPS

Molecular modelling and binding studies with L-selectin revealed a basic motif localized close to the putative carbohydrate-binding site in the lectin domain [9]. To investigate whether this domain could also bind LPS, we first conducted binding experiments with soluble selectins and immobilized LPS. Recombinant human L- and E-selectins, lacking the transmembrane and cytosolic domains, were produced as C-terminal chimeras with the ZZ domain of Protein A [9]. The ZZ domain allows both affinity purification on IgG–agarose and subsequent detection of bound selectin in an ELISA. The binding characteristics of the recombinant selectins were shown previously to be indistinguishable from those of natural L-selectin [9]. Figure 1(a) shows that L-selectin-ZZ, but not E-selectin-ZZ, bound to LPS derived from *E. coli* 055:B5. The binding of L-selectin was dependent on the amount of immobilized LPS and was independent of added calcium ions. The anti-L-selectin antibodies LAM1-3 and MCA 1076 inhibited binding, whereas neither isotype-matched anti-E-selectin antibodies nor non-specific isotype control antibodies had any effect on the interaction (Table 1). Fucoidan also inhibited binding (results not shown).

Table 1 Inhibition of binding of L-selectin-ZZ to LPS by anti-selectin antibodies

L-Selectin-ZZ, in the presence or absence of various antibodies as shown, was added to LPS-coated microtitre plate wells. Binding was determined by measurement of A_{490} (arbitrary units), and results are means ± S.D. of 2 experiments. Values in parentheses indicate the percentage inhibition of binding. Binding of L-selectin-ZZ to LPS was inhibited by the anti-L-selectin antibodies LAM1-3 and MCA 1076, but not by the anti-E-selectin antibodies BBA-2 and MCA 883, or by the isotype-matched control antibody MCA 928.

Antibody	Binding of L-selectin-ZZ to LPS (units)
None	0.615 ± 0.017 (0%)
MCA 1076 (anti-L-selectin antibody)	0.342 ± 0.025 (45%)
LAM1-3 (anti-L-selectin antibody)	0.099 ± 0.055 (84%)
BBA-2 (anti-E-selectin antibody)	0.765 ± 0.068 (0%)
MCA 883 (anti-E-selectin antibody)	0.593 ± 0.003 (4%)
MCA 929 (non-specific antibody)	0.702 ± 0.085 (0%)

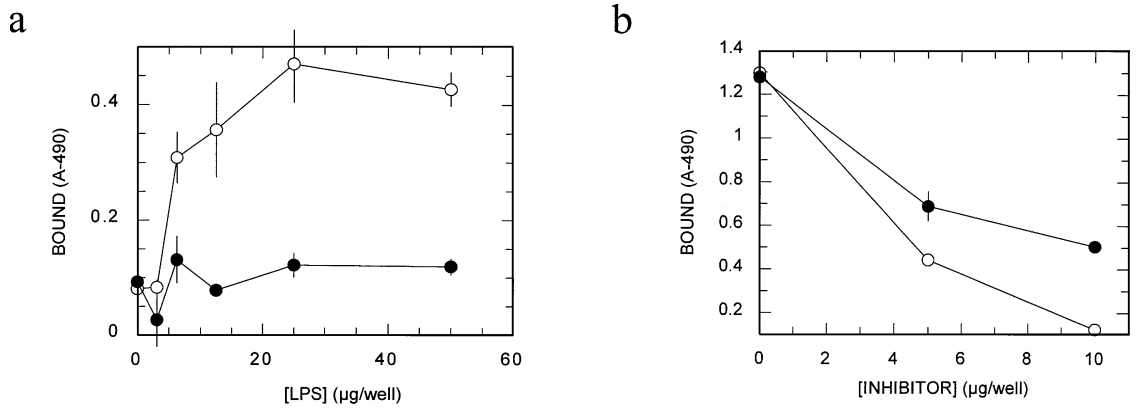

Figure 1 Binding of L-selectin to LPS

(**a**) A constant amount of L-selectin-ZZ (○) or E-selectin-ZZ (●) in Tris/HCl (pH 7.4) containining 150 mM NaCl, HRP-conjugated IgG and 5 mM $CaCl_2$ or 5 mM EDTA was added to microtitre plate wells coated with serial dilutions of LPS (200 μl total volume; maximum concentration of LPS 250 μg/ml). Binding of L-selectin-ZZ, but not E-selectin-ZZ, to LPS was observed. Similar binding of L-selectin was observed in the presence of 5 mM $CaCl_2$ or 5 mM EDTA. (**b**) L-Selectin in the presence or absence of monophosphorylated (○) or diphosphorylated (●) lipid A was added to LPS-coated wells. Both forms of lipid A inhibited the binding of L-selectin to LPS. Binding was determined by measurement of A_{490}.

Table 2 Binding of L-selectin-ZZ to LPS from different strains of bacteria

A constant amount of L-selectin-ZZ or E-selectin-ZZ was added to microtitre plate wells coated with LPS derived from different strains of bacteria. Binding was determined by measurement of A_{490} (arbitrary units), and results are means ± S.D. of two experiments. Binding of L-selectin-ZZ, but not of E-selectin-ZZ, was observed.

Strain of bacteria	Binding to LPS (units)	
	L-Selectin-ZZ	E-Selectin-ZZ
Serratia marcescens	0.391 ± 0.026	0.068 ± 0.003
Klebsiella pneumoniae	0.253 ± 0.022	0.009 ± 0.004
Escherichia coli K-235	0.104 ± 0.008	0.009 ± 0.006
Escherichia coli 0111:B4	0.313 ± 0.009	0.015 ± 0.001
Pseudomonas aeruginosa 10 (Habs)	0.326 ± 0.050	0.015 ± 0.002
Escherichia coli 055:B5	0.323 ± 0.015	0.045 ± 0.002
Shigella flexneri 1A	0.084 ± 0.009	0.020 ± 0.003
Escherichia coli 0127:B8	0.109 ± 0.018	0.042 ± 0.001
Escherichia coli 026:B6	0.190 ± 0.026	0.040 ± 0.013
Vibro cholerae inaba 569 B	0.322 ± 0.036	0.073 ± 0.011
Salmonella typhimurium	0.016 ± 0.010	0.062 ± 0.013
Salmonella typhosa	0.217 ± 0.028	0.060 ± 0.016
Escherichia coli 0128:B12	0.144 ± 0.030	0.070 ± 0.011
Salmonella abortus equi	0.107 ± 0.024	0.046 ± 0.012
Salmonella enteritidis	0.176 ± 0.018	0.076 ± 0.013

We then investigated whether L-selectin was able to bind LPS derived from a variety of bacterial strains. Table 2 shows that 14 types of LPS bound L-selectin-ZZ but not E-selectin-ZZ. The only exception was the LPS from *Salmonella typhomurium*, which did not bind L-selectin-ZZ. The differences in the degree of binding of L-selectin-ZZ to LPS from different strains of bacteria could be due to differences in the polysaccharide present on the lipid A backbone, or could simply reflect differences in the solubility and efficiency of coating of LPS on to microtitre plates. The lipid A core of LPS, unlike the polysaccharide moiety, is highly conserved among diverse bacterial species. To test whether the differences in binding were due to differences in the polysaccharide chains associated with lipid A, the binding of L-selectin-ZZ to LPS from *E. coli* 055:B5 (a bacterial strain showing higher affinity for L-selectin-ZZ) in the presence of lipid A was investigated. Figure 1(b) shows that both the mono- and di-phosphophorylated forms of lipid A inhibited the binding of L-selectin to LPS, indicating that the lipid A group in LPS is required for binding to L-selectin-ZZ. These results indicate that the differential binding of L-selectin-ZZ to LPS from different strains is due to differences in the solubility and coating of LPS.

Binding of L-selectin to lipotechoic acid

It has been reported that monophosphorylated lipid A acts as a prophylactic for sepsis and septic shock induced by Gram-negative [11,12] and Gram-positive [13] bacteria. With Gram-positive bacteria, release of lipotechoic acid from the bacterial cell membrane is associated with septic shock. Figure 2 shows that L-selectin bound to lipotechoic acid (Figure 2a) and that the binding of L-selectin to LPS was inhibited by lipotechoic acid (Figure 2b).

Interaction of FITC-labelled LPS with neutrophils

To establish whether L-selectin is a specific receptor for LPS on L-selectin-expressing cells, we investigated the binding of FITC-labelled LPS to human neutrophils. FACS analysis of freshly isolated neutrophils indicated the expression of high levels of L-selectin and low levels of CD14 (Figure 3a). In the absence of serum, these cells bound FITC-LPS in a concentration-dependent manner (Figure 3b). This binding was completely blocked by co-incubation of neutrophils with FITC-labelled or unlabelled LPS (results not shown). Furthermore, the binding of FITC-LPS to neutrophils was inhibited by the anti-L-selectin antibodies LAM1-3 and Dreg 56, but not by isotype-matched control antibodies (Figure 3c). These results indicate that L-selectin is the major binding site for LPS on neutrophils in the absence of

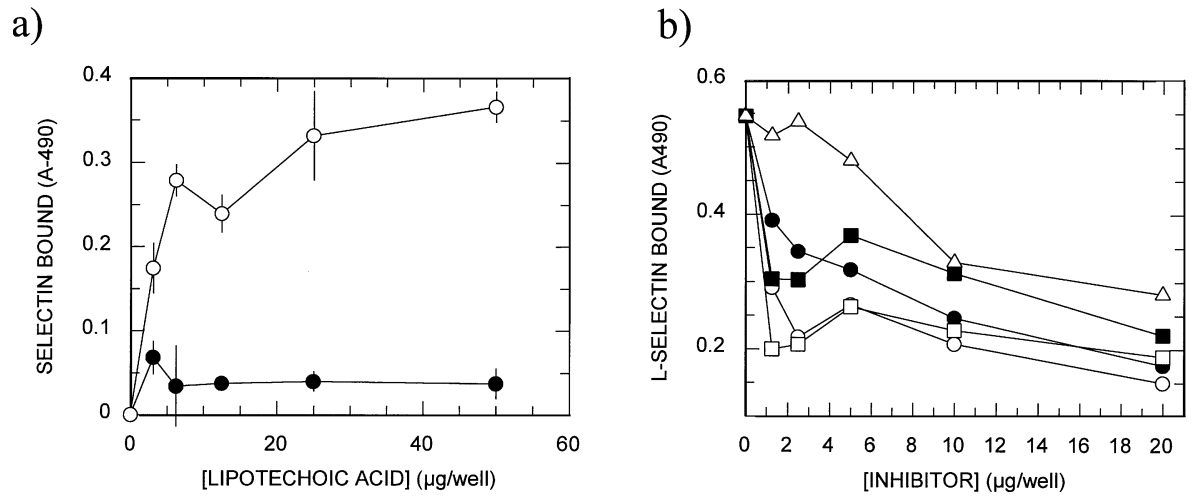

Figure 2 Binding of selectins to lipotechoic acid

(**a**) A constant amount of L-selectin-ZZ (○) or E-selectin-ZZ (●) in Tris/HCl (pH 7.4) containining 150 mM NaCl, HRP-conjugated IgG and 5 mM $CaCl_2$ or 5 mM EDTA was added to microtitre plate wells coated with serial dilutions of lipotechoic acid from *Streptococcus pyogens* (200 μl total volume; maximum concentration of lipotechoic acid 250 μg/ml). Binding of L-selectin-ZZ, but not E-selectin-ZZ, to lipotechoic acid was observed. (**b**) L-Selectin-ZZ binding to LPS-coated microtitre plate wells was determined in the presence or absence of serial dilutions of lipotechoic acid from *Streptococcus pyogens* (○), *Streptococcus faccalis* (●), *Streptococcus mutans* (□) or *Streptococcus sangius* (■), or of LPS (△). Binding of L-selectin-ZZ to LPS was inhibited by all lipotechoic acids and by soluble LPS. Binding was determined by measurement of A_{490}.

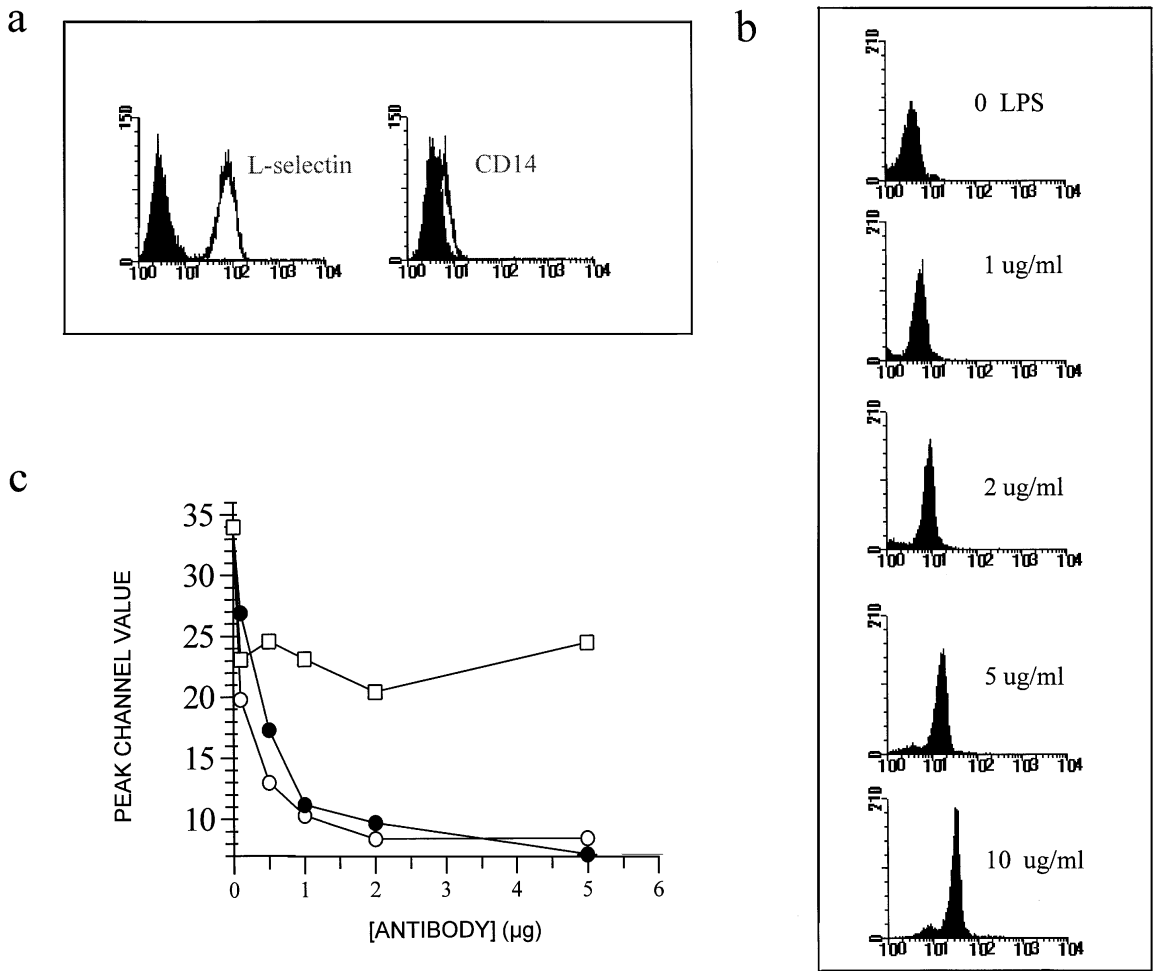

Figure 3 Binding of FITC-labelled LPS to neutrophils

(**a**) Freshly isolated human neutrophils were incubated with either an anti-L-selectin antibody or an anti-CD14 antibody on ice for 30 min, followed by the addition of FITC-conjugated anti-mouse IgG. Expression of L-selectin or CD14 (unshaded curve) compared with the control (shaded curve) is shown. (**b**) Isolated neutrophils were incubated with serial dilutions of FITC-conjugated LPS and the intensity of fluoresence associated with the cells was measured. An increase in fluoresence intensity with increasing concentrations of FITC-labelled LPS (μg/ml) was observed. (**c**) FITC-labelled LPS was incubated with neutrophils in the presence of the anti-L-selectin antibodies LAM1-3 (○) or Dreg 56 (●), or of an isotype-matched control antibody (□). Anti-L-selectin antibodies showed concentration-dependent inhibition of FITC-LPS binding to neutrophils, whereas the control antibody resulted in partial inhibition which was not concentration-dependent.

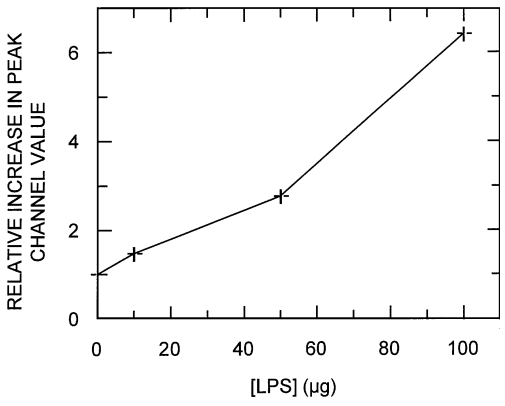

Figure 4 LPS-induced superoxide production in neutrophils

Isolated neutrophils, loaded with 1,2,3-dihydrorhodamine, were incubated with serial dilutions of LPS. The amount of superoxide produced was measured as the peak channel value on a Becton–Dickinson FACScan instrument.

serum, and are consistent with published data showing that anti-CD14 antibodies do not block the binding of FITC-labelled LPS to neutrophils [6].

LPS-induced superoxide production

Recent evidence has shown that neutrophil cell-surface L-selectin is capable of signal transduction, resulting in Ca^{2+} mobilization, increased tyrosine phosphorylation, activation of mitogen-activated protein kinase (MAP kinase) activity and production of superoxide radicals [14]. These responses were elicited by monovalent Fab fragments of anti-L-selectin antibodies as well as by sulphatides. Since similar changes are observed in leucocytes treated with LPS [6,15], we hypothesized that L-selectin may be the signalling receptor responsible for neutrophil activation by LPS. As expected, superoxide production in neutrophils was stimulated in a concentration-dependent manner by LPS (Figure 4). This stimulation of superoxide production was inhibited by the anti-L-selectin antibodies LAM1-3 and TQ1, whereas an

Table 3 Inhibition of LPS-induced superoxide production in neutrophils by anti-L-selectin antibodies

LPS (100 μg/ml) was incubated with neutrophils loaded with 1,2,3-dihydrorhodamine in the presence or absence of anti-L-selectin antibodies (LAM1-3 or TQ1) or an anti-CD31 antibody (same isotype as the anti-L-selectin antibodies). Results are peak channel values (arbitrary units), and are means $\pm$ S.D. of two experiments. Values in parentheses show percentage inhibition of superoxide production. Superoxide production in response to 100 μg/ml LPS was inhibited by 50–63% in the presence of the anti-L-selectin antibodies. No significant effect was observed in the presence of anti-CD31 antibody. Anti-L-selectin antibodies resulted in partial inhibition when 50 μg/ml LPS was used.

Antibody	Superoxide production (units)
None	10.75 $\pm$ 0.9 (0%)
Anti-CD31 antibody	8.29 $\pm$ 0.9 (20%)
LAM1-3 (anti-L-selectin antibody)	4.04 $\pm$ 0.4 (63%)
TQ1 (anti-L-selectin antibody)	5.23 $\pm$ 0.7 (51%)

anti-CD31 antibody, which is of the same isotype as the anti-L-selectin antibodies, was not inhibitory (Table 3).

DISCUSSION

We have shown that L-selectin can bind both LPS and lipotechoic acid, as well as transducing signals resulting in neutrophil activation. Previous workers have proposed that as-yet-unidentified proteins are required to explain the signal-tranducing properties of LPS. For example, although CD14, a glycosyl-phosphatidylinositol-anchored cell-surface protein, has been shown to bind LPS in the presence of serum LBP [15], there is considerable evidence that CD14-independent LPS signalling pathways exist in leucocytes [4,7]. The LPS antagonists lipid IVA and *Rhodobacter sphaeroides* lipid A inhibit LPS-induced cellular signalling by interacting with cell-surface protein(s) distinct from CD14 [7]. Furthermore, it has been shown *in vitro* that high concentrations of LPS can stimulate monocytes and neutrophils to produce cytokines without the involvement of CD14/LBP. Depletion of serum LBP by anti-LBP antibodies had little effect on the survival rate of mice injected with high doses of LPS [6,16,17]. It is possible that the optimal binding of LPS and signalling requires co-operativity between CD14 and a separate receptor which also binds lipid A. We propose that L-selectin fulfils these functions in neutrophils.

The role of L-selectin in the 'tethering' and 'rolling' of leucocytes on the endothelium is well documented. These interactions facilitate subsequent extravasation and accumulation of leucocytes in tissues [8]. While the pathogenesis of septic shock is multifactorial, the syndrome is characterized by the tissue accumulation and activation of leucocytes. The observation that L-selectin-deficient mice are resistant to high doses of LPS may now be explained in two ways: (i) an impairment of L-selectin-mediated adhesion to the endothelium, and (ii) loss of L-selectin-mediated activation of neutrophils by LPS. These findings contribute to our understanding of the pathological mechanisms of septic shock, and suggest that inhibitors of L-selectin may be of therapeutic value in treating this life-threatening condition.

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