Introduction

The host response to lipopolysaccharide (LPS) is crucial in the defence against Gram-negative bacterial infection. Cells of the myeloid lineage are capable of recognizing picomolar quantities of LPS and respond, via several signal transduction cascades, with the release of a wide range of pro-inflammatory cytokines (Rietschel et al., 1994). This response is mediated by recognition of the lipid A component of LPS and involves shuttling of LPS monomers from micelles or bacterial membranes by the proteins LPS-binding protein and soluble CD14 to the cellular receptors for LPS (Pugin et al., 1993). Enterobacterial LPS is recognized by a signalizing complex comprising at least CD14, Toll-like receptor (TLR)-4 and the co-receptor MD-2 (Poltorak et al., 2000). However, recent reports indicate that the LPS of the non-enterobacterial organisms Porphyromonas gingivalis, Leptospira interrogans, Rhizobium sp. Sin-1 and Legionella pneumophila are capable of signalizing independently of TLR4, instead utilizing TLR2-mediated signal transduction (Hirschfeld et al., 2001; Werts et al., 2001; Girard et al., 2003).

To investigate further how widely TLR2- and TLR4-signal- ling LPS are represented among Gram-negative species, we assembled a panel of LPS representing diverse lipid A structures, including the LPS of Escherichia coli, Yersinia pestis, Porphyromonas gingivalis, Chlamydia trachomatis, Pseudomonas aeruginosa and Bacteroides fragilis, and examined the capacity of each to activate cells via the TLR2 or TLR4/MD-2 receptor complexes.

Methods

Preparation of LPS. The LPS of E. coli R1 (=NCTC-13114), B. fragilis NCTC-9343, Pseudomonas aeruginosa PAC-611, C. trachomatis LGV-1 and Porphyromonas gingivalis MPRL-1675 were reconstituted from frozen laboratory stocks previously prepared using either the phenol/chloroform/petroleum (E. coli, B. fragilis, Pseudomonas aeruginosa) or phenol/water method (Porphyromonas gingivalis, C. trachomatis) as described previously (Hancock & Poxton, 1988). The LPS of Y. pestis was a kind gift of P. Oyston (DSTL, Porton Down, UK). All LPS samples (except that of C. trachomatis, of which only 8 µg was available) were repurified to remove protein and lipoprotein contamination according to the method of Hirschfeld et al. (2001). Briefly, LPS samples were adjusted to 0.2 % triethylamine and 0.5 % deoxycholate and subjected to two rounds of phenol/water re-extraction. Aqueous phases were then pooled and adjusted to 75 % ethanol and 30 mM sodium acetate to allow precipitation at –20 °C for 1 h. The LPS was harvested by centrifugation (10 min at 10 000 g), washed in 1 ml cold 100 % ethanol, air dried and resuspended in the original volume of 0.2 % triethylamine. Recovery of LPS was assumed to be 100 %.

HeLa cell transfections and interleukin (IL) 8 promoter assays. LPS signalling via TLR2 or TLR4/MD-2 was assessed using an IL8 promoter assay as previously described (Pridmore et al., 2001). Briefly, HeLa cells were transiently transfected (Superfect; Qiagen) with the luciferase reporter plasmids pIL8-pLUC and pRL-TK (Promega), a CD14 expression plasmid and expression plasmids for human TLR2, human MD-2 or empty vector control. At 24 h following transfection,
cells were challenged with 100 ng LPS ml\(^{-1}\) for a further 6 h. The cells were then lysed and luciferase reporter levels measured using the Dual-Luciferase system (Promega). Data were analysed using Student’s \(t\)-test.

**Challenge of monocytes with LPS and measurement of tumour necrosis factor (TNF-\(\alpha\)).** Peripheral blood from three healthy human volunteers was diluted 1:3 in PBS, layered onto Histopaque-1077 (Sigma) and centrifuged at 400 g for 30 min and the interface cells were removed. These cells were then washed twice in PBS, plated at a concentration of 2 \(\times\) 10\(^5\) monocytes per well in a 96-well plate and washed 1:5 h later to remove non-adherent cells. Tenfold serial dilutions (100 \(\mu\)l) of each LPS in RPMI/10 % human serum (Sigma) were then added to monocytes in duplicate. Four hours later, supernatants were collected and assayed for TNF-\(\alpha\) content using the L929 bioassay (Delahooke et al., 1995). Data were normalized to the percentage of maximum TNF-\(\alpha\) released from each individual due to variability in maximum TNF-\(\alpha\) output between the three volunteers.

### Results and Discussion

Signalling in response to LPS was measured using an NF-\(\kappa\)B-responsive region (the IL8 promoter) linked to firefly luciferase and levels were normalized against constitutive low-level expression of Renilla luciferase. The HeLa cells used in the study have been shown by RT-PCR to express TLR4 but not TLR2 or MD-2 (Pridmore et al., 2001). Signalling through TLR2 was therefore examined by introduction of a TLR2 expression plasmid and through TLR4 by introduction of a plasmid expressing MD-2, which combines with endogenous TLR4 to produce a functional receptor.

Cells transfected with MD-2 responded with significantly higher IL8 promoter activity to *E. coli* LPS (\(P < 0.001\)) and *Y. pestis* LPS (\(P < 0.05\)) than to medium alone (Fig. 1). However, the response of TLR2-transfected cells to these pre-

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**Fig. 1.** TLR2- and TLR4-mediated recognition of LPS. Transfected cells were challenged with 100 ng LPS ml\(^{-1}\) (filled bars) or medium alone (open bars) and induction of the IL8 promoter was measured. Results are means±SEM of three pools of transfected HeLa cells. *, Significant difference between response to LPS and medium alone (\(P < 0.005\), Student’s \(t\)-test). Results are representative of two similar experiments and also of two experiments using 10 \(\mu\)g LPS ml\(^{-1}\).
parisons was not significantly different from the responses to medium alone, suggesting that the protocol employed to remove contaminating lipoprotein (or other TLR2 agonists) from the LPS preparations was successful.

Responses to LPS of Porphyromonas gingivalis, Pseudomonas aeruginosa, B. fragilis and C. trachomatis were seen to occur only in cells transfected with TLR2, and not in those transfected with MD-2 (Fig. 1), indicating that these LPS are recognized by TLR2 and not by TLR4. Statistical analysis showed that the response of TLR2-transfected cells to each of these LPS was significantly different from that made to medium alone ($P < 0.05$). Furthermore, the response of MD-2-transfected cells to each of these LPS was not significantly different from the response to medium alone, indicating that no TLR4 signalling of these LPS could be detected. RT-PCR analysis of the HeLa cells revealed no expression of TLR1, but some expression of TLR6 (data not shown), and therefore a role for heterodimerization of TLR6 with TLR2 in the recognition of these LPS cannot be ruled out.

These findings are consistent with the previously reported ability of Porphyromonas gingivalis LPS to signal via TLR2 (Hirschfeld et al., 2001) and with the ability of B. fragilis and Pseudomonas aeruginosa LPS to signal in macrophages from C3H/HeJ mice, which lack functional TLR4 (Delahouze et al., 1995; Girard et al., 2003). However, recent reports for C. trachomatis serovar E (Heine et al., 2003) and serovar LGV-2 (Prebeck et al., 2003) have shown that these serovars signal via TLR4 and not TLR2. These are interesting observations, as we have also shown that serovar E but not serovar LGV-1 signals via TLR4 (S. Hosseinizadeh and A. Eley, unpublished), suggesting that there might be differences in TLR signalling between serovars of C. trachomatis. It is well recognized that there are differences in pathogenicity between different serovars of Chlamydia, so the differences in TLR signalling are perhaps not entirely surprising. Indeed, the idea that LPS of different serovars might signal via different TLRs is not without precedent, as different strains of Pseudomonas aeruginosa have already been shown to signal via different TLRs (Hajjar et al., 2002). Additionally, it is worth noting that the study of Prebeck et al. (2003) involved challenge of murine macrophages. Murine TLRs can show distinct differences from human TLRs in responding to certain ligands – for example taxol and lipid IVa both stimulate murine TLR4 but do not activate human TLR4. Our study used human cells and human TLR constructs.

Together with the recent discoveries that the LPS of Legionella pneumophila (Girard et al., 2003), Rhizobium spp. (Girard et al., 2003), Leptospira interrogans (Werts et al., 2001) and Porphyromonas gingivalis (Hirschfeld et al., 2001) also signal via TLR2 and not TLR4, our results also begin to challenge the view that most LPS signal, like enterobacterial LPS, via the TLR4/MD-2 complex (Poltorak torak et al., 2000). Indeed, it seems likely, based on current evidence, that, rather than being the exception to the rule, TLR2-signalling LPS may in fact be commonly represented among Gram-negative species.

Challenge of human monocytes with serial tenfold dilutions of each LPS showed E. coli LPS to be a more potent inducer of TNF-$\alpha$ than any of the other LPS investigated in this study. Responses to E. coli LPS occurred at concentrations approximately 100–1000-fold lower than those required to evoke similar TNF-$\alpha$ responses on challenge with the other LPS (Fig. 2). These results are in agreement with earlier observations that TLR2-signalling LPS discovered to date are significantly less potent activators of human cells than enterobacterial LPS (Fig. 2 and Rietschel et al., 1994). By contrast, many of the LPS that have been clearly demonstrated to signal via TLR4, for example those of E. coli (Poltorak et al., 2000), Salmonella Minnesota (Tapping et al., 2000) and Neisseria meningitidis (Pridmore et al., 2001), are widely considered to be among the most strongly active endotoxic agents (Morrison et al., 1999). On the basis of these observations, it could therefore be speculated that expression of TLR2-signalling LPS could be of some advantage to certain organisms where avoidance of a vigorous inflammatory response to LPS may provide a benefit. However, it should also be pointed out that not all TLR4-signalling LPS demonstrate strong biological activity, as Y. pestis LPS was seen to possess a biological activity similar in magnitude to that of the TLR2-signalling LPS tested in our experiments, and we have previously shown that the LPS of some strains of N. meningitidis demonstrate relatively weak TLR4 signalling and concurrent biological activity (Pridmore et al., 2003).

The exact structural features of lipid A that define whether it is recognized by TLR2 or TLR4 can of course only be elucidated by an extensive study of chemically synthesized analogues. However, our report of a further three TLR2-signalling LPS allows some speculation as to which structural features in general might be responsible for discrimination between differing Gram-negative species.
between TLR2 and TLR4. Comparison of the lipid A molecules of different organisms (reviewed by Rietschel et al., 1994; Erridge et al., 2002) reveals that there are essentially four main structural differences between naturally occurring lipid A molecules: (i) the number of phosphates attached to the glucosamine backbone (0, 1 or 2); (ii) the number (3–7) of acyl chains; (iii) the length (C10–C28) of the acyl chains; and (iv) the presence or absence of branched, unsaturated or substituted acyl chains. To aid comparison of these features, a schematic representation of the lipid A molecules from organisms for which structural data are available is presented in Fig. 3.

It becomes clear on comparison of these structures that the TLR4-signalling lipid A molecules discovered to date seem to follow a fairly defined pattern, essentially consisting of a bisphosphorylated diglucosamine backbone substituted with six acyl chains of 12–16 carbons in length. A number of

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**Fig. 3.** Schematic representations of TLR2- and TLR4-signalling lipid A molecules. Sources of structural information for lipid A diagrams were Bhat et al. (1994) (Rhizobium sp.), Karunaratne et al. (1992) (Pseudomonas aeruginosa), Lindberg et al. (1990) (B. fragilis), Qureshi et al. (1997) (C. trachomatis), Rietschel et al. (1994) (E. coli, N. meningitidis), Della Venezia et al. (1985) (Y. pestis) and Zahringer et al. (1999) (Porphyromonas gingivalis, Legionella pneumophila). Dashed lines indicate commonly observed partial substitutions, numbers below acyl chains indicate chain length and Pi represents phosphate substitution. The exact locations of the fatty acid substituents of Y. pestis lipid A remain to be determined, though the predominant form is thought at present to be hexa-acyl.
synthetic lipid A molecules with monophosphate and triacyl architecture have also recently been shown to be capable of signalling via TLR4 (Ogawa et al., 2002; Tamai et al., 2003), though it remains possible that these dimerize in the TLR4/MD-2 complex to constitute the more typical twin phosphate, hexa-acyl format of TLR4-agonist lipid A and hence invoke signalling.

By contrast, TLR2 appears to be capable of recognizing a wider range of potential lipid A structures. Even minor deviations from the typical TLR4-activating lipid A structure appear to result in TLR2 signalling – such as the bisphosphoryl, penta-acyl, C10–12 format of *Pseudomonas aeruginosa* lipid A or the C13–C28 chain length of otherwise bisphosphoryl, hexa-acyl *Legionella pneumophila* lipid A. Consistent with this observation, Hajjar et al. (2002) recently showed that, while the hexa-acyl LPS of *Pseudomonas aeruginosa* adapted to survival in the cystic fibrosis lung is readily recognized by human TLR4, the penta-acyl LPS expressed by non-adapted strains of *Pseudomonas aeruginosa* (as used in this study) is not.

A possible explanation for the reduced bioactivity of *Y. pestis* LPS lies in the observation that not all of it is hexa-acylated, with a significant proportion being tetra-acylated (Kawahara et al., 2002). It is therefore possible that this contaminating tetra-acyl lipid A blocks responses to the TLR4-active hexa-acyl lipid A in the same way that tetra-acyl lipid IVa is a TLR4 agonist in the presence of either branching or non-saturation of the acyl chains. However, since none of these features are present in the presence of either branching or non-saturation of the acyl chains. However, since none of these features are present in...


