Characteristics of lipopolysaccharide interaction with human peripheral-blood monocytes

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The interaction between radioiodinated lipopolysaccharide from *Escherichia coli* 0111:B4 (^{125}I -LPS) and human peripheral-blood monocytes was studied. The association of ^{125}I -LPS with monocytes at 37 °C appeared to depend on binding to the cell membrane with subsequent internalization of the molecule, and was not saturable with time (up to 2 h) or ^{125}I -LPS concentration (up to 10 $\mu\text{g}/\text{ml}$). There was no apparent difference in the behaviour of unlabelled LPS and ^{125}I -LPS with respect to monocyte association. ^{125}I -LPS association with monocytes was inhibited by LPS and O-polysaccharide from *E. coli* 0111:B4 and *Salmonella typhi* 0901, but not by lipid A or polymyxin B. We propose that the mechanism of human monocyte stimulation by LPS involves polysaccharide-dependent binding to the cell membrane followed by internalization of the LPS molecule. We were unable to demonstrate a specific LPS receptor such as that found on murine B-lymphocytes.

INTRODUCTION

Lipopolysaccharide(s) (LPS) from Gram-negative bacterial cell walls exert protean effects on mammalian physiological systems (reviewed by Morrison & Ulevitch, 1978). Several of these effects, such as fever and the acute-phase response, are mediated by LPS-induced release of interleukin 1 (IL-1) from mononuclear phagocytes (Dinarello, 1984), but the mechanism of this process remains unclear. Structurally, LPS isolated from smooth organisms consist of three regions: lipid A, core oligosaccharide and O-polysaccharide (Lüderitz *et al.*, 1984). The biological effects of LPS are reproduced by free lipid A, whereas the O-polysaccharide is immunogenic, but not toxic (Lüderitz *et al.*, 1966). LPS interaction with many mammalian cell types (Morrison & Ulevitch, 1978) has been described, and although a specific LPS receptor on the plasma membrane of resident and elicited rabbit peritoneal macrophages has been described (Haeflner-Cavaillon *et al.*, 1982), there is little consensus on the nature of the interaction of LPS with its target cells. The work reported here represents an attempt to clarify the initial events in the process of monocyte activation by LPS.

MATERIALS AND METHODS

Materials

Phenol-extracted LPS from *Escherichia coli* 0111:B4 was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pronase (EC 3.4.24.4), RNAase (EC 3.1.27.5) and DNAase (EC 3.1.21.1) were supplied by Boehringer (Mannheim, Germany). *Salmonella typhi* 0901 LPS and polymyxin B were from Difco (Detroit, MI, U.S.A.). RPMI 1640 medium was supplied as a powder (Gibco, Grand Island, NY, U.S.A.) and made up in sterile,

pyrogen-free water (Baxter, Johannesburg, South Africa). HBSS and phosphate-buffered saline were from Flow Laboratories (Irvine, Ayrshire, Scotland). Ficoll-Paque and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). Four-well Multi-dishes were supplied by Nunc (Roskilde, Denmark). All other reagents were of analytical grade.

LPS purification and labelling

E. coli 0111:B4 LPS was purified with RNAase and Pronase (Morrison & Leive, 1975) and ^{125}I -labelled with the Bolton–Hunter reagent by a modification of the procedure of Ulevitch (1978). The label was covalently bound to the LPS as determined by gel filtration in the presence of 6 M-guanidinium chloride and polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate and 4 M-urea. The pyrogenicity of the labelled LPS in rabbits was unaffected by the labelling procedure (S. J. C. Warner, unpublished work). The radioiodinated LPS will be referred to throughout as ' ^{125}I -LPS'.

LPS fragments

LPS from *E. coli* 0111:B4 or *S. typhi* 0901 was hydrolysed in 0.1 M-acetic acid at 100 °C for 2.5 h. The lipid A was removed by centrifugation and further purified by chloroform/EDTA extraction (Dancey *et al.*, 1977). O-polysaccharide was purified from the supernatant of the acid-hydrolysis reaction by chromatography on Sephadex G-50 (30 cm $\times$ 2.5 cm) eluted with pyridine/acetic acid/water (2:1:199, by vol.) (Jann *et al.*, 1975). The freeze-dried O-polysaccharide was dissolved at 10 mg/ml in sterile pyrogen-free saline (0.9% NaCl), sterile-filtered (Millex-GV filter, 0.22 μm pore size; Millipore, Bedford, MA, U.S.A.) and stored at 4 °C. Lipid A was solubilized at 1 mg/ml in sterile pyrogen-free saline by the addition of 2 μl of triethylamine/ml and stored at 4 °C.

Abbreviations used: LPS, lipopolysaccharide(s); ^{125}I -LPS, radioiodinated lipopolysaccharide from *Escherichia coli* 0111:B4; IL-1, interleukin 1; DNAase, deoxyribonuclease; RNAase, ribonuclease; HBSS, Hanks balanced salt solution; Dulbecco's phosphate-buffered saline, 137 mM-NaCl/8 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 /2.7 mM-KCl; MNL, mononuclear leucocytes; SDS, sodium dodecyl sulphate.

Peripheral-blood monocyte isolation

Venous blood was obtained with informed consent from healthy donors and treated with the anticoagulant heparin (10 units/ml final concn.). Mononuclear leucocytes (MNL) were isolated by centrifugation (400 g, 20 min, 20 °C) on Ficoll-Paque (Böyum, 1968) and were washed three times by centrifugation in ice-cold phosphate-buffered saline (150 g, 10 min, 4 °C) to decrease platelet contamination (Kragballe *et al.*, 1980). The cells were suspended in RPMI 1640 medium containing 20 mM-Hepes buffer, pH 7.3, 2 mM-glutamine and antibiotics (100 units of penicillin/ml and 100 µg of streptomycin/ml). The cell concentration, determined in a haemocytometer, was adjusted to 7.5×10^6 MNL/ml, and 2.5×10^6 MNL were plated into each well of Nunc four-well Multi-dishes. After incubation for 90 min at 37 °C in an humidified atmosphere of 5% CO₂/air (1:19) the non-adherent cells were removed by shaking the dishes, aspirating the medium and washing twice with 1.5 ml of warm (37 °C) HBSS. The viability of the monocyte monolayers thus obtained was determined by the fluorescein diacetate/Acridine Orange method (De Mulder *et al.*, 1981). Monolayer purity was assessed by ingestion of latex beads (0.91 µm diameter; Sigma) and non-specific esterase staining (Yam *et al.*, 1971).

Determination of monolayer-associated LPS

RPMI 1640 (0.3 ml) containing from 0.1 to 10 µg of ¹²⁵I-LPS/ml was added to wells containing monocyte monolayers. The cells were then incubated at 37 °C in an atmosphere of CO₂/air (1:19) for various time periods. Incubation was terminated in less than 5 s by aspiration of the medium and rapid washing with 1 ml of ice-cold 0.15 M-NaCl. Monolayers were solubilized by overnight incubation at room temperature with 0.5 ml of 1 M-NaOH. The cell digest was counted for radioactivity in a Packard Auto Gamma counter at 60% efficiency.

Quantification of LPS association with monolayers

The amount of cell-associated ¹²⁵I-LPS was calculated from the specific radioactivity. The c.p.m. bound at a given time were corrected for the c.p.m. determined after the addition and immediate removal of ¹²⁵I-LPS. Incubations of ¹²⁵I-LPS with cells were for 60 min at 37 °C, except where otherwise stated. All experiments were performed on quadruplicate wells, and each experiment was repeated with cells from at least three donors. Results are given as means $\pm$ S.E.M.

SDS/polyacrylamide-gel electrophoresis

Monocyte monolayers were incubated with 10 µg of ¹²⁵I-LPS/ml at 37 °C for 60 min and washed with 0.15 M-NaCl. Portions (20 µl) of the supernatant and control ¹²⁵I-LPS that had been incubated without cells were each added to a well containing a monocyte monolayer. This procedure was followed to rule out any possible effect of cellular proteins on the electrophoresis of the ¹²⁵I-LPS. The contents of each well were prepared for electrophoresis by nuclease digestion of monolayers, the protocol of O'Farrell (1975) being used. A 0.25 ml portion of RNAase (50 µg/ml) was added to the cells. After three freeze-thaw cycles, DNAase was added (final concn. 50 µg/ml), and the solution was kept on ice for 15 min. The contents of each well were freeze-dried and heated at 100 °C for 5 min in SDS sample buffer.

Electrophoresis was performed on 14% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and 4 M-urea (Tsai & Frasch, 1982). The gels were dried and autoradiographed on Kodak X-Omat AR film at -70 °C.

RESULTS

Monocyte purity and viability

The monolayers contained more than 95% viable cells, at least 85% of which stained positively for non-specific esterase and were capable of latex-bead phagocytosis.

Dependence of ¹²⁵I-LPS-monocyte interaction on endocytosis

The association of ¹²⁵I-LPS (0.1 µg/ml) with monocyte monolayers was determined at 4 °C in the presence of 0.1% (w/v) NaN₃ and 20 mM-NaF, conditions which prevent endocytosis (Gregory *et al.*, 1980). The amount of cell-associated ¹²⁵I-LPS after 60 min of incubation was decreased to $48 \pm 4.9\%$ ($n = 3$) of control values determined at 37 °C in the absence of inhibitors. We conclude that, at 37 °C, the LPS is internalized by the monocytes, an ATP-dependent pathway being utilized. Thus the amount of LPS detected on the monocytes at 37 °C represents both internalized and plasma-membrane-bound ligand. We have therefore chosen to use the term 'cell-associated' to describe this LPS, as neither of the terms 'binding' or 'uptake' is solely applicable to the process of LPS interaction with monocytes.

Time-dependence of ¹²⁵I-LPS association with monocytes (Fig. 1)

The association of ¹²⁵I-LPS (0.1 µg/ml) with the monolayers increased rapidly during the first 15 min of incubation and continued to increase, although at a slower rate, up to 2 h after initiation of uptake. In one experiment the association process was continuing 4 h after initiation (results not shown). Thus the uptake of ¹²⁵I-LPS by the cells was not saturable with time.

Fig. 1. Time-dependence of ¹²⁵I-LPS-monocyte association

Wells containing monocyte monolayers were incubated with 0.1 µg of ¹²⁵I-LPS/ml at 37 °C. The amount of cell-associated material at the time points indicated was determined as described in the Materials and methods section. Bars represent S.E.M. ($n = 4$).

Fig. 2. Concentration-dependence of ^{125}I -LPS and unlabelled LPS association with monocytes

Monocyte monolayers were incubated with ^{125}I -LPS (●), or with unlabelled LPS (○) containing $0.1\ \mu\text{g}$ of ^{125}I -LPS/ml, for 60 min at 37°C . The amount of cell-associated LPS was determined from the specific radioactivity and is given as ng/well. The S.E.M. is encompassed by the symbol ($n = 4$).

Concentration-dependence of ^{125}I -LPS-monocyte association

The association of ^{125}I -LPS with monocytes was not saturable with increasing concentration over the range 0.1 – $10\ \mu\text{g}/\text{ml}$ and, irrespective of LPS concentration, approx. 1% of the LPS added to the cells was taken up after 60 min at 37°C (Fig. 2). Cells were incubated with $0.1\ \mu\text{g}$ of ^{125}I -LPS/ml, together with unlabelled LPS to bring the total LPS concentration to between $0.2\ \mu\text{g}/\text{ml}$ and $20\ \mu\text{g}/\text{ml}$. The amount of cell-associated LPS was calculated from the resultant specific activity and net c.p.m. associated with the cells. There was no significant difference between the amount of cell-associated LPS determined by this method and that measured in the presence of equivalent concentrations of ^{125}I -LPS alone. Experimental points determined by both methods fall on a single line when plotted on a graph of LPS concentration versus the amount of cell-associated LPS (Fig. 2). There is thus no apparent difference in behaviour between unlabelled LPS and ^{125}I -LPS with respect to association with monocytes.

Effect of LPS, LPS-derived fragments and polymyxin B on ^{125}I -LPS-monocyte association (Table 1)

The presence of unlabelled LPS ($10\ \mu\text{g}/\text{ml}$) or O-polysaccharide ($100\ \mu\text{g}/\text{ml}$) from *E. coli* 0111:B4 or *S. typhi* 0901 decreased the amount of cell-associated ^{125}I -LPS compared with control incubations without these substances. Lipid A ($10\ \mu\text{g}/\text{ml}$) had no significant effect on the amount of cell-associated ^{125}I -LPS. Pretreatment of ^{125}I -LPS ($0.1\ \mu\text{g}/\text{ml}$) with polymyxin B ($10\ \mu\text{g}/\text{ml}$), a cationic polypeptide antibiotic which binds lipid A with high affinity (Morrison & Jacobs, 1976), did not affect the ability of ^{125}I -LPS to associate with monocytes. The concentration-dependence of the effects of unlabelled *E. coli* LPS and O-polysaccharide is shown in Figs. 3 and 4. Preincubation of monocytes with unlabelled LPS ($10\ \mu\text{g}/\text{ml}$), O-polysaccharide ($100\ \mu\text{g}/\text{ml}$) or lipid A ($10\ \mu\text{g}/\text{ml}$), followed by washing of the cells and replacement with medium containing $0.1\ \mu\text{g}$ of ^{125}I -LPS/ml alone, had no effect on ^{125}I -LPS cell association compared with control cells pre-incubated in medium alone (results not shown).

SDS/polyacrylamide-gel electrophoresis of monocyte-associated ^{125}I -LPS

The electrophoretic patterns obtained were in accordance with those found by Tsai & Frasch (1982). There were no detectable differences between the ^{125}I -LPS associated with monocytes, that remaining in the incubation supernatant, and control material incubated at 37°C without cells.

DISCUSSION

The results presented imply that the interaction of LPS with human monocytes is partly dependent on cellular energy production, and is not saturable with time up to 2 h, nor with LPS concentration over the range 0.1 – $10\ \mu\text{g}/\text{ml}$. Homologous and heterologous LPS and O-polysaccharide, but not lipid A or polymyxin B, inhibit the association of LPS with monocytes. These findings suggest that the interaction of LPS with the monocyte membrane is largely determined by the O-polysaccharide moiety of the molecule. Other data provide support for this hypothesis. The rate of phagocytosis of *Salmonella* organisms by a murine macrophage-like cell line is dependent on the O-polysaccharide structure of the LPS (Liang-Takasaki *et al.*, 1983), and mannose residues are

Table 1. Effect of competitors on ^{125}I -LPS association with human monocytes

Cell monolayers were incubated for 60 min at 37°C with $0.1\ \mu\text{g}$ of ^{125}I -LPS/ml in the presence of the competing substances tabled below. Cell-associated ^{125}I -LPS was determined as described in the Materials and methods section. Results are given as means $\pm$ S.E.M. for the numbers of determinations shown in parentheses. Statistical significance of differences between experimental groups and controls incubated in the absence of competitors: *, not significant ($P > 0.15$); †, $P < 0.0025$ against control.

Competitor	Cell-associated ^{125}I -LPS (% of control)
Lipid A ($10\ \mu\text{g}/\text{ml}$)	91.0 ± 7.3 (4)*
<i>E. coli</i> LPS ($10\ \mu\text{g}/\text{ml}$)	67.6 ± 4.9 (5)†
<i>E. coli</i> O-polysaccharide ($100\ \mu\text{g}/\text{ml}$)	69.6 ± 4.7 (5)†
<i>S. typhi</i> LPS ($10\ \mu\text{g}/\text{ml}$)	60.7 ± 0.7 (3)†
<i>S. typhi</i> O-polysaccharide ($100\ \mu\text{g}/\text{ml}$)	64.7 ± 1.3 (3)†
Polymyxin B ($10\ \mu\text{g}/\text{ml}$)	105.0 ± 5.6 (6)*

Fig. 3. Effect of unlabelled *E. coli* LPS on ^{125}I -LPS association with monocytes

Monolayers were incubated with $0.1 \mu\text{g}$ of ^{125}I -LPS/ml for 60 min at 37°C in the presence of various concentrations of unlabelled *E. coli* LPS. Data represent the percentage of cell-associated radioactivity in the absence of unlabelled LPS. Bars represent S.E.M. ($n = 3$).

Fig. 4. Effect of unlabelled *E. coli* O-polysaccharide on ^{125}I -LPS association with monocytes

Monolayers were incubated for 60 min at 37°C with various concentrations of *E. coli* O-polysaccharide. Data represent the percentage of cell-associated radioactivity determined in the absence of O-polysaccharide. Bars represent S.E.M. ($n = 3$).

important in the binding of *Haemophilus influenzae* LPS to murine peritoneal macrophages (Raichvarg *et al.*, 1982). A lectin-like LPS receptor has been demonstrated on the membrane of rabbit peritoneal macrophages (Haefner-Cavaillon *et al.*, 1982), and binding of tritiated *Bordetella pertussis* LPS to this receptor was inhibited by unlabelled LPS and O-polysaccharide, as well as by LPS from heterologous organisms.

By contrast, several investigators have suggested that hydrophobic interactions between lipid A and membrane lipids determine the interaction of LPS with cell membranes (Hammerling & Westphal, 1967; Ciznar & Shands, 1971; Watson & Riblet, 1975; Kabir & Rosenstreich, 1977). Ionic interactions between LPS and leucocytes have also been proposed (Gimber & Rafter, 1969). Davies *et al.* (1978) described cyclical binding of *E. coli* LPS to erythrocytes from several mammalian species, including man, as well as to human mononuclear leucocytes, and murine peritoneal macrophages and lymphocytes. Regular cycles of adsorption and desorption

were noted in all cases. A similar phenomenon was described in the binding of *E. coli* LPS to porcine lymphocytes (Symons & Clarkson, 1979). Davies *et al.* (1978) proposed a model of LPS binding to cell membranes in which their observations were explained in terms of LPS subpopulations with various lipid and polysaccharide compositions. The molecular heterogeneity of LPS is well established (Goldman & Leive, 1980), and it was subsequently shown that both murine macrophages (Jacobs *et al.*, 1983) and spleen cells (Goodman *et al.*, 1984) selectively bind lipid A-rich subunits in LPS aggregates. It was reported (Davies *et al.*, 1978; Davies & Stewart-Tull, 1981) that macrophages took up 10-fold more LPS than non-phagocytic cells, and it was suggested that this might be due to internalization of the LPS. Our data provide further support for this hypothesis, in that inhibition of endocytosis decreased the amount of cell-associated LPS. Bona (1973) has provided direct electron-microscopic evidence for the endocytosis of LPS by guinea-pig macrophages. We found no evidence for a specific, saturable LPS receptor such as has been proposed to exist on the surface of murine lymphocytes (Morrison, 1983; Mergenhagen & Pluznik, 1984), or rabbit peritoneal macrophages (Haefner-Cavaillon *et al.*, 1982). This may reflect a genuine difference between human monocytes and these cells, or alternatively may be a reflection of the immaturity of the monocyte as a macrophage progenitor.

Several factors may influence the outcome of investigations into the interaction of LPS with cell membranes. The physico-chemical properties of LPS depend on such variables as the bacterial culture conditions, extraction method, and bivalent-cation content (Galanos & Lüderitz, 1975), and LPS may be contaminated with protein and RNA, depending on the extraction procedure and subsequent purification (Lüderitz *et al.*, 1966). The molecular-size heterogeneity of LPS aggregates precludes the determination of classical binding constants in analysing the interaction of LPS with putative receptors. Larsen & Sullivan (1984) have, erroneously in our opinion, assumed an arbitrary M_r of 1000000 in the determination of binding constants for the interaction of ^3H -labelled *S. typhi* LPS with human monocytes. These authors also ignored the possibility of active uptake of LPS by the phagocytes, a phenomenon that would confound conventional analysis of ligand-receptor interactions. Rapid separation of cell-associated and free LPS is essential in order to avoid underestimation of the amount of the former, as the dissociation half-time of LPS from rabbit peritoneal macrophages was determined to be 45 s (Haefner-Cavaillon *et al.*, 1982). Our studies have utilized a well-characterized, highly purified ^{125}I -labelled LPS preparation whose specific activity permits the detection of less than 1 ng of cell-bound LPS. The behaviour of this LPS with regard to monocyte association appears to be no different from that of unlabelled LPS. Incubations were terminated in less than 5 s by aspiration of the medium and washing with cold saline and are thus likely to reflect accurately the amount of cell-associated LPS. On the basis of our results, we propose that LPS initially binds via a carbohydrate-dependent interaction to the monocyte surface and is then internalized. We have no evidence for a specific saturable LPS receptor, as has been described on the surface of murine lymphocytes or rabbit peritoneal macrophages (Coutinho *et al.*, 1978; Haefner-Cavaillon *et al.*, 1982).

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