Porins from *Salmonella enterica* serovar Typhimurium induce TNF- α , IL-6 and IL-8 release by CD14-independent and CD11a/CD18-dependent mechanisms

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Lipopolysaccharide (LPS) of Gram-negative bacteria and several surface components of Gram-positive bacteria utilize CD14 and CD11a/18 as cellular receptors to induce expression and release of cytokines. Of the surface components of Gram-negative bacteria, porins exhibit a biological activity similar to that of LPS. The results in this paper show that the mechanism of stimulation by porins of THP-1 cells enriched in CD14 receptor after treatment with 1,25-dihydroxyvitamin D₃ (vitamin D₃) is independent of this receptor, but is partially dependent on CD11a/18 integrins.

Keywords: *Salmonella typhimurium*, lipopolysaccharide, cytokines, integrins, cellular receptors

INTRODUCTION

CD14 is a glycosyl phosphatidylinositol-linked 55 kDa protein present on the surface of macrophages and polymorphonuclear leukocytes, and it functions as the cell surface receptor for lipopolysaccharide (LPS). LPS is known to interact with CD14 in inducing expression of cytokines. The binding of LPS to CD14 is facilitated by an LPS-binding protein present in plasma (Gegner *et al.*, 1995; Hailman *et al.*, 1994; Haziot *et al.*, 1996; Juan *et al.*, 1995; Ulevitch & Tobias, 1994, 1995; Viriyakosol & Kirkland, 1995; Wright *et al.*, 1990). This receptor is present at high density on the cell membrane (mCD14) of monocytes and macrophages, and at low density on polymorphonuclear leukocytes. It is also found as a soluble protein (sCD14) in human serum and urine (Antal-Szalmás *et al.*, 1997; Ulevitch & Tobias, 1994). CD14 lacks transmembrane and cytokine-binding domains and is not believed to have intrinsic signalling capabilities. Toll-like receptor 4 (TLR4) appears to be a very important LPS signal transducer (Hoshino *et al.*, 1999). It is thought that, also, Toll-like receptor 2 (TLR2), a transmembrane protein (Tabeta *et al.*, 2000), functions as a signal transducer upon LPS binding by CD14 in activating NF- κ B and the expression of NF- κ B-controlled genes which encode cytokines (Chaudhary *et al.*, 1998; Yang *et al.*, 1998). Peptidoglycan and lipoteichoic acid from Gram-positive bacteria can also utilize CD14 as a receptor (Cleveland *et al.*, 1996;

Crauwels *et al.*, 1997; Kusunoki *et al.*, 1995; Weidemann *et al.*, 1994). This has also been demonstrated for lipoarabinomannan of *Mycobacterium tuberculosis* (Zhang *et al.*, 1993), rhamnose-glucose polymers from *Streptococcus mutans* (Soell *et al.*, 1995), and mannuronic acid polymers from *Pseudomonas* species (Espevik *et al.*, 1993).

β_2 leukocyte integrins (CD11a/CD18, CD11b/CD18 and CD11c/CD18) may also participate in LPS signalling (Ingalls & Golenboch, 1995; Ingalls *et al.*, 1997). This family of receptors are heterodimeric cell surface glycoproteins composed of a CD11 and a CD18 subunit. These integrins mediate cell adhesion to endothelial cell ligands such as intracellular adhesion molecules.

A number of studies have shown that porins from several Gram-negative bacteria also stimulate cells to produce and secrete cytokines (Galdiero *et al.*, 1993, 1995, 2001; Henderson *et al.*, 1996; Iovane *et al.*, 1998; Wilson *et al.*, 1998).

The precise mechanisms by which LPS activates cells have not yet been elucidated (Tabeta *et al.*, 2000). One proposed model (Wright, 1995) suggests that CD14 functions as an albumin-like carrier molecule that binds a large variety of molecules without recognition specificity, and that it can then transfer these molecules to unidentified recognition/cell-activating molecules in the cell membrane. According to this model, and considering the similarity between the biological effects

of LPS and porins, and the utilization of CD14 and CD11a/18 as receptors also by components of Gram-positive bacteria, the aim of the present study was to test the hypothesis that CD14 receptors and CD11a/18 integrins may also function in signalling cytokine release following porin stimulation.

METHODS

Bacterial strains. The bacterial strain used was *Salmonella enterica* serovar Typhimurium SH5014 grown in Nutrient Broth (Difco) for 18–24 h at 37 °C under agitation. Cells were harvested at the end of the exponential growth phase, and outer membranes were prepared from cell envelopes following protocols described by Nikaido & Vaara (1987).

Porin preparation. *S. enterica* serovar Typhimurium strain SH5014 was used to extract and purify porins. Porins were isolated from the lysozyme/EDTA envelopes as described by Nurminen (1985). Briefly, 1 g of envelopes was suspended in 2% Triton X-100 in 0.01 Tris/HCl (pH 7.5, containing 10 mM EDTA); after the addition of trypsin (10 mg envelopes g⁻¹), the pellet was dissolved in SDS buffer (4%, w/v, SDS in 0.1 M sodium phosphate, pH 7.2), and applied to an Ultragel ACA 34 column equilibrated with 0.25% SDS buffer. The fraction containing proteins, identified by A₂₈₀, was extensively dialysed and checked by SDS-PAGE according to Laemmli (1970). The purity of the porin preparation was checked by SDS-PAGE, which revealed two bands, with molecular masses of 34 and 36 kDa. The protein content of the porin preparation was determined by the Lowry method and by the *Limulus* amoebocyte lysate assay (*Limulus* test) (Thye Yin *et al.*, 1972). The *Limulus* test showed the presence of LPS at 50 pg porins µg⁻¹. The LPS concentration in the porin preparation was estimated to be < 0.005% (w/w). In addition, polymyxin B (Sigma Aldrich) was incubated with porins to neutralize the biological activity of traces of LPS that could be present in the preparation. The porins were incubated with polymyxin B at room temperature for 1 h in a ratio of 1:10; LPS was incubated with polymyxin B in a ratio of 1:100 (Morrison & Jacobs, 1976). LPS, porins and the polymyxin B mixture were used in pyrogen-free phosphate-buffered saline (PBS).

LPS preparation. LPS-R was isolated from *S. enterica* serovar Typhimurium SH5014 with phenol/chloroform/petroleum ether as described by Galanos *et al.* (1969). Briefly, liquid phenol (90 g dry phenol plus 11 ml water/chloroform/petroleum ether in a volume ratio of 2:5:8) was added to 1 g dried bacteria. After 2 min homogenization, bacteria were centrifuged and extracted twice. The supernatants were filtered through filter paper and treated as described by Galanos *et al.* (1969).

Cell line. The human promyelomonocytic cell line THP-1 (ATCC TIB-202) (Tsuchiya *et al.*, 1980) was cultured at 37 °C in a moist atmosphere of 5% CO₂ in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Labtek, Eurobio). To induce adherence to plastic and expression of CD14, cultures were grown in 6-well plates in the presence of 0.1 µM 1,25-dihydroxyvitamin D₃ (vitamin D₃) (Calbiochem-Novabiochem). After 72 h, cultures were fed by adding 0.5 vol. medium containing vitamin D₃ and incubated for another 24 h (Kitchens *et al.*, 1992). In some serum-dependence experiments cells were washed four times with PBS and cultured in serum-free medium for 12 h before experimentation.

Western blot analysis of CD14 and CD11a protein in vitamin D₃-treated and non-treated cells. Cell pellets were resuspended in 50 µl lysis buffer containing 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 12 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT. After three freeze-thaw cycles, cell lysates were centrifuged at 12000 g for 30 min, and the supernatant was saved. Samples (50 µg per lane) were separated by SDS-10% PAGE and transferred to nitrocellulose membrane. Anti-CD14 clone MEM-18, anti-CD14 clone UCHM-1 and anti-CD11a clone 38 mAbs were used. After several washes, the membrane was developed with an enhanced luminol reagent (Du-Pont, NEN) and exposed to Kodak X-Omat film.

Cell stimulation and cytokine release. All assays were carried out using 3 × 10⁶ THP-1 cells ml⁻¹. Porins (50–5000 ng ml⁻¹) and LPS (10–1000 ng ml⁻¹) were prepared in pyrogen-free distilled water and then added to suspensions of THP-1 cells at a range of concentrations. These were then incubated for 24 h at 37 °C in an atmosphere of 5% CO₂. After incubation the samples were centrifuged at 1800 r.p.m. at 4 °C for 10 min and the supernatants were collected and stored at –70 °C. All samples were assayed for the presence of cytokines (TNF-α, IL-6 and IL-8) by ELISA, according to the manufacturer's instructions (Roche Diagnostic). Cell viability was determined by measuring leakage of lactate dehydrogenase activity from cells into the supernatant, using a kit purchased from Roche Diagnostic.

Inhibition of CD14 and CD11a/18 binding. Vitamin D₃-treated THP-1 cells (4 × 10⁶ cells ml⁻¹) were incubated for 30 min at 4 °C with a range of dilutions of mAbs against CD14 or CD11a/18 (anti-CD14 clones MEM 18, isotype IgG, and UCHM-1, isotype IgG 2a; anti-CD11a clone 38, isotype IgG2a; and anti-CD18 clone MeM18, isotype IgG1) (Cymbus Biotechnology) and with appropriate non-immune isotype controls. This preparation was subsequently incubated with porins (50–5000 ng ml⁻¹) or LPS (10–1000 ng ml⁻¹). Cultures were incubated for 24 h at 37 °C in a humidified atmosphere (5% CO₂ and 95% air). Vitamin D₃-untreated cells were used to monitor CD11a/18 expression. In some experiments, we used as stimulus a mixture of porins plus polymyxin B. At the end of the incubation period, cells were centrifuged at 400 g at 4 °C for 10 min, and the supernatants were aliquoted and stored at –70 °C until they were assayed for the presence of TNF-α, IL-6 and IL-8.

Antibodies. Two mouse mAbs against human CD14 were used: MEM 18 (isotype IgG) and UCHM-1 (isotype IgG 2a). Anti-CD11a clone 38 isotype IgG2a and anti-CD18 clone MeM18 isotype IgG1 were utilized. Negative control immunoglobulins for the inhibitors experiments were purified mouse IgG1 and IgG2a (Cymbus Biotechnology).

Reproducibility of results. The results are expressed as mean values ± SD. The differences between the effects of anti-CD14, anti-CD11a/18 and control mAbs were analysed by the paired two-tailed sample *t*-test. The level of significance was set at 0.05.

RESULTS

Purity of porin preparation

The purification protocols, and methods used to discount the likely contamination by LPS in porin preparation, have been extensively described in previous work (Galdiero *et al.*, 1990, 1995).

To determine whether traces of LPS were present, we carried out a *Limulus* test using as a standard a LPS

Fig. 1. Pattern of *S. enterica* serovar Typhimurium SH5014 porins and LPS on SDS-PAGE. (a) Lane 1, molecular mass standards (Amersham Pharmacia Biotech) (phosphorylase B, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α -lactalbumin, 14.4 kDa); lane 2, *S. enterica* serovar Typhimurium porins (10 μ g). The gel was stained with Coomassie blue. (b) Lane 1, *S. typhimurium* LPS standard (Sigma-Aldrich); lane 2: *S. enterica* serovar Typhimurium SH5014 LPS (10 μ g). The gel was stained with silver nitrate.

solution which gave a *Limulus* test value of 0.1 EU ml⁻¹. The LPS concentration in the porin preparation was estimated to be < 0.005% (w/w). These trace amounts of LPS did not induce any biological activity under our experimental conditions (data not shown). The purity of the porin preparation from *S. enterica* serovar Typhimurium SH5014 was checked by SDS-PAGE. Coomassie blue staining revealed the presence of two protein species, with molecular masses of 34 and 36 kDa (Fig. 1a).

The pattern of LPS preparation was shown by SDS-PAGE followed by silver nitrate staining (Tsai & Frasch, 1982) (Fig. 1b). In all of the tests performed, porins which had been incubated with polymyxin B gave the same results as porins alone (data not shown).

CD14 protein expression correlates with vitamin D₃ treatment of cells

To determine if the CD14 expression could also be detected by changes in protein levels, Western blot analysis was performed on both vitamin D₃-treated cells

Fig. 2. Western blot analysis of CD14 and CD11a protein levels in vitamin D₃-treated cells and non-treated cells. Proteins (50 μ g) were separated by SDS-10% PAGE and transferred to nitrocellulose membrane. For detection of CD14 protein, the anti-CD14 mAbs MEM-18 and UCHM-1 were used; for detection of CD11a protein, the anti-CD11a mAb clone 38 was used; immune complexes were detected by enhanced luminol reagent (Du-Pont, NEN) as described by the manufacturer. The blots are representative of three independent experiments. Plots of densitometer scans (arbitrary units) are shown below the blots.

and non-treated cell extracts. As indicated in Fig. 2, vitamin D₃-treated cell extracts showed a strong cross-reactivity to anti-CD14 mAb MEM 18 and anti-CD14 mAb UCHM-1.

Effect of anti-CD14 mAbs on the release of TNF- α , IL-6 and IL-8 by vitamin D₃-treated THP-1 cells stimulated by porins or by LPS

To determine the involvement of CD14 in porin-mediated expression of cytokines, we used anti-CD14 mAbs. LPS was used as a reference to evaluate the effect of anti-CD14 mAbs in the assays performed in this study. All experiments were carried out using the well-characterized THP-1 human monocytic cell line (Tsuchiya *et al.*, 1980).

To induce cell maturation and expression of surface CD14, THP-1 cells were pretreated with vitamin D₃. Exposure to vitamin D₃ induces adherence to plastic and expression of high levels of cell-surface CD14 (Fleit & Kobasiuk, 1991). More than 95% of adherent cells expressed CD14 as detected by mAbs (data not shown). Vitamin D₃-treated THP-1 cells were incubated with *S. enterica* serovar Typhimurium porins or LPS. After 24 h, the production of TNF- α , IL-6 and IL-8 in the culture supernatants was determined by ELISA. The production of these cytokines was markedly higher in culture supernatants of THP-1 cells that had been

Fig. 3. Secretion of TNF- α , IL-6 and IL-8 by vitamin D₃-treated THP-1 cells stimulated with LPS or porins. Cytokine levels were measured by capture ELISA (see Methods). The data are the mean $\pm$ SE of duplicate determinations for three independent experiments ($P < 0.05$).

stimulated with porins or LPS than in the unstimulated cells. Increasing the concentration of either porins or LPS led to increased levels of cytokine release (Fig. 3). Cytokine release was evident with 50 ng porins ml⁻¹ or 10 ng LPS ml⁻¹; maximum production was achieved with 1000 ng LPS ml⁻¹ or 5000 ng porins ml⁻¹. In some experiments cytokine release was assayed under serum-free conditions. In this case, release of all three cytokines was induced by porins but not by LPS (data not shown).

Cytokine release was not due to LPS contamination of the porin preparation, since the addition of polymyxin B had no effect on porin-induced cytokine production, under conditions in which it completely blocked cytokine production by LPS at concentrations ranging from 10 to 1000 ng ml⁻¹ (data not shown). The lowest concentration of LPS able to induce cytokine release was 10 ng ml⁻¹, which is much higher than the amount of LPS present in our porin preparations.

To examine whether cytokine production induced by porins is dependent on CD14, vitamin D₃-treated THP-1 cells were preincubated with a range of concentrations of anti-CD14 mAbs, clone MEM 18 and clone UCHM-1, for 30 min at 4 °C.

After 24 h stimulation with porins (50–5000 ng ml⁻¹) or LPS (10–1000 ng ml⁻¹), supernatants were harvested, and TNF- α , IL-6 and IL-8 activity was determined by

ELISA (Table 1). As expected, both anti-CD14 mAbs blocked the LPS-mediated production of TNF- α , IL-6 and IL-8 in a dose-dependent manner. Independently of their isotype (IgG or IgG 2a), the mAbs used showed inhibiting activity. Both anti-CD14 mAbs failed to inhibit cytokine production induced by porins at any of the dilutions tested. The anti-CD14 mAbs alone did not induce TNF- α , IL-6 and IL-8 production in THP-1 cells. The control mAbs which were used as isotype controls could not reduce the LPS-induced cytokine production. A non-specific stimulus, bovine serum albumin, did not induce any cytokine liberation (data not shown).

Effect of anti-CD11a/18 mAbs on the release of TNF- α , IL-6 and IL-8 by THP-1 cells stimulated with porins or LPS

THP-1 cells were preincubated with anti-CD11a and anti-CD18 mAbs and cells were subsequently treated with porins (50–5000 ng ml⁻¹) or LPS (10–1000 ng ml⁻¹). As controls, either non-immune isotype mAbs or a buffer were used. Preincubation with anti-CD11a and anti-CD18 mAbs decreased LPS-induced release of TNF- α , IL-6 and IL-8. Preincubation with anti-CD11a/CD18 was able to decrease slightly, but nonetheless significantly, porin-mediated release of TNF- α , IL-6 or IL-8. These results are shown in Table 2.

The ability to inhibit TNF- α , IL-6 and IL-8 release was stronger when THP-1 cells were incubated with 10 ng LPS ml⁻¹ and 50 ng porins ml⁻¹. The inhibition of the release of those cytokines become much lower at higher concentrations of either LPS or porins, probably due to non-specific stimulation. In LPS-treated cells, the use of mAbs to CD11a reduced TNF- α release by about 50 %, and IL-6 and IL-8 release by about 25–35 %. The use of mAbs to CD18 reduced the release of the three cytokines by about 25–35 %.

In porin-treated cells, both anti-CD11a and anti-CD18 mAbs reduced the release of the three cytokines by about 20–25 %.

DISCUSSION

The aim of our study was to investigate whether porins, like LPS, induce cytokine production mediated by the CD14 receptor or by CD11a/18 integrins, following monocyte activation. *In vivo*, porins and LPS exhibit similar effects (Galdiero *et al.*, 1994). *In vitro*, both agents are strong macrophage and polyclonal B-cell activators (Doe *et al.*, 1978; Goodman & Sultz, 1979a, b). The results presented in this study show that porin-mediated induction of TNF- α , IL-6 and IL-8 was independent of CD14 and only partially dependent on CD11a/18. We showed that the addition of anti-CD14 antibodies resulted in a significant suppression of LPS-induced cytokine production, while the addition of anti-CD14 mAbs was not able to significantly suppress cytokine induction in cells stimulated by porins. Serum-associated LPS-binding protein, which forms complexes

Table 1. Effect of anti-CD14 mAbs MEM 18 and UCHM-1 on production of TNF- α , IL-6 and IL-8 by vitamin D₃-treated THP-1 cells stimulated by LPS or porins

Vitamin D₃-treated THP-1 cells were stimulated for 24 h with LPS or porins in the presence of anti-CD14 mAbs MEM 18 or UCHM-1, or the corresponding control mAbs.

Stimulus (ng ml ⁻¹)	Mean concentration (pg ml ⁻¹) $\pm$ SD*											
	TNF- α †				IL-6†				IL-8†			
	Control mAb	MEM 18 mAb	Control mAb	UCHM mAb	Control mAb	MEM 18 mAb	Control mAb	UCHM mAb	Control mAb	MEM 18 mAb	Control mAb	UCHM mAb
LPS												
(1000)	4050 $\pm$ 63.6	2400 $\pm$ 48.9 (40.8)	4100 $\pm$ 64.0	2500 $\pm$ 50.0 (39.1)	2300 $\pm$ 47.9	1500 $\pm$ 38.7 (34.8)	2200 $\pm$ 46.9	1650 $\pm$ 40.6 (25.0)	5100 $\pm$ 71.4	2100 $\pm$ 45.8 (58.9)	5200 $\pm$ 72.1	2000 $\pm$ 44.7 (61.6)
(100)	2800 $\pm$ 52.9	940 $\pm$ 30.6 (66.5)	2900 $\pm$ 53.8	800 $\pm$ 28.2 (72.5)	1150 $\pm$ 33.9	600 $\pm$ 24.4 (47.9)	1200 $\pm$ 34.6	550 $\pm$ 23.4 (54.2)	3500 $\pm$ 59.1	800 $\pm$ 28.2 (77.2)	3700 $\pm$ 60.8	850 $\pm$ 29.1 (77.1)
(10)	300 $\pm$ 17.3	30 $\pm$ 5.4 (90.0)	320 $\pm$ 17.8	40 $\pm$ 6.3 (87.5)	750 $\pm$ 27.3	130 $\pm$ 11.4 (82.7)	800 $\pm$ 28.2	160 $\pm$ 12.6 (80.0)	950 $\pm$ 30.8	50 $\pm$ 7.0 (94.8)	1050 $\pm$ 32.4	100 $\pm$ 10.0 (90.5)
Porins												
(5000)	2800 $\pm$ 52.9	2700 $\pm$ 51.9 (3.6)	2900 $\pm$ 53.8	2950 $\pm$ 54.3 (0)	1450 $\pm$ 38.0	1400 $\pm$ 37.4 (3.5)	1500 $\pm$ 38.7	1400 $\pm$ 37.4 (6.7)	3800 $\pm$ 61.6	3700 $\pm$ 60.8 (2.7)	3700 $\pm$ 60.8	3600 $\pm$ 60.0 (2.8)
(500)	1300 $\pm$ 36.0	1200 $\pm$ 34.6 (7.7)	1200 $\pm$ 34.6	1100 $\pm$ 33.1 (8.4)	700 $\pm$ 26.4	760 $\pm$ 27.5 (0)	750 $\pm$ 27.3	785 $\pm$ 28.0 (0)	2000 $\pm$ 44.7	1900 $\pm$ 43.5 (5.0)	1800 $\pm$ 42.4	1700 $\pm$ 41.2 (5.6)
(50)	400 $\pm$ 20.0	358 $\pm$ 18.9 (10.5)	300 $\pm$ 17.3	263 $\pm$ 16.2 (12.3)	140 $\pm$ 11.8	126 $\pm$ 11.2 (10.0)	150 $\pm$ 12.2	137 $\pm$ 11.7 (8.6)	500 $\pm$ 22.3	450 $\pm$ 21.2 (10.0)	400 $\pm$ 20.0	350 $\pm$ 18.7 (12.5)

* The percentage inhibition is shown in parentheses.

† Cytokine production by LPS-stimulated cells in the presence of anti-CD14 mAbs significantly ($P < 0.05$) less than with the corresponding control mAb.

Table 2. Effect of anti-CD11/18 mAbs on production of TNF- α , IL-6 and IL-8 by THP-1 cells stimulated by LPS or porins

THP-1 cells were stimulated for 24 h with LPS or porins in the presence of anti-CD11 mAb or anti-CD18 mAb and the corresponding control mAbs.

Stimulus (ng ml ⁻¹)	Mean concentration (pg ml ⁻¹) $\pm$ SD*											
	TNF- α †				IL-6†				IL-8†			
	Control mAb	CD11 mAb	Control mAb	CD18 mAb	Control mAb	CD11 mAb	Control mAb	CD18 mAb	Control mAb	CD11 mAb	Control mAb	CD18 mAb
LPS												
(1000)	4200 $\pm$ 64.8	3600 $\pm$ 60.0 (14.2)	4150 $\pm$ 64.4	3700 $\pm$ 60.8 (10.8)	2200 $\pm$ 46.9	1800 $\pm$ 42.4 (18.1)	2200 $\pm$ 46.9	1900 $\pm$ 43.5 (13.6)	5000 $\pm$ 70.7	4400 $\pm$ 66.3 (12.0)	5100 $\pm$ 71.4	4300 $\pm$ 65.5 (15.6)
(100)	2700 $\pm$ 51.9	2200 $\pm$ 46.9 (18.5)	2800 $\pm$ 52.9	2450 $\pm$ 49.4 (12.5)	1200 $\pm$ 34.6	950 $\pm$ 30.8 (20.8)	1100 $\pm$ 33.1	865 $\pm$ 29.4 (21.3)	3600 $\pm$ 60.0	2800 $\pm$ 52.9 (22.2)	3700 $\pm$ 60.8	2700 $\pm$ 51.9 (27.0)
(10)	350 $\pm$ 18.7	160 $\pm$ 12.6 (54.2)	300 $\pm$ 17.3	220 $\pm$ 14.8 (26.6)	700 $\pm$ 26.4	520 $\pm$ 22.8 (25.7)	750 $\pm$ 27.3	500 $\pm$ 22.3 (33.3)	1000 $\pm$ 31.6	650 $\pm$ 25.4 (35.0)	1100 $\pm$ 33.1	720 $\pm$ 26.8 (35.0)
Porins												
(5000)	2700 $\pm$ 51.9	2440 $\pm$ 49.3 (9.6)	2900 $\pm$ 53.8	2600 $\pm$ 50.9 (10.3)	1400 $\pm$ 37.4	1280 $\pm$ 35.7 (8.5)	1550 $\pm$ 39.3	1380 $\pm$ 37.1 (10.9)	3900 $\pm$ 62.4	3460 $\pm$ 58.8 (11.2)	3800 $\pm$ 61.6	3340 $\pm$ 57.7 (12.1)
(500)	1350 $\pm$ 36.7	1200 $\pm$ 34.6 (11.1)	1100 $\pm$ 33.1	950 $\pm$ 30.8 (13.6)	800 $\pm$ 28.2	700 $\pm$ 26.4 (12.5)	700 $\pm$ 26.4	600 $\pm$ 24.4 (14.2)	1900 $\pm$ 43.5	1650 $\pm$ 40.6 (13.1)	1800 $\pm$ 42.4	1600 $\pm$ 40.0 (11.1)
(50)	300 $\pm$ 17.3	230 $\pm$ 15.1 (23.3)	350 $\pm$ 18.7	270 $\pm$ 16.4 (22.8)	200 $\pm$ 14.1	150 $\pm$ 12.2 (25.0)	150 $\pm$ 12.2	110 $\pm$ 10.4 (26.6)	600 $\pm$ 24.4	450 $\pm$ 21.2 (25.0)	500 $\pm$ 22.3	400 $\pm$ 20.0 (20.0)

* The percentage inhibition is shown in parentheses.

† Cytokine production in the presence of anti-CD11/18 mAbs significantly ($P < 0.05$) less than with the corresponding control mAb.

with LPS through high-affinity attachment to the lipid A moiety, catalyses LPS recognition by mCD14, resulting in the generation of an LPS-induced proinflammatory signal (Galdiero *et al.*, 1990, 1993, 1994). The presence of serum had no influence on the ability of porins to stimulate cytokine production. This suggests that a different mechanism is induced in this process. These data confirm that the capacity of porins to induce

cytokine release is an intrinsic property, and is not due to the presence of trace amounts of LPS.

During our studies we have also confirmed that the biological effect persists after treatment of porins with polymyxin B, while it is neutralized when LPS is treated with polymyxin B.

Our data show an involvement of CD11a/CD18

integrins in signal transmission in porin-stimulated cells. The inhibition of those receptors induces only a small decrease in cytokine release, which may, however, be biologically significant. It is possible that responsive cells may have other porin-binding sites.

Porin-specific receptors are still unknown. Therefore, it is possible that porin stimulation is not due to binding to specific receptors, but is a consequence of the perturbation of the cell membrane lipoproteic phase, induced during absorption or porin penetration (Galdiero *et al.*, 1998; Tufano *et al.*, 1984). *In vivo*, during Gram-negative infection, porins and LPS do not act as single components, but act together as a lipoproteic complex (Hellman *et al.*, 2000; Vesý *et al.*, 2000).

Different Gram-negative bacteria have been shown to release several proteins that could act in combination with LPS to activate macrophages (Betz & Morrison, 1977; Galdiero *et al.*, 1988; Goldman *et al.*, 1981; Goodman & Sultz, 1979a, b; Morrison *et al.*, 1976; Sultz & Goodman, 1976). Endotoxin protein preparations have been shown to contain at least 12 proteins, ranging in size from 5 to 80 kDa (Goldman *et al.*, 1981). The predominant proteins present in endotoxin preparations were the porins, protein II and lipoprotein (Goldman *et al.*, 1981), the most abundant protein in the outer membrane of bacteria in the *Enterobacteriaceae* (Evans & Pollack, 1993; Sultz & Goodman, 1976). The LPS-associated protein complex can probably use different mechanisms of cell activation, which may or may not include specific receptors, as a consequence of the prevailing amounts of LPS or porins in such a complex. Whole bacterial cells, which express on their surface LPS and porins, use both CD14 (van Furth *et al.*, 1999) and CD11a/18 (Vazque-Torres & Ferric, 2000) when interacting with leukocytes. Our results show that purified porins and LPS interact in different ways with the cell surface, both leading to cellular stimulation. Our data indicate that CD14 and CD11a/18 are involved in cytokine responses to LPS, but only CD11a/18 is involved in those to porins, and to a much lesser extent. Anti-CD14 mAbs and anti-CD11a/18 mAbs are capable of blocking LPS uptake by cells expressing these receptors and thereby prevent the LPS-induced release of proinflammatory cytokines. The same mAbs are not able to neutralize the biological activity of the porins.

In view of our findings, adjunctive treatment of severe Gram-negative bacterial infection, with antibodies to specific receptors, may not be straightforward.

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Received 21 March 2001; revised 6 June 2001; accepted 13 June 2001.