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Anti-CD14 antibodies reduce responses of cultured human endothelial cells to endotoxin

E. J. U. VON ASMUTH, M. A. DENTENER, V. BAŽIL,* M. G. BOUMA, J. F. M. LEEUWENBERG & W. A. BUURMAN *Department of Surgery, Biomedical Center, University of Limburg, Maastricht, The Netherlands and *Harvard University, Department of Biochemistry and Molecular Biology, Cambridge, Massachusetts, U.S.A.*

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SUMMARY

Lipopolysaccharide (LPS) activates both myeloid and endothelial cells. Whereas CD14 has been shown to be involved in LPS recognition by myeloid cells, the mechanism responsible for the strong response of endothelial cells to LPS remains to be elucidated. The role of CD14 in this process was studied using CD14-specific antibodies (Ab). Anti-CD14 Ab inhibited LPS-induced interleukin-6 (IL-6) release and E-selectin expression by cultured human umbilical vein endothelial cells (HUVEC). Messenger RNA encoding IL-6 and E-selectin was reduced in parallel. The inhibitory effect of anti-CD14 Ab was epitope dependent, maximal at low LPS concentrations and dropping with increasing LPS doses. Anti-CD14 Ab did not affect endothelial cell activation induced by IL-1β, tumour necrosis factor-α (TNF-α) and phorbol 12-myristate 13-acetate (PMA). IL-6 release and E-selectin expression of HUVEC were strongly reduced when LPS activation was performed in the absence of serum, indicating involvement of serum components in LPS activation of HUVEC. Nevertheless, anti-CD14 Ab also blocked LPS-induced HUVEC activation in the absence of serum. Although the role of serum components in LPS activation remains to be elucidated, CD14 seems to be a key mediator in LPS-induced activation of endothelial cells.

INTRODUCTION

Endothelial cells are immunologically active cells and respond strongly to lipopolysaccharide (LPS) presence. *De novo* expression of the leucocyte adhesion molecule E-selectin,[1,2] and the release of interleukin-6 (IL-6),[3,4] a cytokine that is involved in inflammatory reaction, are part of this response. The mechanism of LPS-induced activation of endothelial cells, however, has not been clarified.

CD14 has been shown to be involved in LPS activation of monocytes,[5–7] alveolar macrophages[7] and neutrophils.[8,9] CD14 binds LPS complexed with an acute phase protein, designated LPS binding protein (LBP),[5,6] or with 'septin', a group of serum proteins that, in co-operation, facilitate LPS interaction with this receptor.[10] It seems that CD14 is also able to recognize LPS directly in the absence of serum.[7,8,11,12] It is generally accepted that LBP and 'septin' opsonize LPS, enhancing its affinity to CD14. CD14 is a glycosylphosphatidylinositol-anchored protein[13,14] that has originally been considered as a monocyte/macrophage-specific surface marker.[15] CD14 has also been reported to be expressed on neutrophils[9,16] and B cells,[17,18] although to a lesser extent compared to monocytes. Weak interaction of anti-CD14 monoclonal antibodies (mAb) with endothelial cells has been described previously,[19–21] suggesting that small amounts of CD14 are also present on endothelial cells. These data prompted us to study the role of CD14 in LPS-induced activation of endothelial cells.

Abbreviations: BCS, bovine calf serum; HS, human serum; HUVEC, human umbilical vein endothelial cells; LBP, LPS binding protein; LPS, lipopolysaccharide.

Correspondence: Dr W. A. Buurman, Dept. of Surgery, Biomedical Center, University of Limburg, PO Box 616, 6200 MD Maastricht, The Netherlands.

MATERIALS AND METHODS

Reagents and mAb

Recombinant human (rh) tumour necrosis factor-α (TNF-α) was kindly provided by BASF/Knoll AG (Ludwigshafen, Germany); rhIL-1β was a kind gift of Dr Gillis (Immunex, Seattle, WA); LPS (phenol extract, chromatographically purified from *Escherichia coli*, serotype 055:LB5) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St Louis, MO). Preparations of TNF, IL-1β and PMA were tested for endotoxin contamination by the chromogenic limulus amoebocyte lysate assay (Coatest, Kabi Diagnostica, Nyköping, Sweden), and were found to contain less than 5 pg/ml LPS in final solutions. The following CD14-reactive Ab were used: MEM-15 and MEM-18,[22] both IgG1; Cris6,[16] IgG1, kindly provided by Dr Vilella (Servei d'Immunologia, Hospital Clinic, Villarroel, Barcelona, Spain); UCHM1,[19] IgG2a, kindly pro-

vided by Dr Beverly (ICRF Human Tumor Immunology Group, University College Hospital, London, U.K.); BL-467,[23] IgG1 kindly provided by Dr Fiebig (Section of Biosciences, University of Leipzig, Leipzig, Germany). Rabbit anti-CD14 Ab were isolated from serum derived from CD14-immunized rabbits. Monoclonal antibody MOPC21, IgG1, a non-specific control mAb, was kindly provided by Celltech (Slough, U.K.).

Cells

Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase treatment of the human umbilical vein. The cells were cultured in fibronectin-coated (fibronectin kindly provided by Dr van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA) in RPMI-1640 (Gibco, Paisley, U.K.), supplemented with 10% pooled 0·2 μm filtered heat-inactivated human serum (HS) derived from the local blood bank, 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT), 50 μg/ml heparin (Sigma), 30 μg/ml endothelial growth supplement (Collaborative Research Incorporated, Bedford, MA) and antibiotics. Endothelial cells were characterized by their pavement-like monolayer morphology, by positive staining with mAb hec7 directed against PECAM-1,[24] generously provided by Dr Muller (Rockefeller University, NY), and by positive staining with the anti-E-selectin mAb ENA1[25] after 4 hr of incubation with TNF-α.

Induction of cytokine release

HUVEC of passage 3 were seeded at 10^4 cells/well in fibronectin-coated 96-well flat-bottom tissue culture plates (Costar) in culture medium, 1 day prior to stimulation. HUVEC were rinsed twice with RPMI-1640 and the medium was replaced by the appropriate agents in 200 μl/well RPMI-1640 supplemented with antibiotics, without growth factor, and with 10% HS (not heat-inactivated), or 0·1% BSA (endotoxin contamination $<0{\cdot}1$ ng/g; Sigma) in experiments in which serum-free medium was employed. Media were tested for endotoxin contamination in the chromogenic limulus amoebocyte lysate assay, and were found to contain less than 5 pg/ml LPS. Supernatants were harvested and kept at −20° until use in the IL-6 ELISA. Endothelial cell monolayers were washed, fixed with 0·05% glutaraldehyde for 10 min at room temperature, and kept at 4° until use in the E-selectin ELISA. Human monocytes were isolated as described previously[7] and incubated with the indicated reagents at 10^4 cells/well in flat-bottom tissue culture plates in 200 μl/well RPMI-1640 supplemented with 10% HS and antibiotics.

IL-6 ELISA

The IL-6 concentration in the culture supernatant was determined using a previously described sandwich ELISA for IL-6.[26] In short, 96-well immuno maxisorp plates (Nunc, Roskilde, Denmark) were coated with a newly developed murine mAb 5E1, specific for human IL-6. Recombinant human IL-6 (a generous gift from Professor Sebald, Physiologisch-Chemisches Institut der Universität, Würzburg, Germany) was used for standard titration curves. Test samples were added. Plates were incubated with polyclonal rabbit anti-human IL-6 Ab, followed by peroxidase-conjugated goat anti-rabbit IgG (Jackson, Westgrove, PA). *O*-phenylene-diamine (Sigma) was added as a substrate and photospectometry was performed at 492 nm. Standard titration curves were not influenced by the presence of serum in the sample buffer. The IL-6 ELISA had a lower detection limit of 10 pg/ml IL-6.

Detection of E-selectin expression

E-selectin expression was determined by ELISA using the E-selectin specific mAb ENA1,[25] followed by peroxidase-conjugated goat anti-mouse IgG. *O*-phenylene-diamine (Sigma) was added as a substrate and photospectometry was performed at 492 nm.

Preparation and analysis of RNA

HUVEC was grown and stimulated, as already described, in 150 cm^2 tissue culture flasks (Costar). Total cellular RNA was isolated as described previously.[27] RNA samples were treated as recommended by Amersham and applied onto a N^+ Membrane (Amersham International, Amersham, U.K.), using a Biodot apparatus (BioRad, Richmond, CA). cDNA probes were labelled with [^{32}P]dCTP using the random primer labelling kit of Boehringer Mannheim (Mannheim, Germany) and hybridized to the blot (10^6 c.p.m./ml) as described previously.[28] The IL-6 probe [1·3 kilobase (kb) HIII-*Eco*RI fragment] was kindly provided by Dr Aarden (CLB, Amsterdam, The Netherlands), the E-selectin probe (1·0 kb *Eco*RI–*Bg*lII fragment) was kindly provided by Celltech and the actin probe (1·3 *Pst* fragment) was kindly provided by Dr Berkvens (University of Leiden, Leiden, The Netherlands). Labelled bands were visualized by autoradiography.

RESULTS

Anti-CD14 Ab inhibit LPS-induced HUVEC responses

HUVEC monolayers were incubated with 1 μg/ml LPS in RPMI-1640 enriched with 10% HS and antibiotics. The influence of co-incubation with five murine anti-CD14 mAb, MEM-18, Cris6, UCHM1, MEM-15 and BL-467, and of serum of CD14-immunized rabbits (IgG concentration 10 μg/ml), on IL-6 release and on E-selectin expression by HUVEC was investigated. MEM-18, Cris6, MEM-15 and rabbit polyclonal anti-CD14 antibodies prevented LPS-induced IL-6 release and E-selectin expression by 80% or more. UCHM1 inhibited HUVEC responses less effectively, while BL-467 as well as a non-reactive control mAb did not influence HUVEC responses (Table 1). As a control, the same panel of Ab was tested for their influence on 100 pg/ml LPS-induced TNF and IL-6 release by human monocytes. The LPS-induced release of both cytokines was completely prevented by co-incubation with MEM-18 and Cris6, while MEM-15 and rabbit anti-CD14 Ab reduced both TNF and IL-6 release by more than 60% (Table 1). In parallel to the effect on LPS-induced HUVEC activation, mAb BL-467 hardly influenced LPS-induced monocyte TNF and IL-6 release (Table 1). None of the anti-CD14 antibodies induced HUVEC IL-6 release, HUVEC E-selectin expression or monocyte cytokine release in the absence of LPS (data not shown). MEM-18, which inhibited HUVEC IL-6 release and E-selectin expression at 0·2 μg/ml, with a maximal inhibition at 2 μg/ml (data not shown), was selected for further experiments.

Next, the influence of MEM-18 on HUVEC IL-6 and E-selectin mRNA expression induced by incubation with 1 μg/ml LPS for 2 hr, was determined. In the presence of MEM-18, substantially reduced amounts of mRNA encoding IL-6 and E-

Table 1. Anti-CD14 antibodies can inhibit LPS-induced responses of both HUVEC and human monocytes

	HUVEC		Human monocytes	
	IL-6 (ng/ml)	E-selectin (A 492 nm)	TNF (ng/ml)	IL-6 (ng/ml)
Unstimulated	0·1 ± 0·1	0·02 ± 0·02	0·02 ± 0·02	0·3 ± 0·1
LPS	11·2 ± 0·7	1·6 ± 0·08	1·4 ± 0·2	8·4 ± 0·5
LPS + MEM-18	0·9 ± 0·2	0·12 ± 0·04	0·1 ± 0·1	0·04 ± 0·01
LPS + Cris6	0·5 ± 0·2	0·09 ± 0·04	0·1 ± 0·1	0·02 ± 0·01
LPS + UCHM1	2·7 ± 0·7	0·28 ± 0·05	0·87 ± 0·08	5·0 ± 0·4
LPS + MEM-15	1·2 ± 0·2	0·22 ± 0·04	0·50 ± 0·04	3·2 ± 0·4
LPS + BL-467	12·4 ± 1·5	1·6 ± 0·1	0·97 ± 0·05	7·2 ± 0·6
LPS + rabbit anti-CD14	1·4 ± 0·3	0·19 ± 0·05	0·12 ± 0·07	1·5 ± 0·1
LPS + MOPC21	10·8 ± 1·0	1·5 ± 0·1	1·2 ± 0·2	8·3 ± 0·4

Influence of 10 μg/ml mAb and rabbit polyclonal Ab reactive with CD14 on HUVEC IL-6 release and E-selectin expression and monocyte TNF and IL-6 release induced by LPS. MOPC21, a non-reactive mAb of the IgG1 subclass, was used as a control. Cells were incubated in presence of LPS (monocytes with 100 pg/ml and HUVEC with 1 μg/ml LPS) and antibodies for 5 hr (to determine maximal E-selectin expression) and 18 hr (to determine cytokine release). Data are expressed as mean ± SD of four measurements.

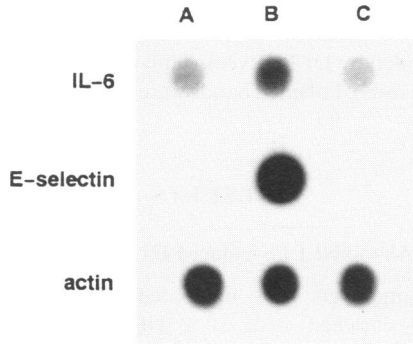

Figure 1. MEM-18 inhibits LPS-induced IL-6 and E-selectin mRNA expression in HUVEC. Messenger RNA was isolated from untreated HUVEC (A) and HUVEC stimulated for 2 hr with 1 μg/ml LPS in the absence (B) or presence (C) of 5 μg/ml MEM-18, in medium containing 10% HS. Parallel blots were run and hybridized with actin probe to compare the amount of RNA in different samples.

selectin were detected (Fig. 1), indicating interference of MEM-18 with LPS-induced IL-6 release and E-selectin expression on a pre-transcriptional level.

To investigate whether anti-CD14 Ab specifically inhibited HUVEC responses to LPS, we compared HUVEC responses to LPS, IL-1β, TNF-α and PMA in the presence and absence of MEM-18. Whereas MEM-18 induced a major reduction in E-selectin expression and IL-6 release in response to 5 ng/ml LPS and 5 μg/ml LPS, no clear influence was found in the responses of HUVEC monolayers stimulated with IL-1β, TNF-α and PMA (Fig. 2).

Inhibition of LPS-induced HUVEC IL-6 release and E-selectin expression is not abrogated in the absence of serum

To investigate whether serum presence is a prerequisite for CD14-mediated LPS activation of HUVEC, HUVEC monolayers were rinsed intensively with serum-free medium before the addition of LPS, to ensure maximal removal of soluble serum components. Monolayers were subsequently incubated in the presence or absence of 10% HS with LPS concentrations ranging from 1 ng/ml to 100 μg/ml in RPMI-1640 supplemented with 0·1% BSA. In the presence of serum, MEM-18 reduced IL-6 release and E-selectin expression in response to 1–100 ng/ml LPS for 90% or more. This reduction was partly overcome by stimulation with higher concentrations of LPS (Fig. 3). As reported previously[29] HUVEC showed diminished responses to LPS in the absence of serum (Fig. 3). MEM-18, however, still effectively reduced both LPS-induced IL-6 release and E-selectin expression in the absence of serum in the incubation medium (Fig. 3). The use of special serum-free endothelial cell culture medium (Endothelial-SFM, Gibco) instead of RPMI supplemented with bovine serum albumin (BSA), and additional attempts to remove serum proteins, either by repeating the wash procedure or by 24-hr culture in serum-free medium before washing and activation in new serum-free medium, did not prevent the inhibitory effects of MEM-18 co-incubation in the above-described experiment (data not shown).

DISCUSSION

We demonstrated that certain anti-CD14 Ab inhibited LPS-induced activation of cultured HUVEC, measured by induction of: (1) IL-6 release; (2) E-selectin surface expression, and (3) IL-6 and E-selectin mRNA expression. We also found that anti-CD14 mAb reduced LPS-induced IL-8 release, and surface expression of ICAM-1 and VCAM-1 (data not shown). On the basis of these results, we conclude that CD14 participates in the response of endothelial cells to LPS. A recent study of Frey *et al.*,[30] also demonstrates CD14 involvement in endothelial cell activation by LPS. This finding extends the immunological importance of CD14 in terms of its role in LPS-induced activation of not only haemopoietic, but also non-hemopoietic cells. Like others, we have found clear differences in the capacities of anti-CD14 Ab to block LPS-induced cellular activation.[5,30] Differences in both epitope specificity and affinity

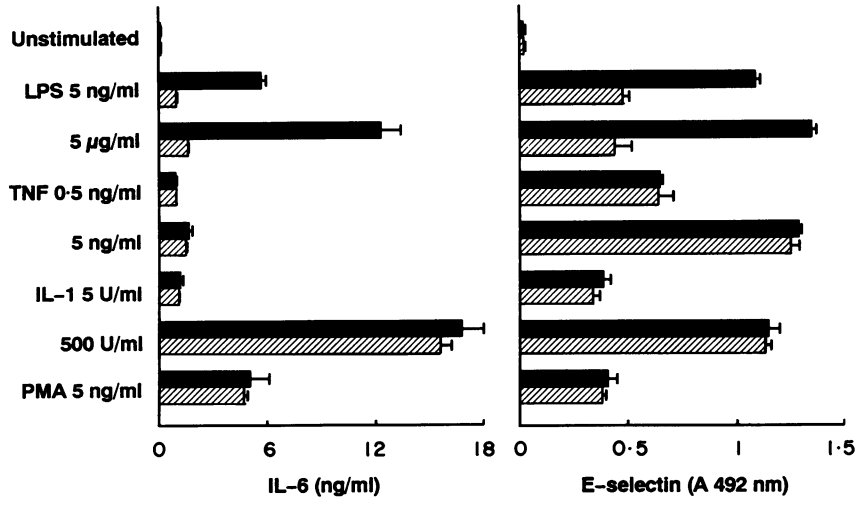

Figure 2. MEM-18 inhibits LPS-induced, but not TNF-, IL-1β- or PMA-induced IL-6 release and E-selectin expression. HUVEC were incubated with the indicated reagents in the absence (■) and presence (▨) of 5 μg/ml MEM-18. E-selectin expression and IL-6 release were measured after 5 and 18 hr respectively as indicated in Materials and Methods. Data are expressed as means ± SD of four measurements.

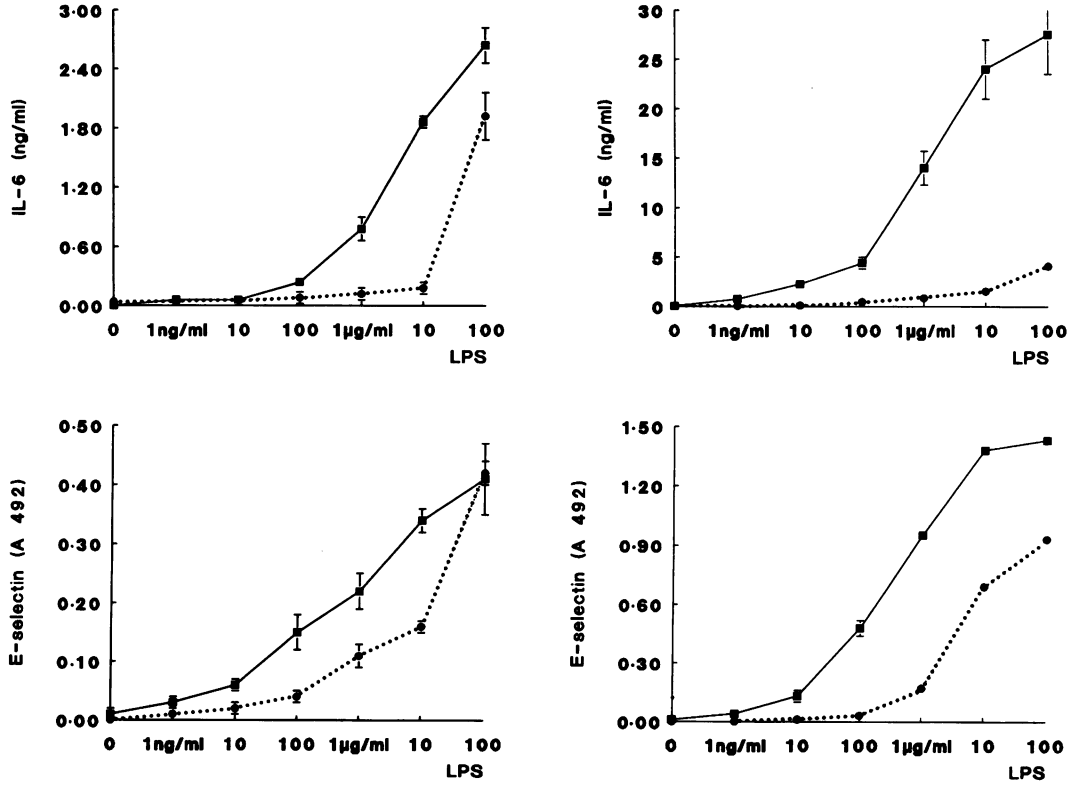

Figure 3. MEM-18 inhibits LPS-induced IL-6 release and E-selectin expression both in the absence (left graphs) and presence (right graphs) of 10% HS. HUVEC were incubated with different concentrations of LPS in the absence (■) or presence of (●) 5 μg/ml MEM-18 for 5 hr (to determine maximal E-selectin expression) and 18 hr (to determine IL-6 release) in the presence or absence of serum. Data are expressed as means ± SD of four measurements (where SD bars are not shown, they fall within the symbol).

of the individual anti-CD14 Ab may have contributed to this variation. Capacities of anti-CD14 Ab to block LPS-induced HUVEC activation were, however, similar to the capacities of these Ab to block LPS-induced activation of monocytes, indicating that the same CD14 epitope is involved in the activation process. The finding that anti-CD14 mAb do not inhibit endothelial cell responses to cytokines and PMA demonstrates that CD14 is specifically involved in the LPS-induced activation.

There are still several questions to be answered. First, what is

the role of CD14 in cellular activation by LPS? The fact that anti-CD14 mAb are able to inhibit LPS-induced activation of HUVEC in the absence of serum supports the concept that CD14 is able to interact directly with LPS.[7,8,11,12,30] It can, however, not be excluded that some serum proteins like LBP may remain bound to the endothelial cell surface and mediate LPS–CD14 interactions, despite the efforts to wash these proteins away. Another conclusion drawn from this observation is that CD14 must be associated with the endothelial cell membrane. The next question concerns the origin of CD14 present on endothelial cells. One possibility is that CD14 represents an endogenous membrane molecule. Since soluble CD14 is found in large amounts in normal plasma,[22,31] association of exogenous CD14 with a counter receptor on the surface of endothelial cells would be another option, which has recently been claimed.[30] Attempts to prevent association of exogenous CD14 with the endothelial cell surface by culturing HUVEC monolayers under serum-free conditions before LPS activation did not, however, prevent the inhibitory effect of anti-CD14 mAb. Whatever the answer, the quantity of cell surface-associated CD14 on HUVEC seems to be fairly low, as we were not able to detect surface CD14 using either flow cytometry analysis or precipitation from detergent lysate of surface iodinated HUVEC (data not shown). In contrast, we obtained positive results using both these methods for detection of surface CD14 on monocytes (data not shown). However, in addition to the functional evidence for the presence of CD14 on endothelial cells, weak staining of endothelial cells by anti-CD14 mAb has been reported.[19-21] Finally, it is not clear which serum proteins are involved in CD14-dependent activation of endothelial cells in the presence of serum. For monocytes, LBP and 'septin' have been identified as serum components which enhance CD14-mediated LPS activation.[5,6,10] The fact that the LPS-induced response of endothelial cells in the presence of serum is much stronger compared to serum-free conditions, clearly indicates that serum factors such as LBP participate in this process. The experiments addressing the questions mentioned above are currently being addressed in our laboratory.

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