

Acinetobacter baumannii lipopolysaccharides are potent stimulators of human monocyte activation via Toll-like receptor 4 signalling

Clett Erridge,^{1†} Olga L. Moncayo-Nieto,^{2†} Robert Morgan,² Michelle Young² and Ian R. Poxton²

Correspondence
Ian R. Poxton
i.r.poxton@ed.ac.uk

¹Department of Bioscience, 204 George Street, University of Strathclyde, Glasgow G1 1XW, UK

²Medical Microbiology, Centre for Infectious Diseases, University of Edinburgh College of Medicine and Veterinary Medicine, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK

Acinetobacter baumannii is a major nosocomial pathogen and frequent cause of hospital-acquired pneumonia, surgical wound infections and sepsis. As very little is known of the endotoxic potential of *A. baumannii* lipopolysaccharide (LPS) with respect to human cells or of its ability to stimulate inflammatory signalling via human Toll-like receptors (TLRs), the biological activity of these endotoxins was investigated in human monocytic THP-1 cells and in TLR-deficient HEK-293 cells transfected with human TLR2 and TLR4 constructs. Endotoxins derived from five clinical isolates of *A. baumannii* and one of *Acinetobacter* 'genomospecies 9' showed high potency, which was comparable to that of *Escherichia coli* strain R1 NCTC 13114 LPS, in the induction of the *Limulus* amoebocyte reaction and interleukin 8 and tumour necrosis factor alpha release from THP-1 cells. Whole UV-killed cells of *A. baumannii* and *Acinetobacter* 'genomospecies 9' stimulated both TLR2- and TLR4-dependent signalling, whereas pure endotoxins of all investigated strains induced signalling via TLR4, but not TLR2.

Received 7 July 2006
Accepted 15 October 2006

INTRODUCTION

Acinetobacter baumannii is an opportunistic bacterial pathogen that has been increasingly identified as a cause of hospital-acquired pneumonia, wound infections and sepsis (Forster & Daschner, 1998; Maragakis *et al.*, 2004; Koprnova *et al.*, 2001). *A. baumannii* is notable for the frequent development of antimicrobial resistance (Jain & Danziger, 2004; Hanlon, 2005) and is an occasional cause of a fulminant form of community-acquired pneumonia associated with a particularly high mortality rate (Leung *et al.*, 2006; Chen *et al.*, 2001). In some individuals, *A. baumannii* infection can induce systemic inflammatory response syndrome (SIRS) together with septic shock, disseminated intravascular coagulation (DIC) and acute respiratory distress syndrome (ARDS) (Leung *et al.*, 2006; Chen *et al.*, 2001). The mechanisms responsible for the unusually severe systemic inflammatory reaction in response to *A. baumannii* infection in some individuals

remain poorly understood. However, as this opportunistic organism does not produce any known cytotoxins and is thought to possess only a limited number of virulence factors (Andrews, 1986), it has been suggested that the fulminant course of disease might be related to an exaggerated host response to endotoxin [lipopolysaccharide (LPS)] released from the outer membrane (Leung *et al.*, 2006).

The ability of endotoxins from other bacteria, notably those of *Escherichia coli*, *Neisseria meningitidis* and *Salmonella enterica* serovar Minnesota, to induce systemic inflammation in response to low-dose challenge has been well established (Pier *et al.*, 1981; Brandtzaeg *et al.*, 2001; Taveira da Silva *et al.*, 1993; Rietschel *et al.*, 1994). When present in the blood, endotoxins from these organisms stimulate circulating white blood cells to release pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF- α) and interleukin 1 β (IL1 β), thereby contributing to a wide range of deleterious effects, including DIC, ARDS, shock and multiple organ failure (Opal *et al.*, 1999; Wakefield *et al.*, 1998). Detection of these endotoxins occurs via the pattern-recognition receptors Toll-like receptor (TLR)-4 and MD-2 (Hirschfeld *et al.*, 2000; Poltorak *et al.*, 1998), though the endotoxins of certain strains of several non-enterobacterial species, such as *Bacteroides fragilis*, *Pseudomonas aeruginosa*,

†These authors contributed equally to this work.

Abbreviations: ARDS, acute respiratory distress syndrome; DIC, disseminated intravascular coagulation; FCS, fetal calf serum; IL, interleukin; ITS, inter-spacer; LAL, *Limulus* amoebocyte lysate; LPS, lipopolysaccharide; SIRS, systemic inflammatory response syndrome; TLR, Toll-like receptor; TNF, tumour necrosis factor.

Porphyromonas gingivalis and *Rhodobacter sphaeroides*, interact only very weakly or not at all with human TLR4/MD2 and have low biological activity (Lindberg *et al.*, 1990; Goldberg & Pier, 1996; Coats *et al.*, 2003; Kirkland *et al.*, 1991; Erridge *et al.*, 2004).

As very little is known of the endotoxic potential of *A. baumannii* LPS with respect to human cells or of its ability to stimulate signalling via human TLRs, the biological activity of *Acinetobacter* endotoxins was investigated in human cells and their interactions with human TLR2 and TLR4 were studied using endotoxins extracted from six well-characterized clinical isolates of *A. baumannii*.

METHODS

Identification of strains. A total of six strains from an established library of clinically derived *Acinetobacter* cultures maintained at the Royal Infirmary of Edinburgh Clinical Microbiology Laboratory (Edinburgh, UK) was investigated. Three strains were obtained originally from blood cultures, two were from sputum and one was from broncho-alveolar lavage (Table 1). Strains were identified provisionally by API 20NE and Vitek (bioMérieux) prior to molecular characterization.

16S ribosomal gene and ITS region sequencing. PCR was performed on boiled lysates of each *A. baumannii* culture. To amplify the 16S ribosomal region, primer sequences provided by E. Kuijper (Leiden University Medical Centre, Leiden, The Netherlands) were used: P0 243BAC (5'-GGCTCAGATTGAACGCTGGC-3') and P4 249BAC (5'-TCGTTGCGGGACTTAACCCAAC-3'). Conditions were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 23 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. Products were sequenced using primers P1 244BAC (5'-TAACACATGCAAGTCGAACG-3'), P2 245BAC (5'-CCCATTGTGCAATATTC-3') and P3 248BAC (5'-GGATTAGATACCTCGTAGTCC-3') and P4 249BAC (5'-TCGTTGCGGGACTTAACCCAAC-3'). NCBI nucleotide BLAST analysis was performed and sequence alignments made with BLOEDIT software. PCR was performed as described by Chang *et al.* (2005) to amplify the inter-spacer (ITS) region, which includes small fragments of the 16S rRNA and the 23S rRNA genes, using universal primers 1512F (5'-GTCGTAACAAGGTAGCCGTA-3') and 6R (5'-GGGTTYCCCCRTTCRGAAT-3') (where Y is C or T and R is A or G). Conditions were: 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. PCR products were sequenced using primers for the ITS (5'-ACGAAAGATT-3' and 3'-GGGGTTGTAT-5') and analysis performed as above.

UV irradiation of bacteria. Bacteria were grown in 25 ml nutrient broth for 24 h, centrifuged at 5 000 g for 5 min, then washed twice in 0.9% saline. Thin films of bacterial suspensions were exposed to bactericidal UV radiation for 40 min in glass Petri dishes. Viability was assessed by culturing UV-exposed cells on blood agar for 48 h.

LPS extraction. Endotoxins of each strain were extracted by the aqueous phenol extraction method originally developed by Westphal & Luderitz (1954) with modifications as described by Hancock & Poxton (1988). Samples were run on 10% SDS-PAGE gel and silver stained to show chemotype as described by Hancock & Poxton (1988). For the removal of protein and lipoprotein contaminants from crude primary endotoxin extracts, samples were repurified according to the method of Hirschfeld *et al.* (2000). Briefly, concentrations of triethylamine and sodium deoxycholate in the LPS samples were adjusted to 0.2 and 0.5%, respectively, and samples were subjected to two rounds of aqueous phenol re-extraction. Aqueous phases were then pooled and ethanol and sodium acetate concentrations were adjusted to 75% and 30 mM, respectively, 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 repurified LPS was assumed to be 100%. Following SDS-PAGE and electro-transfer to nitrocellulose membranes, colloidal gold staining of repurified endotoxin confirmed that lipoprotein contaminants could not be detected following re-extraction (data not shown).

Cells and transfection assays. THP-1 and HEK-293 cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) or Dulbecco's modified Eagle's medium/10% FCS (Sigma), respectively. For transfection assays, HEK-293 cells were plated in 96-well plates at 8×10^3 cells per well and transfected after 24 h using Genejuice (Novagen) according to the manufacturer's instructions. Amounts of construct per well were 30 ng plasmids expressing human TLR2 or 30 ng human TLR4 co-expressing MD-2 (Invivogen), 30 ng pCD14 (a kind gift from Professor C. Gregory, University of Edinburgh, Edinburgh, UK) and 10 ng luciferase reporter construct driven by the NF- κ B-dependent E-selectin promoter cloned into pGL3 (Promega) as described by Schindler & Baichwal (1994), with the balance made up with empty pCDNA3. Cells were grown for 2-3 days post-transfection prior to 18 h challenge. Reporter levels were normalized to co-transfected renilla expression (Promega Dual-Glo). Promoter expression is represented as the mean (\pm SD) of triplicate determinations of the degree of induction of the normalized signal relative to cells cultured in medium alone. Endogenous expression of TLRs in HEK-293 was ruled out by RT-PCR (data not shown). Repurified LPS of *E. coli* core-type R1 NCTC 13114 served as positive control for TLR4 signalling and repurified LPS of *Porphyromonas gingivalis* MPRL-1675 or the synthetic bacterial lipopeptide mimetic Pam₃CSK₄ (Invivogen) was used as positive control for TLR2 signalling.

Table 1. Source and identification of strains used in this study

Strain (MPRL no.)	Source	Identification	
		API 20NE	16S-23S ITS sequencing
4800	Blood	<i>A. lwoffii</i>	<i>Acinetobacter</i> genomospecies 9
4801	Blood	<i>A. baumannii</i>	<i>Acinetobacter</i> genomospecies 13TU
4802	Sputum	<i>A. baumannii</i>	<i>Acinetobacter</i> genomospecies 13TU
4803	Blood	<i>A. baumannii</i>	<i>Acinetobacter</i> genomospecies 13TU
4808	Sputum	<i>A. baumannii</i>	<i>Acinetobacter</i> genomospecies 13TU
4809	Broncho-alveolar lavage	<i>A. baumannii</i>	<i>Acinetobacter</i> genomospecies 13TU

Challenge of THP-1 cells and determination of IL8 and TNF- α expression. THP-1 cells were plated at 5×10^4 cells per well in 96-well plates and differentiated with 0.1 μ M dihydroxy-vitamin D3 for 72 h before challenge with 0.1–100 ng ml⁻¹ of each endotoxin preparation in triplicate in RPMI supplemented with 1% FCS. As pilot experiments showed that maximal cytokine release was achieved in response to 10 ng *E. coli* LPS ml⁻¹, this was the maximum concentration examined for this endotoxin (data not shown). Supernatant TNF- α and IL8 concentrations were measured at 4 and 18 h, respectively, by ELISA (R&D). All results are presented as means of triplicate wells and are representative of at least three independent experiments.

Limulus amoebocyte lysate (LAL) assay. Limulus amoebocyte lysate (Chromo-LAL; Quadratech) assay was performed in triplicate on samples of *A. baumannii* strain 4801 and *E. coli* R1 LPS serially diluted 3.2-fold in pyrogen-free water. Chromogen development was halted after 20 min with 1% H₂SO₄.

Statistical analysis. All results were compared by ANOVA (Tukey's test). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Identification of bacterial strains

Accurate identification of *A. baumannii* is unreliable by conventional phenotypic methods (Gerner-Smidt *et al.*, 1991). Therefore, strains that were provisionally identified by API 20NE and Vitek were confirmed by 16S and 16S–23S ITS region RNA gene sequencing. API 20NE suggested that five of the six library strains were *A. baumannii*, but that strain 4800 was *Acinetobacter lwoffii* (Table 1). 16S rRNA gene sequencing confirmed that all strains were *Acinetobacter* species, though as sequencing of this region cannot discriminate between *Acinetobacter* species, the 16S–23S ITS was investigated as this highly conserved region provides further discrimination between *Acinetobacter* species (Chang *et al.*, 2005). Five of the six strains were identified as *Acinetobacter* genomic species 13TU, which is part of the *A. baumannii* complex (Chang *et al.*, 2005), whereas strain 4800 was reclassified as *Acinetobacter* 'genomospecies 9'.

LPS chemotype

The six LPS preparations on silver-stained 10% SDS-PAGE gels were identical in appearance and gave patterns typical of R-form LPS. Bands were only found at the front of the gel, in contrast to the *E. coli* LPS control, which gave a typical smooth ladder pattern (results not shown). In the non-purified samples, faint bands were apparent that were probably proteins or lipoproteins. These disappeared after repurification and were absent in blots stained with colloidal gold. LPS of many *A. baumannii* strains are smooth in character when stained with silver (Pantophlet *et al.*, 1999) and the ladder pattern can also be revealed by Western blotting with specific antibodies to the O-antigen. Without such antibodies, we cannot be certain that the LPSs were not smooth as the sugars of the O-antigen may be resistant to periodate oxidation, the first step of silver staining.

A. baumannii LPS is a potent stimulator of the LAL reaction

As the biological activity of *A. baumannii* LPS remains relatively poorly investigated, endotoxin of strain 4801 was examined using the LAL assay. This assay has been widely used to examine the potency of other endotoxins (Rietschel *et al.*, 1994), thereby allowing comparison with endotoxins of other bacteria. Unlike many other non-enterobacterial endotoxins, which demonstrate very low activity in this assay compared to *E. coli* LPS (Rietschel *et al.*, 1994; Lindberg *et al.*, 1990; Kirkland *et al.*, 1991), the activity of the endotoxin of *A. baumannii* strain 4801 was roughly comparable in this assay to that of *E. coli* (Fig. 1), which is the endotoxin most widely used to standardize the assay.

A. baumannii LPS is a potent inducer of pro-inflammatory cytokine expression in THP-1 cells

A. baumannii LPS has been shown to stimulate inflammatory signalling in murine cells (Garcia *et al.*, 1999; Knapp *et al.*, 2006). However, the potency of certain types of LPS in murine systems occasionally differs dramatically from the potency of the same endotoxins when applied to human cells (Hajjar *et al.*, 2002; Akashi *et al.*, 2001). For this reason, and because monocytes are thought to play a key role in the recognition of endotoxin and pathology of sepsis (Wakefield *et al.*, 1998), the biological activity of *A. baumannii* LPS was examined in the human monocytic cell-line THP-1. The minimum concentration required by *E. coli* and each *Acinetobacter* endotoxin to stimulate significant ($P < 0.05$) IL8 release from these cells was 0.1 ng ml⁻¹, except for LPS of strains 4800 and 4808, which stimulated significant IL8 release at 1 ng ml⁻¹ (Fig. 2a). Similarly, the minimum concentration required by each of the *Acinetobacter* endotoxins to stimulate significant TNF- α release was the same as that of *E. coli* LPS, 1 ng ml⁻¹ (Fig. 2b).

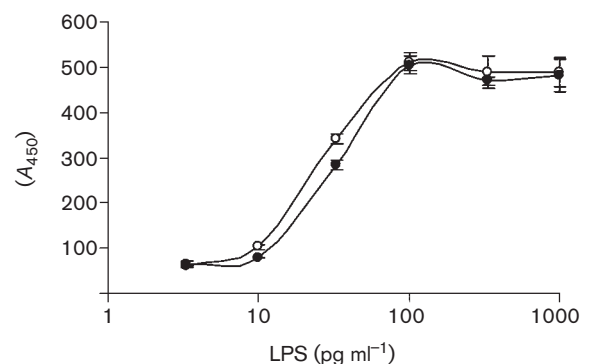


Fig. 1. Comparison of the ability of *Acinetobacter* and *Escherichia coli* endotoxins to stimulate the LAL reaction. The LAL assay was performed on serial dilutions of *A. baumannii* strain 4801 and R1 LPS in pyrogen-free water. Results are presented as means of triplicate measurements (\pm SD) and are representative of three similar experiments. \circ , *E. coli* LPS; \bullet , *A. baumannii* LPS.

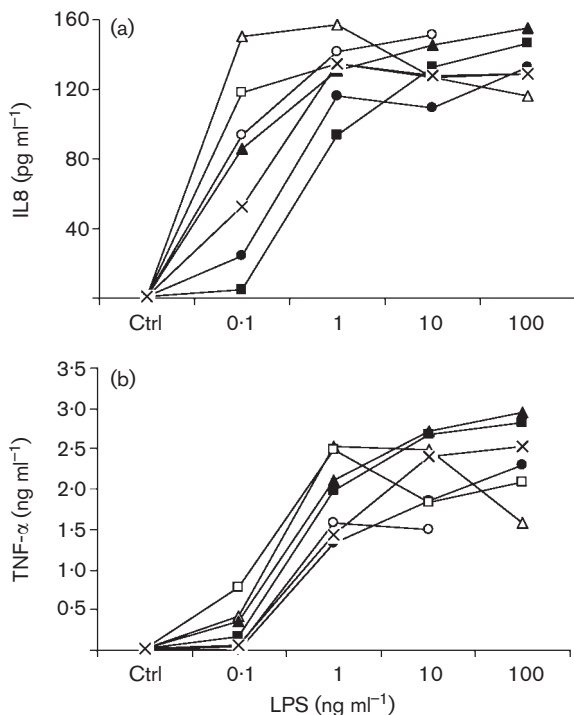


Fig. 2. Comparison of *Acinetobacter* and *Escherichia coli* endotoxin-induced IL8 and TNF- α release from human monocytic cells. Human monocytic THP-1 cells were challenged with medium alone (ctrl) or with 0.1–100 ng ml⁻¹ of each endotoxin preparation. Supernatant IL8 (a) and TNF- α (b) concentrations were measured at 4 and 18 h, respectively, by ELISA. Results are presented as means of triplicate wells and are representative of at least three independent experiments. ○, *E. coli*; ●, 4800; △, 4801; ▲, 4802; □, 4803; ■, 4808; X, 4809.

As several previous studies of the relative potency of endotoxins have used the minimum concentration required to stimulate cells as a marker of bioactivity (Lindberg *et al.*, 1990; Goldberg & Pier, 1996; Erridge *et al.*, 2004), the relative potency of *Acinetobacter* endotoxin determined in these assays can be compared with those of other non-enterobacterial endotoxins. For example, in a previous study using similar experimental systems and the same control *E. coli* LPS, it was shown that certain non-enterobacterial endotoxins, such as those of *B. fragilis* and *Pseudomonas aeruginosa*, stimulate human monocyte activation only at concentrations equal to or greater than 10–100 ng ml⁻¹ (Erridge *et al.*, 2004). Since concentrations of *A. baumannii* LPS as low as 0.1 ng ml⁻¹ were sufficient to induce some degree of cellular activation, it is concluded that the potency of *A. baumannii* LPS is closer to that of *E. coli* LPS than it is to less active endotoxins such as those of *B. fragilis*. Together with the results from the LAL assay and the findings of earlier studies, these data suggest that LPS of both *A. baumannii* and *Acinetobacter* ‘genomospecies 9’ fall into the group of the most highly active endotoxins, which includes those of *E. coli*, *S. enterica* Minnesota and *N. meningitidis*

(Rietschel *et al.*, 1994; Brandtzaeg *et al.*, 2001; Taveira da Silva *et al.*, 1993). This observation therefore supports the hypothesis that *A. baumannii* endotoxin may contribute to the particularly strong inflammatory responses observed during certain forms of *A. baumannii* infection (Leung *et al.*, 2006; Chen *et al.*, 2001). Indeed, it may be of relevance that comparatively low concentrations of both *A. baumannii* and ‘genomospecies 9’ endotoxin induced release of the inflammatory cytokine TNF- α , production of which is thought to be a major contributor to the pathology of SIRS, sepsis and DIC (Wakefield *et al.*, 1998) (Fig. 2b).

Role of human TLR2 and TLR4 in the detection of UV-killed *A. baumannii*

Detection of bacterial infection is achieved by the use of pattern recognition receptors. A major family of these, the TLRs, play a critical role in the host defence against bacteria (Underhill & Ozinsky, 2002). In particular, TLR2 and TLR4 are the most significant determinants of host detection of and defence against bacterial infection (Takeuchi *et al.*, 1999). Therefore, the further potential pathways for the production of pro-inflammatory cytokines by *A. baumannii* and ‘genomospecies 9’ and the potential of each to stimulate cellular signalling via human TLR2 and TLR4 were investigated.

Although a recent study has identified that TLR4 is the major receptor required for clearance of *A. baumannii* infection in the mouse (Knapp *et al.*, 2006), it should be noted that the specificities of human and murine TLR4/MD2 are occasionally seen to be quite different. For example, the partial lipid-A structure lipid-IVa is a potent activator of murine TLR4, yet is an inhibitor of human TLR4 signalling (Akashi *et al.*, 2001) and whereas penta-acyl *Pseudomonas aeruginosa* LPS is an agonist of murine TLR4/MD2, it is an antagonist of human TLR4/MD2 (Hajjar *et al.*, 2002). Therefore, to avoid such species-specific disparities, *A. baumannii*-induced signalling was investigated by transfection of the TLR-deficient human cell-line HEK-293 with human TLR2 and TLR4 constructs.

Using this assay, it was found that whole UV-killed *A. baumannii* and ‘genomospecies 9’ stimulated both TLR2- and TLR4-dependent signalling, although they did not stimulate NF- κ B signalling in the absence of TLRs (Fig. 3). There were no significant differences between the strains in terms of capacity to induce TLR2 stimulation, although the TLR4 signal induced by strain 4801 was significantly lower than that induced by the other strains. Interestingly, a recent study has shown that TLR4 is critical to bacterial clearance in a murine model of *A. baumannii* infection (Knapp *et al.*, 2006). Moreover, the surprising finding was also made that functional TLR2 appeared to reduce the magnitude of host inflammatory responses to bacterial challenge (Knapp *et al.*, 2006). Our findings suggest that, in human cells at least, whole *A. baumannii* stimulates pro-inflammatory signalling, at least as measured by NF- κ B signalling, via both TLR2- and TLR4-dependent recognition of these bacteria.

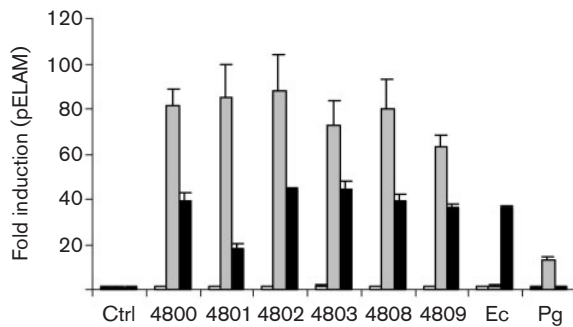


Fig. 3. Determination of TLR-signalling pathways initiated by whole UV-killed *Acinetobacter*. TLR-deficient HEK-293 cells were transfected with CD14 alone (white bars) or with CD14 plus TLR2 (grey bars) or TLR4/MD2 (black bars). Cells were then challenged with 10^5 UV-killed bacteria ml^{-1} for 18 h. The degree of induction of a co-transfected NF- κ B-sensitive reporter construct (pELAM) is shown, relative to cells cultured in medium alone (ctrl). Results are presented as means of triplicate wells and are representative of at least three independent experiments. Ec, Repurified *E. coli* LPS; Pg, repurified *Porphyromonas gingivalis* LPS.

The proposal that TLR2-dependent anti-inflammatory pathways may exist that modify responses to these bacteria (Knapp *et al.*, 2006) will require further investigation before this issue can be clarified.

A. baumannii and ‘genomospecies 9’ endotoxins stimulate human TLR4-dependent signalling

As *Acinetobacter* endotoxin has been proposed to be a possible virulence factor for this organism (Leung *et al.*, 2006), the role of TLR signalling in response to endotoxins of the chosen strains was investigated. A recent study has shown that *A. baumannii* LPS stimulates signalling in murine cells via TLR4 (Knapp *et al.*, 2006) though, as discussed earlier, it should be noted that endotoxins that are agonists of the murine TLR4/MD2 complex are occasionally antagonists of human TLR4/MD2 (Hajjar *et al.*, 2002; Akashi *et al.*, 2001). Moreover, there is evidence that TLR2 may play a more prominent role in the detection of certain non-classical endotoxins in human cells, in a manner that is independent of lipoprotein contamination (Darveau *et al.*, 2004; Werts *et al.*, 2001; Erridge *et al.*, 2004).

For these reasons, the potential of *A. baumannii* and ‘genomospecies 9’ LPS to stimulate human TLR2 and TLR4 signalling was investigated. All of the *Acinetobacter* endotoxins stimulated TLR4-dependent signalling, though it was found that crude endotoxin extracts from four of the six strains also stimulated significant TLR2-dependent signalling (Fig. 4a). Early studies of enterobacterial LPS revealed that the TLR2-dependent signal in crude endotoxin preparations can be due to lipoprotein contamination and that repurification of these crude extracts by phenol-water

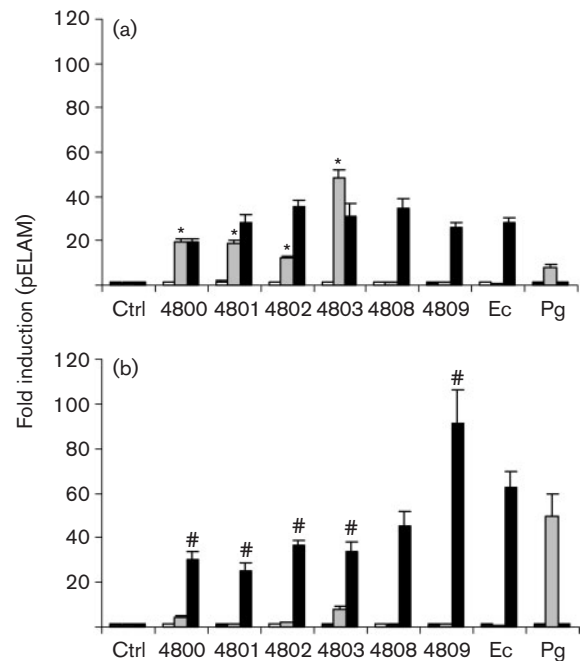


Fig. 4. Determination of TLR-signalling pathways initiated by *A. baumannii* endotoxin. TLR-deficient HEK-293 cells were transfected with CD14 alone (white bars) or with CD14 plus TLR2 (grey bars) or TLR4/MD2 (black bars). Cells were then challenged with 10 ng ml^{-1} of crude extracts of *Acinetobacter* endotoxin (a) or with 10 ng ml^{-1} of the same endotoxins after phenol-water re-extraction (b) for 18 h. The degree of induction of a co-transfected NF- κ B-sensitive reporter construct (pELAM) is shown, relative to cells cultured in medium alone (ctrl). Results are presented as means of triplicate wells and are representative of at least three independent experiments. Ec, Repurified *E. coli* LPS; Pg, repurified *Porphyromonas gingivalis* LPS. *TLR2 signal significantly higher than cells cultured in medium alone ($P < 0.05$), #TLR4 signal significantly different from TLR4 response to $10 \text{ ng E. coli LPS ml}^{-1}$ ($P < 0.05$).

re-extraction removed TLR2-stimulating contaminants completely (Hirschfeld *et al.*, 2000). It is likely that the crude endotoxin extracts of strains 4800–4803 contained more TLR2-stimulating contaminants than extracts of strains 4808 and 4809, as no statistically significant TLR2 signal remained following repurification (Fig. 4b). The differences in TLR2 signalling between the crude preparations are more likely to reflect variation in purification efficiency than levels of TLR2 stimulants present in the parent bacteria as TLR2 responses to each strain of whole bacteria were equivalent (Fig. 3). Likewise, statistical analysis of the TLR4 dependent signalling of the repurified endotoxins showed that strains 4800–4803 stimulated significantly less TLR4 signalling than *E. coli* LPS, whereas that of strain 4809 stimulated significantly more TLR4 signalling than *E. coli* LPS (Fig. 4b). However, given that there was no significant difference between TLR4 signalling of the crude extracts and that of *E. coli* LPS (Fig. 4a), it is

likely that the differences between the repurified endotoxins represent variation in efficiency of LPS recovery during the repurification process rather than genuine differences in potency of the native endotoxins.

Nevertheless, these results place the endotoxins of the examined *Acinetobacter* strains in the group of endotoxins that interact strongly with human TLR4/MD2, which previous studies have indicated include those of *E. coli*, *S. enterica* and *N. meningitidis* (Trent *et al.*, 2006), but not those of *B. fragilis*, *Porphyromonas gingivalis* or *Pseudomonas aeruginosa* (Coats *et al.*, 2003; Erridge *et al.*, 2004). It is possible that the strong interaction of the *Acinetobacter* endotoxins with TLR4/MD2 contributes to the potent toxicity of these endotoxins in human cells (Coats *et al.*, 2005; Rietschel *et al.*, 1994).

To our knowledge, this study is the first to investigate the biological activity and receptor utilization of *Acinetobacter* endotoxins in human cells. Evidence is presented to demonstrate that the endotoxins of both *A. baumannii* and *Acinetobacter* 'genomosppecies 9' are potent stimulators of inflammatory signalling in human monocytic cells and that responses to these bacteria are dependent on both TLR2 and TLR4. If, as expected, an exaggerated innate immune response to the endotoxins of these organisms contributes to the pathology of *Acinetobacter* infection, further investigation of the interaction of these endotoxins with the TLR4/MD2 complex may be warranted as a potential drug target for the amelioration of inflammatory signalling.

ACKNOWLEDGEMENTS

We are grateful to Professor Sebastian Amyes for assistance in sourcing strains, Professor Christopher Gregory for CD14 construct, Dr Kate Templeton for her assistance with PCR and sequencing protocols, and Stella Yan and Rebecca Lamb for assistance in the preparation of preliminary results.

REFERENCES

- Akashi, S., Nagai, Y., Ogata, H., Oikawa, M., Fukase, K., Kusumoto, S., Kawasaki, K., Nishijima, M., Hayashi, S. & other authors (2001). Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition. *Int Immunol* **13**, 1595–1599.
- Andrews, H. J. (1986). *Acinetobacter* bacteriocin typing. *J Hosp Infect* **7**, 169–175.
- Brandtzaeg, P., Bjerre, A., Ovstebo, R., Brusletto, B., Joo, G. B. & Kierulf, P. (2001). *Neisseria meningitidis* lipopolysaccharides in human pathology. *J Endotoxin Res* **7**, 401–420.
- Chang, H., Wei, Y., Dijkshoorn, L., Vanechoutte, M. T., Tang, C. T. & Chang, T. C. (2005). Species-level identification of isolates of the *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* complex by sequence analysis of the 16S-23S rRNA gene spacer region. *J Clin Microbiol* **43**, 1632–1639.
- Chen, M. Z., Hsueh, P. R., Lee, L. N., Yu, C. J., Yang, P. C. & Luh, K. T. (2001). Severe community-acquired pneumonia due to *Acinetobacter baumannii*. *Chest* **120**, 1072–1077.
- Coats, S. R., Reife, R. A., Bainbridge, B. W., Pham, T. T. & Darveau, R. P. (2003). *Porphyromonas gingivalis* lipopolysaccharide antagonizes *Escherichia coli* lipopolysaccharide at Toll-like receptor 4 in human endothelial cells. *Infect Immun* **71**, 6799–6807.
- Coats, S. R., Pham, T. T., Bainbridge, B. W., Reife, R. A. & Darveau, R. P. (2005). MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize *Escherichia coli* lipopolysaccharide at the TLR4 signaling complex. *J Immunol* **175**, 4490–4498.
- Darveau, R. P., Pham, T. T., Lemley, K., Reife, R. A., Bainbridge, B. W., Coats, S. R., Howald, W. N., Way, S. S. & Hajjar, A. M. (2004). *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun* **72**, 5041–5051.
- Erridge, C., Pridmore, A., Eley, A., Stewart, J. & Poxton, I. R. (2004). Lipopolysaccharides of *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. *J Med Microbiol* **53**, 735–740.
- Forster, D. H. & Daschner, F. D. (1998). *Acinetobacter* species as nosocomial pathogens. *Eur J Clin Microbiol Infect Dis* **17**, 73–77.
- Garcia, A., Salgado, F., Solar, H., Gonzalez, C. L., Zemelman, R. & Onate, A. (1999). Some immunological properties of lipopolysaccharide from *Acinetobacter baumannii*. *J Med Microbiol* **48**, 479–483.
- Gerner-Smidt, P., Tjernberg, I. & Ursing, J. (1991). Reliability of phenotypic tests for identification of *Acinetobacter* species. *J Clin Microbiol* **29**, 277–282.
- Goldberg, J. B. & Pier, G. B. (1996). *Pseudomonas aeruginosa* lipopolysaccharides and pathogenesis. *Trends Microbiol* **4**, 490–494.
- Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B. & Miller, S. I. (2002). Human Toll-like receptor 4 recognises host-specific LPS modifications. *Nat Immunol* **3**, 354–359.
- Hancock, I. C. & Poxton, I. R. (1988). *Bacterial Cell Surface Techniques*. Chichester: Wiley.
- Hanlon, G. W. (2005). The emergence of multidrug resistant *Acinetobacter* species: a major concern in the hospital setting. *Lett Appl Microbiol* **41**, 375–378.
- Hirschfeld, M., Ma, Y., Weis, J. H., Vogel, S. N. & Weis, J. J. (2000). Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol* **165**, 618–622.
- Jain, R. & Danziger, L. H. (2004). Multidrug-resistant *Acinetobacter* infections: an emerging challenge to clinicians. *Ann Pharmacother* **38**, 1449–1459.
- Kirkland, T. N., Qureshi, N. & Takayama, K. (1991). Diphosphoryl lipid A derived from lipopolysaccharide (LPS) of *Rhodopseudomonas sphaeroides* inhibits activation of 70Z/3 cells by LPS. *Infect Immun* **59**, 131–136.
- Knapp, S., Wieland, C. W., Florquin, S., Pantophlet, R., Dijkshoorn, L., Tshimbalanga, N., Akira, S. & van der Poll, T. (2006). Differential roles of CD14 and Toll-like receptors 4 and 2 in murine *Acinetobacter* pneumonia. *Am J Respir Crit Care Med* **173**, 122–129.
- Koprnova, J., Svetlansky, I., Babel'a, R., Bilikova, E., Hanzen, J., Zuscakova, I. J., Milovsky, V., Masar, O., Kovacicova, G. & other authors (2001). Prospective study of antibacterial susceptibility, risk factors and outcome of 157 episodes of *Acinetobacter baumannii* bacteremia in 1999 in Slovakia. *Scand J Infect Dis* **33**, 891–895.
- Leung, W. S., Chu, C. M., Tsang, K. Y., Lo, F. H., Lo, K. F. & Ho, P. L. (2006). Fulminant community-acquired *Acinetobacter baumannii* pneumonia as a distinct clinical syndrome. *Chest* **129**, 102–109.
- Lindberg, A. A., Weintraub, A., Zahringer, U. & Rietschel, E. T. (1990). Structure-activity relationships in lipopolysaccharides of *Bacteroides fragilis*. *Rev Infect Dis* **12**, S133–S141.

- Maragakis, L. L., Cosgrove, S. E., Song, X., Kim, D., Rosenbaum, P., Ciesla, N., Srinivasan, A., Ross, T., Carroll, K. & Perl, T. M. (2004).** An outbreak of multidrug-resistant *Acinetobacter baumannii* associated with pulsatile lavage wound treatment. *JAMA* **292**, 3006–3011.
- Opal, S. M., Scannon, P. J., Vincent, J. L., White, M., Carroll, S. F., Palardy, J. E., Parejo, N. A., Pribble, J. P. & Lemke, J. H. (1999).** Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. *J Infect Dis* **180**, 1584–1589.
- Pantophlet, R., Brade, L. & Brade, H. (1999).** Identification of *Acinetobacter baumannii* strains with monoclonal antibodies against the O antigens of their lipopolysaccharides. *Clin Diagn Lab Immunol* **6**, 323–329.
- Pier, G. B., Markham, R. B. & Eardley, D. (1981).** Correlation of the biologic responses of C3H/HEJ mice to endotoxin with the chemical and structural properties of the lipopolysaccharides from *Pseudomonas aeruginosa* and *Escherichia coli*. *J Immunol* **127**, 184–191.
- Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M. & other authors (1998).** Defective LPS signaling in C3H/HEJ and C57Bl/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085–2088.
- Rietschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zahringer, U., Seydel, U. & other authors (1994).** Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J* **8**, 217–225.
- Schindler, U. & Baichwal, V. R. (1994).** Three NF- κ B binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. *Mol Cell Biol* **14**, 5820–5831.
- Takeuchi, O., Hoshino, K., Kawei, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. & Akira, S. (1999).** Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* **11**, 443–451.
- Taveira da Silva, A. M., Kaulbach, H. C., Chuidian, F. S., Lambert, D. R., Suffredini, A. F. & Danner, R. L. (1993).** Brief report: shock and multiple-organ dysfunction after self-administration of *Salmonella* endotoxin. *N Engl J Med* **328**, 1457–1460.
- Trent, M. S., Stead, C. M., Tran, A. X. & Hankins, J. V. (2006).** Diversity of endotoxin and its impact on pathogenesis. *J Endotoxin Res* **12**, 205–223.
- Underhill, D. M. & Ozinsky, A. (2002).** Toll-like receptors: key mediators of microbe detection. *Curr Opin Immunol* **14**, 103–110.
- Wakefield, C. H., Barclay, G. R., Fearon, K. C. H., Goldie, A. S., Ross, J. A., Grant, I. S., Ramsay, G. & Howie, J. C. (1998).** Proinflammatory mediator activity, endogenous antagonists and the systemic inflammatory response in intra-abdominal sepsis. *Br J Surg* **85**, 818–825.
- Werts, C., Tapping, R. I., Mathison, J. C., Chuang, T. H., Kravchenko, V., Saint Girons, I., Haake, D. A., Godowski, P. J., Hayashi, F. & other authors (2001).** Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol* **2**, 346–352.
- Westphal, O. & Luderitz, O. (1954).** Chemische erforschung von lipopolysaccharides gramnegativer bakterium. *Agnew Chem* **66**, 407–417.