INFECTION AND IMMUNITY, May 1996, p. 1762–1769
0019-9567/96/$04.00+0
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Vol. 64, No. 5

Human Monocyte CD14 Is Upregulated by Lipopolysaccharide

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Received 25 July 1995/Returned for modification 12 September 1995/Accepted 9 February 1996

Membrane CD14 is involved in lipopolysaccharide (LPS)-induced monocyte activation; it binds LPS, and antibodies against CD14 block the effects of low-dose LPS. It is unknown how LPS regulates its own receptor CD14 in vitro. Therefore, we investigated the effects of LPS on CD14 mRNA and membrane and soluble CD14 (mCD14 and sCD14, respectively) in human monocytes and macrophages. No changes were observed during the first 3 h of LPS stimulation. After 6 to 15 h, LPS weakly reduced CD14 mRNA and mCD14 and transiently enhanced sCD14 release. A 2-day incubation with LPS caused increases in the levels of CD14 mRNA (2-fold), mCD14 (2-fold), sCD14 (1.5-fold), and LPS-fluorescein isothiocyanate binding (1.5-fold); a 5-h incubation with LPS was sufficient to induce the late effects on mCD14 and sCD14. The maximal effect on mCD14 and sCD14 was reached with ≥1 ng of LPS per ml; the proportional distribution of the two sCD14 isoforms was not modified by LPS. Besides rough and smooth LPS, lipid A, heat-killed *Escherichia coli*, lipoteichoic acid, and *Staphylococcus aureus* cell wall extract (10 μg/ml) caused similar increases of mCD14. The LPS effect was blocked by polymyxin B but not by anti-tumor necrosis factor alpha, anti-interleukin-6, anti-gamma interferon, and anti-LPS-binding protein. LPS-induced tumor necrosis factor alpha production was abolished after a second 4-h challenge. In contrast, the LPS-induced increases CD14 mRNA, mCD14, and sCD14 were stronger and appeared earlier after a second LPS challenge. In conclusion, CD14 is transcriptionally upregulated by LPS and other bacterial cell wall constituents.

CD14 is a myeloid membrane glycoprotein which serves as a receptor for complexes of lipopolysaccharide (LPS) and LPS-binding protein (LBP) (31, 34). Membrane CD14 (mCD14) is involved in LPS-induced cytokine production, since this effect is blocked by pretreatment with antibodies against CD14 (6). In addition to the membrane-bound form, CD14 exists in two soluble isoforms (sCD14) (1). These molecules are released from stimulated and unstimulated myeloid cells (2, 17). sCD14 also binds LPS and transmits its action to cells which are devoid of mCD14, such as endothelial or epithelial cells (9). The precise role of CD14 in LPS-induced signal transduction is not yet clear. mCD14 is a glycosyl-phosphatidylinositol-anchored protein and as such lacks the intracellular sequence which is required for signalling (11). Moreover, the structure which serves as the receptor for complexes of LPS with sCD14 is not identified. It is unknown to what extent CD14 participates in LPS uptake, since this also can take place independently of CD14 (23). Because of their LPS-binding capacity, both mCD14 and sCD14 play a major role in the pathophysiology of endotoxemia and sepsis. The behavior of the CD14 receptor after LPS stimulation is an important factor within this field which has still not been investigated.

Earlier results regarding in vitro LPS effects on human CD14 are contradictory. In whole blood, 0.1 to 1 ng of LPS per ml enhances mCD14 expression in monocytes independently of protein synthesis. This effect is observed only 0.5 to 3 h after LPS stimulation (24). Other reports show that high LPS doses (100 ng/ml) downregulate mCD14 in human monocytes within 3 to 18 h (2, 33). In human mononuclear bone marrow cells, an 18-h incubation with LPS induces an increase of LPS-fluorescein isothiocyanate (FITC) binding and mCD14 expression (27). Similarly, in the monocytic cell line Mono-Mac 6, CD14 expression is enhanced by culture with LPS (16, 37). CD14 regulation in humans cannot be extrapolated from mouse studies, since mouse CD14 differs from human CD14 in at least two aspects. First, mouse blood plasma does not contain sCD14, whereas in humans, normal sCD14 concentrations of 2 to 3 μg/ml are measured (1, 8). Second, mouse Kupffer cells express little mCD14 and mCD14 expression increases upon in vivo LPS challenge. Human Kupffer cells have strong CD14 surface expression but their response to LPS challenge is unknown (8, 25). Up to now, human studies have focused on mCD14. Transcription of CD14 and sCD14 release in normal monocytes and macrophages have not been studied simultaneously. Neither the mechanism of receptor changes nor the effects of LPS relative to mCD14-mediated functions have been investigated.

Therefore, we performed time course and dose-response studies of the effects on CD14 mRNA, mCD14, and sCD14 of a single or repeated LPS stimulations in human monocytes and monocyte-derived macrophages. We studied the specificity and the mechanism of the CD14 changes and compared the LPS action on CD14 expression with that on cytokine release and on LPS uptake.

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MATERIALS AND METHODS

Reagents. *Escherichia coli* ATCC 25922 was grown overnight in Mueller-Hinton broth at 37°C and heat killed by 15-min boiling. LPS preparations (*Salmonella enterica* serovar typhimurium and LPS-FITC), lipoteichoic acid (from *Enterococcus faecalis*), bovine serum albumin (BSA), and laurylsarcosine were purchased from Sigma Chemical Co., (St. Louis, Mo.). *E. coli* Re LPS, *S. enterica* serovar abortusequi smooth LPS, lipid A, and *Staphylococcus aureus* cell wall extract were kind gifts from C. Galanos (Freiburg i.Br., Germany). Anti-tumor necrosis factor alpha (TNF-α) monoclonal antibodies (MAbs) were donated by Knoll (Ludwigshafen, Germany), and anti-gamma interferon (IFN-γ) antibodies were donated by H. Gallati (Hoffmann-La Roche, Basel, Switzerland). Goat anti-human anti-LBP antiserum and the CD14 cDNA probe was kindly provided

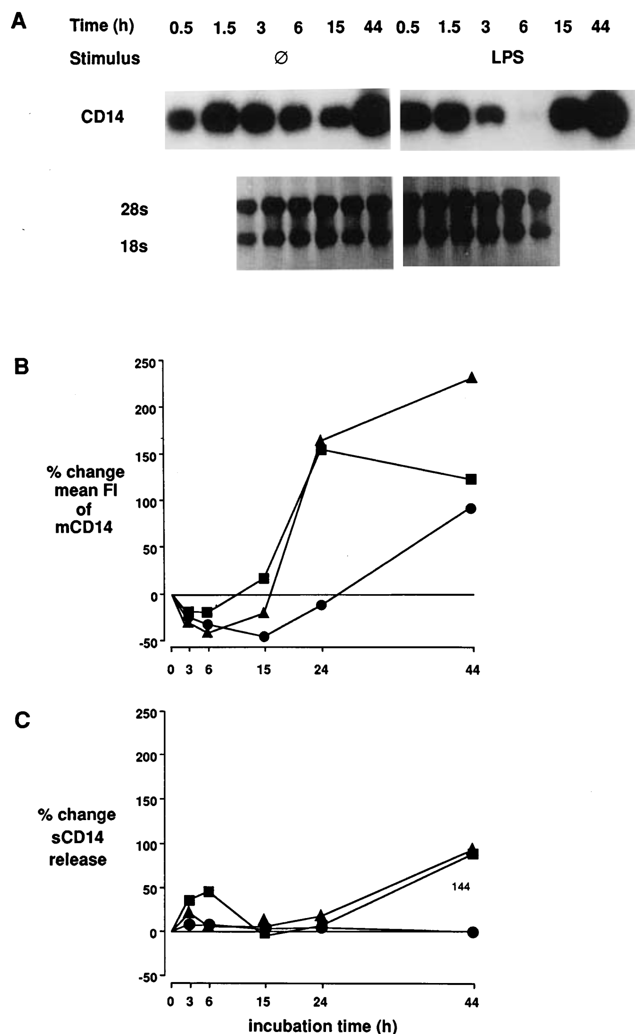

FIG. 1. Time course of LPS effects on monocyte CD14. (A) Northern blots of CD14 mRNA after 0.5-, 1.5-, 3-, 6-, 15-, and 44-h incubations of purified monocytes in medium with 5% heat-inactivated human serum alone (∅) or with 10 ng of *S. enterica* serovar typhimurium LPS per ml. Quantities of 18S and 28S RNA measured at the same time points are depicted as a control. Data from one of three similar experiments are shown. (B) mCD14. Results are expressed as percent change of mean FI in PBMC (●), purified monocytes (▲), or monocyte-derived macrophages (■) treated for 3 to 44 h with 10 ng of *S. enterica* serovar typhimurium LPS per ml as compared with untreated cells. (C) sCD14. Results are expressed as percent change of release induced by incubation of PBMC (●), monocytes (▲), or monocyte-derived macrophages (■) for 3 to 44 h with 10 ng of *S. enterica* serovar typhimurium LPS per ml compared with untreated cells. In panels B and C, data are means of two or three experiments.

by P. Tobias (Scripps Research Institute, La Jolla, Calif.), and Ficoll-Paque (density [d] = 1.077 g/ml), Percoll (d = 1.130 g/ml), and protein G-Sepharose (4-Fast-Flow) were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Polymyxin B was purchased from Behring Diagnostics (La Jolla, Calif.). The nitrocellulose membranes, molecular mass standards, alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG), and the chemiluminescent substrate adamantyl dioxetane phenyl phosphate were purchased from Bio-Rad (Glattbrugg, Switzerland). [^{32}P]dCTP (3,000 μCi/mmol) was purchased from Amersham; all other chemicals were purchased from Merck. All media and buffers were made up in pyrogen-free H_2O and filtered through a Diaflo PM 10 ultrafiltration membrane (molecular weight, >10,000) (Amicon Corporation, Danvers, Mass.) in order to eliminate endotoxin. The concentration of endotoxin in all reagents was below 0.01 ng/ml, as tested by the *Limulus* lysate assay (Coatest; Chromogenix, Mölndal, Sweden).

Preparation and culture of peripheral blood monocytes. Monocytes were purified as previously described (18, 20). Briefly, heparinized venous blood from healthy volunteers was deprived of platelet-rich plasma, diluted with phosphate-buffered saline (PBS), and layered on Ficoll-Paque for density gradient centrifugation. The peripheral blood mononuclear cells (PBMC) were harvested at the interface, washed, and resuspended in PBS. In order to obtain large numbers of monocytes, cells were elutriated in a Curamé 3000 centrifuge (Heraeus, Zürich, Switzerland). In experiments with small numbers of monocytes, cells were purified on a preformed continuous Percoll density gradient (20). Monocytes were cultured in RPMI medium supplemented with gentamicin (Life Technologies, Basel, Switzerland) and 5% heat-inactivated (30 min at 56°C, which does not destroy LBP bioactivity) human serum (pool of five samples from healthy individuals with blood group AB from the blood donor bank) in hydrophobic Teflon wells (Heraeus) for 0.5 to 44 h with or without stimuli. Monocyte-derived macrophages were obtained by culturing monocytes on Teflon membranes for 1 week with intermediate feeding of medium with 5% AB serum.

MAb and F(ab′)$_2$ fragments. The anti-CD14 MAbs 63D3 (IgG1, ATCC HB 44) and 3C10 (IgG2b, ATCC TIB 228) and a control mouse IgG2b MAb (ATCC, TIB 94) were isolated from hybridoma culture supernatants by protein G affinity chromatography under sterile and endotoxin-free conditions. 3C10 and 63D3 have strong and weak potencies, respectively, in blocking the LPS-induced superoxide response (19). The anti-CD14 antibody Leu-M3, which does not block LPS-induced TNF-α release, was purchased from Becton Dickinson (Mountain View, Calif.). My4-FITC was from Coulter (Luton, Great Britain), and the F(ab′)$_2$ fragment of fluoresceinated goat anti-mouse Ig was from Dianova GmbH (Hamburg, Germany).

Immunofluorescent staining and flow cytometry of cells. Monocytes were harvested from the culture by placing the dishes on ice and washing and adjusting the cells to a concentration of 10^6 cells/100 μl in Hanks balanced salt solution. After the Fc receptors were blocked with normal rabbit serum, the anti-CD14 antibody Leu-M3 was added for 30 min at 4°C. Cells were then washed and incubated for 30 min at 4°C with the fluoresceinated F(ab′)$_2$ fragment of goat anti-mouse Ig. After washing and fixation in 0.2% paraformaldehyde, cells were analyzed in a fluorescence-activated cell sorter. Fluorescence was gated from monocytes as judged by their forward and side scatter. LPS-FITC binding was measured after staining for 30 min at 37°C with 1 μg of LPS-FITC per 10^6 cells either in 5% AB serum or in medium with 2% human serum albumin (for negative control binding in the absence of LBP). We have previously reported that mCD14 expression and sCD14 release increased in unstimulated monocytes during in vitro culture (20). Therefore, in the present study we measured the percent changes of mean fluorescence intensity (FI) in LPS-treated cells compared with simultaneously incubated untreated cells (the value of untreated cells was taken to be 0% change).

Assay for sCD14. Concentrations of sCD14 in culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). Ninety-six-well polystyrene plates were coated overnight at room temperature with 2-μg/ml anti-CD14 antibody 63D3 in 0.1 M sodium phosphate buffer, pH 6.5. Plates were washed, and nonspecific binding was blocked for 1 h at room temperature with 0.2 M Tris-HCl buffer (pH 7.5) containing 1% BSA. Standards (0.3 to 10 ng of recombinant sCD14 or sCD14 per ml isolated from serum by affinity chromatography) or samples and peroxidase-coupled 3C10 (500 ng/ml, diluted in 0.1 M Tris buffer [pH 6.5] and 5% fetal calf serum) were added and incubated for 24 h at room temperature. Plates were washed, and captured sCD14 was detected with tetramethylbenzidine. The A_{450} was read in an ELISA reader (Molecular Devices, Palo Alto, Calif.). Heat inactivation of normal serum at 56°C for 30 min destroyed sCD14 immunoreactivity.

Sodium dodecyl sulfate (SDS)-PAGE and Western blotting (immunoblotting). Culture supernatants were denatured by boiling in sample buffer. Denatured proteins and prestained molecular mass standards were electrophoresed in 7.5% polyacrylamide gels under nonreducing conditions and were transferred on Immunlite membranes presoaked in 25 mM Tris–192 mM glycine transfer buffer (pH 8.3) (TBS) by using a wet transblot system. Membranes were incubated overnight at 4°C in TBS containing 7.5% dry milk to reduce nonspecific protein binding and were washed twice with TBS-Tween 0.05% (TTBS). They were then incubated for 3 h at room temperature on a rocking platform with anti-CD14 MAb 3C10 and diluted 1:400 in TTBS containing 1% dry milk. After three washes in TTBS, the membranes were incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG in TTBS containing 1% dry milk. The membranes were then washed three times with TTBS, incubated for 5 min with 5 ml of chemiluminescent substrate solution, and exposed to Kodak X-Omat films for 10 to 30 min.

Northern (RNA) blot analysis. Northern blotting was performed as previously described (14). Briefly, monocytes (3 × 10^6 per well) were lysed in 150 μl of guanidinium isothiocyanate buffer containing 0.5% *N*-laurylsarcosine, 25 mM sodium citrate, and 0.1 M 2-mercaptoethanol at pH 7; lysis was followed by addition of 15 μl of 3 M sodium acetate, pH 4. Total cellular RNA was recovered from monocytes by phenol-chloroform extraction and isopropanol precipitation. Five micrograms of denatured RNA was size fractionated on a 2% agarose gel. The sample buffer contained 39% formamide, 15% formaldehyde, 8% ethidium bromide, and 8% 10× morpholinepropanesulfonic acid buffer. One microliter of running buffer was added to 10 μl of sample containing 2.5-mg/ml bromphenol blue, 50% glycerin, and 10 mM EDTA. Capillary blot transfer onto nylon membranes was performed in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7) overnight. RNA was fixed by UV cross-linking and baking for 2 h at 80°C. Prehybridization was performed for 1 h at 65°C in 2× SSC–2% Den-

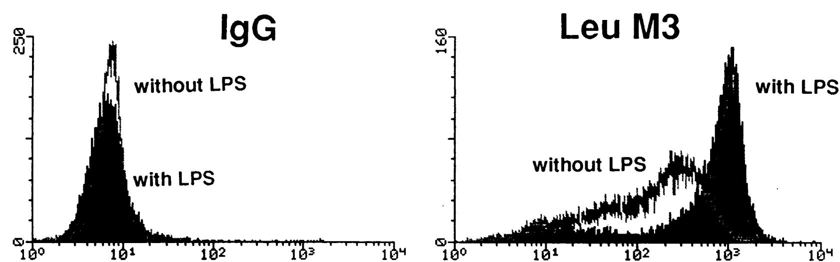

FIG. 2. mCD14 expression in response to LPS. Representative histograms of mCD14 fluorescence (Leu M3) and isotype control (IgG) staining in unstimulated and LPS-treated (10 ng of LPS *S. enterica* serovar typhimurium per ml) monocyte cultures after a 44-h incubation.

hardt's solution–2.5% dextran sulfate–0.1% SDS–0.1% $Na_2H_2P_2O_7$–2 mM EDTA–30-μg/ml salmon sperm DNA. Fifty nanograms of the cDNA probe was random prime labeled by incubation with a mixture containing 5 μl of [^{32}P]dCTP (10 μCi/10 μl), 10 μl of reagent mix, and 1 μl of Klenow enzyme. The probe was isolated on a Sephadex G-50 column. The blots were then hybridized with the labeled probe overnight at 65°C in the buffer described above. After three washes, once each in 2× SSC, 1× SSC, and 0.4× SSC, the blots were dried and exposed to Kodak X-ray films with an intensifying screen at −70°C.

RESULTS

Kinetics of LPS effects on CD14. PBMC, purified monocytes, and monocyte-derived macrophages were incubated with 10 ng of LPS per ml for various time periods between 0.5 and 44 h, and the effects on CD14 mRNA, mCD14 expression, and sCD14 release were assessed. In Fig. 1, results are expressed as percent change in LPS-treated cells compared with simultaneously cultured untreated cells. Figure 1A shows that the level of CD14 mRNA was reduced after 6 h but increased at 15 to 44 h in LPS-treated monocytes compared with unstimulated monocytes. This phenomenon was consistently found with monocytes from three different volunteers. The amounts of RNA analyzed in control and LPS cultures were similar in all samples, as shown by gels of 18S and 28S RNA. Figure 1B shows that mCD14 protein expression was weakly reduced by LPS treatment for a few hours and strongly enhanced by a 2-day incubation with LPS. The maximal reduction was 50%. The reduction was stronger and lasted longer in PBMC than in monocytes and macrophages. The increase in mCD14 expression appeared after 1 day in purified monocytes and macrophages but only after 2 days in PBMC. After 44 h, there was a ≥2-fold increase in the level of mCD14. Figure 2 shows representative histograms of untreated and LPS-treated monocytes after a 44-h exposure. As shown in Fig. 1C, the sCD14 release from monocytes and macrophages doubled after a 2-day LPS incubation. Spontaneous sCD14 release amounted to 66, 93, and 419 ng/10^6 cells per 44 h in PBMC, monocytes, and macrophages, respectively. Interestingly, in monocytes, and more clearly in macrophages, the late effect of LPS was preceded by an early transient enhancement of sCD14 release during the first 3 to 6 h of LPS stimulation. To detect very early changes, we measured (in separate experiments) mCD14 expression in PBMC or whole blood after a 15- to 180-min incubation with 10 ng of LPS per ml. As expected from the mRNA results, we did not find any immediate LPS-induced changes in mCD14 expression (data not shown).

In view of the late increases in mCD14 and sCD14 expression, it was interesting to know the minimal necessary time of incubation with LPS. For this purpose, monocytes were exposed to LPS for either 30 min or 5 h, and the supernatant was then replaced by fresh medium for the next 43.5 or 39 h, respectively, before CD14 measurement. There was no significant change in mean CD14 fluorescence when the LPS was washed off after 30 min. In contrast, the 5-h incubation of cells with LPS, washing, and further culture in medium was sufficient for a significant increase in mean CD14 fluorescence to occur (149% ± 20%; $n = 6$). In addition, similar to its action on mCD14, LPS enhanced sCD14 release only when it was added to the cells for more than 30 min (46% ± 7%; $n = 6$). These observations indicate that a prolonged contact of LPS with cells was necessary for the LPS effects on mCD14 and sCD14.

Dose-response curve of LPS effects on CD14. Our kinetic studies showed a weak downregulation of monocyte mCD14 until 15 h of stimulation with LPS and a strong upregulation after a 2-day incubation with LPS. In order to know the potency of the LPS effects, mCD14 expression and sCD14 release were measured after incubation of cells with increasing concentrations of LPS for either 15 h (early effect) or 44 h (late effect). As shown in Fig. 3, the LPS responsiveness of the three cell populations differed between the early and late effects. After 15 h, 100 ng of LPS per ml caused a nonsignificant decrease of mCD14 in PBMC and in monocytes and a slight increase in macrophages. The late effect of LPS on mCD14 was stronger in monocytes and in macrophages than in PBMC. In monocytes, it was maximal with ≥1 ng of LPS per ml. In macrophages, it was further increased by LPS concentrations up to 100 ng/ml. The increase in mCD14 was independent of the type of anti-CD14 antibody used to detect the membrane antigen. The increases in mCD14 expression were similar with the antibodies that block function, i.e., 3C10 (101%) or My-4 (112%), and those that do not block LPS binding or function, i.e., Leu-M3 (136%) and RoMo1 (111%). LPS-induced changes in sCD14 release were minimal in PBMC. In monocytes, a 44% increase of sCD14 release was observed after a 44-h incubation with 1 ng of LPS per ml. LPS-treated macrophages released a much larger maximal amount of sCD14 (383%) after 40 h.

Effect of LPS on sCD14 isoforms. sCD14 is released from monocytes in two isoforms of 49 and 55 kDa; the former is produced by limited proteolysis and the latter is directly derived from the intracellular space (4, 7, 15, 21). Since we found only the larger isoform in the sera of sepsis patients with high levels of serum sCD14 (21), we investigated whether LPS affects the generation of the CD14 isoforms in vitro. For this purpose, we compared sCD14 in Western blots of monocyte culture supernatants from LPS- and medium-treated cells. In supernatants of both LPS-treated and control cells, the 55-kDa form predominated; in both samples, only a very weak 49-kDa band was detected (data not shown).

Specificity of LPS effects on CD14. The specificity of the LPS effects on CD14 was tested by comparing various LPS preparations. The results are shown in Table 1. A 44-h incubation with 10 ng of *S. enterica* serovar typhimurium LPS per ml

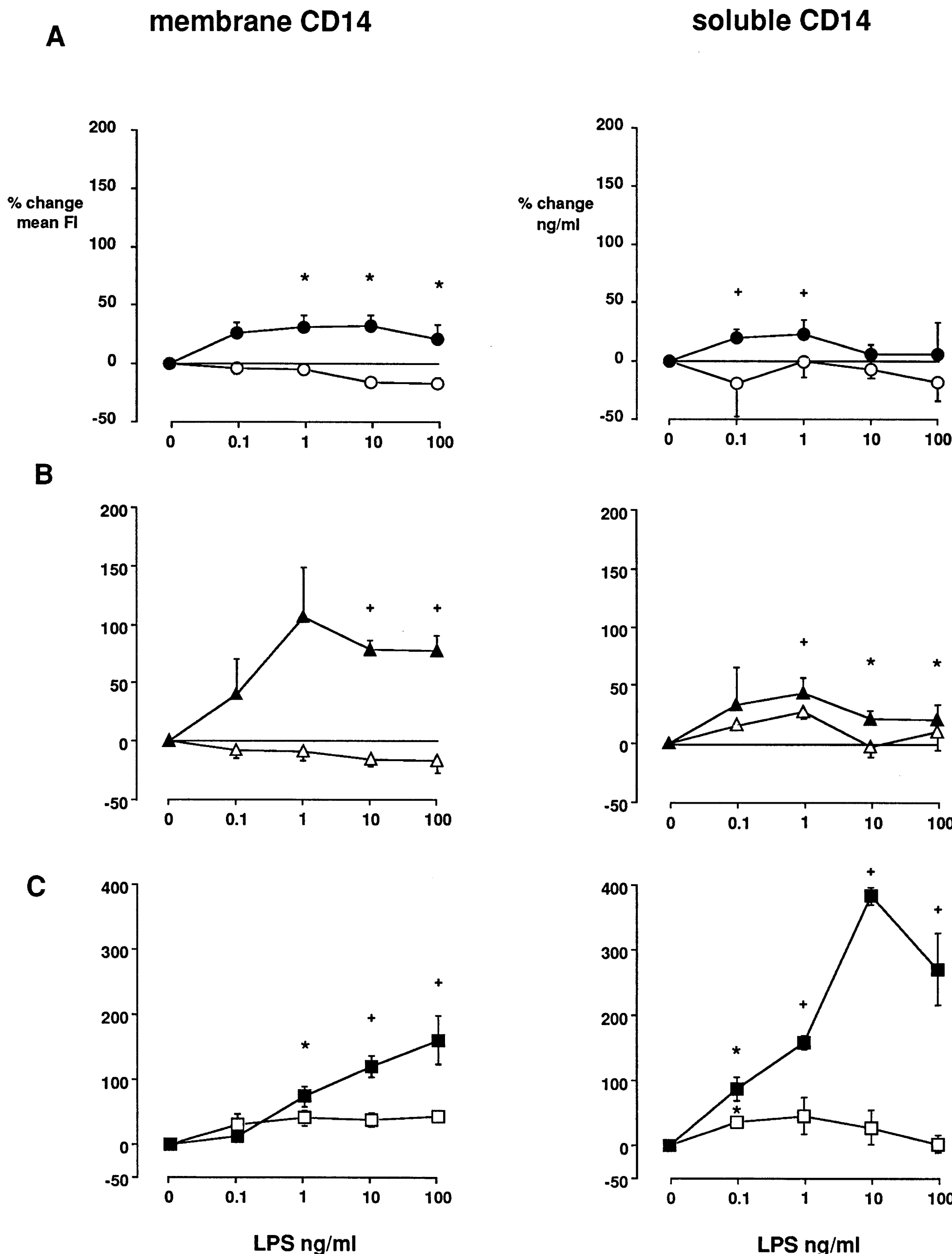

FIG. 3. Dose-response curves of LPS effects on mCD14 and sCD14. PBMC (A), purified monocytes (B), and monocyte-derived macrophages (C) were incubated for 15 h (open symbols) or 44 h (closed symbols), either with or without 0.1 to 100 ng of *S. enterica* serovar typhimurium LPS per ml. After the incubation period, the mean FI of mCD14 and the amount of sCD14 release (ng/ml) were measured by immunofluorescence and ELISA, respectively. Data are means ± standard errors of the means of 3 to 14 experiments and are expressed as percent change of mCD14 and sCD14 in LPS-treated cells as compared with untreated cells. Statistical analysis was performed by using the unpaired t test. *, $P < 0.05$; +, $P < 0.01$.

markedly increased mCD14 expression in monocytes. At the same concentration, the *S. enterica* serovar abortusequi smooth-LPS preparation and the *E. coli* Re rough-LPS preparation caused smaller increases of mCD14. Lipid A was still less potent and enhanced mCD14 expression only at a high dose (1 μg/ml). Heat-killed *E. coli* in a concentration yielding 10 ng of LPS per ml (corresponding to 10^5 CFU/ml) induced an effect similar to that of the purified LPS preparations. Other bacterial cell wall constituents were tested after they were shown to be free of LPS. Lipoteichoic acid and *S. aureus* cell wall extract similarly enhanced mCD14, although a higher concentration (10 μg/ml) was required. The 2-day incubation with 10 μg of *S. aureus* cell wall extract per ml led also to an enhanced expression of CD14 mRNA (data not shown). The kinetic studies and the dose-response curves (Fig. 1 and 3) indicate that the LPS effect on sCD14 was weaker than that on mCD14. Results in Table 1 confirm this finding. After a 2-day incubation with 10 ng of *S. enterica* serovar typhimurium LPS per ml, sCD14 release was enhanced by 46% ± 8%. The other smooth- and rough-LPS preparations, as well as heat-killed *E.*

TABLE 1. Effects of different bacterial stimuli on mCD14 expression and CD14 release from monocytes[a]

Stimulus	Dose (μg/ml)	mCD14 % change mean FI[b]	sCD14 % change release[b]
S. enterica serovar typhimurium LPS	0.01	123 ± 22	46 ± 8
S. enterica serovar abortusequi LPS	0.01	70 ± 11	10 ± 14
E. coli Re LPS	0.01	55 ± 11	11 ± 11
Lipid A	0.1	19 ± 29	19 ± 23
	1.0	111 ± 17	56 ± 18
Heat-killed *E. coli*	0.01[c]	48 ± 4	35 ± 25
Lipoteichoic acid	10	51 ± 9	26 ± 18
Lipoteichoic acid + polymyxin B	10/10	43 ± 11	30 ± 10
S. aureus cell wall extract	10	77 ± 15	40 ± 15
S. aureus + polymyxin B	10/10	95 ± 17	39 ± 7

[a] Purified monocytes (2×10^6) were incubated for 44 h with the stimuli in medium with 5% AB serum. The supernatant was harvested and frozen for sCD14 determination, and the cells were collected on ice and stained with Leu-M3 by indirect immunofluorescence.

[b] Percent change of mCD14 expression (mean FI) and sCD14 release in treated compared with untreated cells. Data are means ± standard errors of the means from 26 experiments with *S. enterica* serovar typhimurium LPS and from 3 to 6 experiments with the other stimuli.

[c] LPS concentration as determined in 10^5 CFUs of heat-killed *E. coli* ATCC 25922 by a *Limulus* assay.

coli, did not enhance sCD14 liberation. In contrast, lipid A, lipoteichoic acid, and *S. aureus* cell wall extract caused a modest increase of sCD14 release. The effects of the gram-positive cell wall components were not due to LPS since they were not inhibited by polymyxin B.

Other markers of LPS effects on CD14. Since mCD14 is not only regulated by but also binds LPS in the presence of serum containing LBP (29), the measurement of fluoresceinated-LPS binding is another means to assess the effect of LPS on mCD14. Therefore, LPS-FITC binding was quantified after 15- and 44-h incubations of monocytes with 10 ng of *S. enterica* serovar typhimurium LPS per ml and washing. At 15 h, the intensities of LPS fluorescence were similar in LPS-pretreated and untreated cells (29% ± 3% versus 28% ± 7%, $n = 3$). In contrast, at 44 h LPS-pretreated monocytes had a 1.6-fold increase in LPS-FITC binding (55 ± 7 versus 34 ± 8, $n = 3$).

Mechanism of LPS effects on CD14. For the blocking experiments we investigated the question of whether the changes in mCD14 expression were due to LPS itself or to an intermediate product induced by LPS. The results are summarized in Table 2. To document that LPS was the primary causative agent, polymyxin B was preincubated with LPS before addition to the cultures. Polymyxin B alone did not increase CD14 but prevented the action of LPS on mCD14. Because LPS is known to induce TNF-α and interleukin-6 (IL-6) within 4 h (5, 10), and since we have shown above that LPS induced the late CD14 changes when the stimulus was present for 5 h, we investigated the potential role of TNF-α or IL-6 in the LPS effect on mCD14. Table 2 shows that neither anti-TNF-α nor anti-IL-6 was able to block the increase in mCD14 expression. IFN-γ is released from lymphocytes in PBMC in response to LPS (3) and is known to downregulate mCD14 (18). Therefore, IFN-γ may counteract the effect of LPS in PBMC. Indeed, in PBMC the effect of LPS was much larger in the presence of anti-IFN-γ (373% ± 51%) than in its absence (55% ± 6% [data not shown]). Since monocytes do not produce IFN-γ, anti-IFN-γ did not modulate the LPS-induced changes in mCD14 expression in purified monocytes. Although LBP is required for rapid high-affinity binding of LPS to human monocyte CD14 and for induction of cytokines at low LPS doses (12), the slowly appearing increase in mCD14 did not require the presence of LBP, since the increase was similar whether LPS was added in serum with or without anti-LBP. Many LPS effects are blocked by anti-CD14 antibodies (6, 22). We therefore tested whether the LPS action on its own receptor was abolished by preincubation of monocytes with anti-CD14 antibodies. We have shown that the LPS effect on CD14 was similar whether Leu-M3 or 3C10 was used. We therefore used for these blocking experiments and for staining the antibody 3C10, which blocks LPS-induced functions (34). At a concentration of 50 μg of 3C10 per ml, 41% of the LPS-effect was blocked. In contrast, 50 μg of control mouse IgG per ml did not modify LPS activity.

LPS-induced CD14 changes and adaptation. It is known that CD14 is involved in LPS-induced TNF-α production (34). However, it is not clear whether the upregulation of mCD14 modulates the TNF-α response. This question was addressed by two types of experiments. First, the time course of TNF-α production induced by 10 ng of LPS per ml was compared with that of the CD14 changes. The TNF-α response was already detectable after 1.5 h (410 versus 14 pg/ml in unstimulated cells). The response increased between 3 and 6 h, was maximal after 15 h (14,467 versus 58 pg/ml in unstimulated cells), and declined up to 44 h (9,311 versus 0 pg/ml in unstimulated cells). Obviously this was a more rapid time course than the one for mCD14 expression in response to LPS (Fig. 1). Therefore, the upregulation of mCD14 follows the downregulation of TNF-α. In a second set of experiments, we investigated the CD14 changes in conditions under which the TNF-α response was adapted. For this purpose, monocytes were incubated for 44 h in medium alone or with a low dose of LPS (10 ng/ml), washed, and then challenged with a high LPS dose (100 ng/ml) for another 4 h. The results of these experiments are shown in Fig. 4. Figure 4A shows the TNF-α release in LPS-pretreated and untreated cultures. Adaptation was observed, since the 4-h rechallenge with a high dose of LPS induced 39 times less TNF-α than was in cells pretreated with medium alone. The LPS effect on CD14 mRNA and CD14 protein was different from that on TNF-α, and the response was maintained in cells after a second LPS stimulation (Fig. 4B and C). mRNA ex-

TABLE 2. Effects of polymyxin B and antibodies on LPS-induced CD14 changes in monocytes[a]

Blocker	Dose[b]	% change mean FI[c]		No. of expt
		Without LPS	With LPS[d]	
None		0	123 ± 22	28
Polymyxin B	10	2 ± 18	16 ± 18[e]	5
Anti-TNF	10	−5 ± 10	150 ± 67	4
Anti-IL-6	10	−4 ± 15	129 ± 49	4
Anti-IFN-γ	10	−7 ± 16	87 ± 35	4
Anti-LBP	1:10	14	146	2
Anti-CD14	50	40 ± 13	73 ± 40[e]	5
Control mouse IgG	50	−14 ± 10	121 ± 31	4

[a] Monocytes were preincubated for 10 min at 37°C with polymyxin B or antibodies before addition of LPS culture for 44 h. Supernatants were harvested for analysis of sCD14, and cells were stained by indirect immunofluorescence with the anti-CD14 antibody Leu-M3 and analyzed with a fluorescence-activated cell sorter.

[b] Units are micrograms per milliliter except for 1:10 concentration of anti-LBP. Goat anti-human anti-LBP antiserum (1:10) was preincubated with serum for 10 min at 37°C, and then cells were cultured with or without LPS in 5% of the pretreated serum.

[c] Data are means ± standard errors of the means.

[d] *S. enterica* serovar typhimurium LPS (10 ng/ml).

[e] Significantly different at the 5% level from value with LPS alone (paired *t* test).

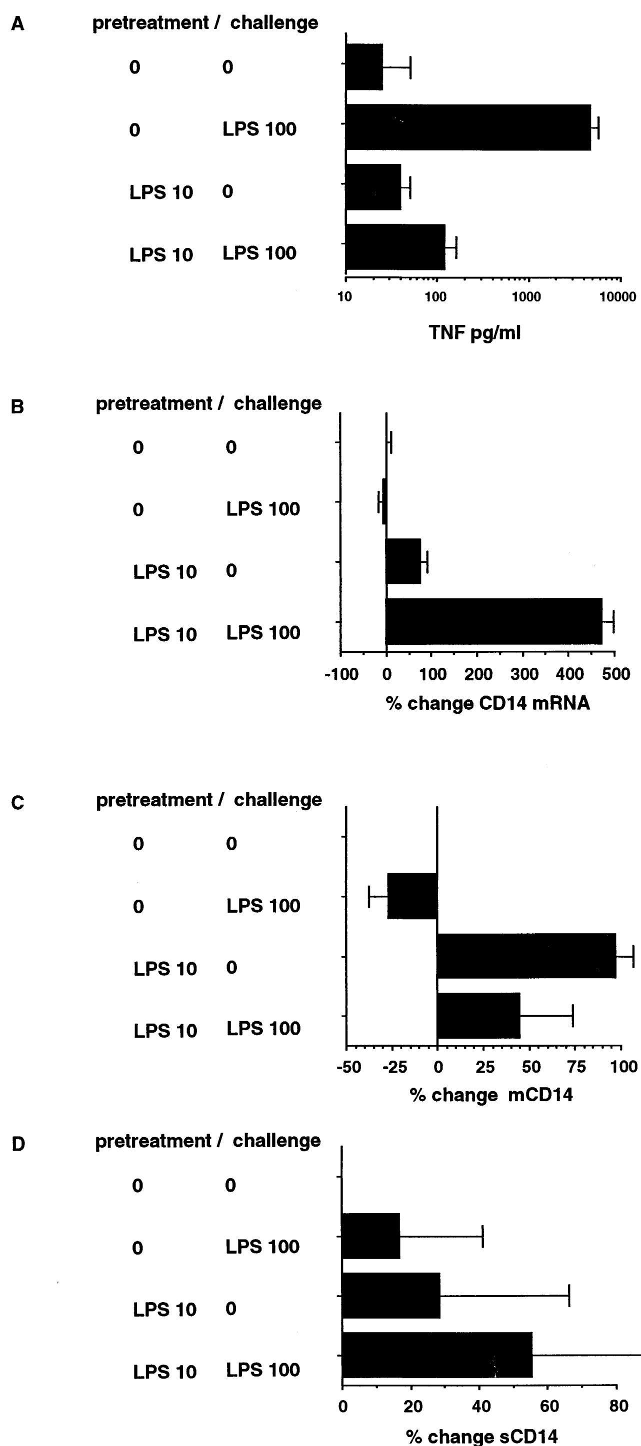

FIG. 4. LPS-induced TNF-α release and CD14 expression. (A) TNF-α release from untreated monocytes and monocytes treated and/or challenged with LPS. (B, C, and D) Changes in CD14 expression in monocytes pretreated with 10 ng of *S. enterica* serovar typhimurium LPS per ml for 44 h and challenged with 100 ng of LPS per ml for 4 h. (B) Percent changes of CD14 mRNA; (C) percent change in mean FI of mCD14; (D) percent change in quantitative sCD14 release in cells pretreated and/or challenged with LPS compared with cells treated and challenged with medium alone. Data are means ± standard errors of the means of four experiments.

pression was strongly increased in LPS-pretreated and challenged cells compared with those cells which were only pretreated or only challenged. Similarly, mCD14 expression was higher in cells which had been exposed to LPS twice than in cells which had only a 4-h LPS challenge. Finally, even sCD14 release was higher in those cells which had been stimulated twice with LPS than in those cells which had only been exposed to high-dose LPS during challenge (Fig. 4D). In order to test the influence of LPS uptake on the differential responses of TNF-α and CD14, endotoxin concentrations were measured in the supernatants collected during the adaptation experiments. After a 4-h treatment with 10 ng of LPS per ml, 1.45 ng of LPS per ml (i.e., 14.5% of the initial amount) was detected in the monocyte supernatant. The amount of LPS remaining in the supernatant of monocytes after a 4-h incubation with 100 ng of LPS per ml was 13.8 ± 1.9 ng/ml (i.e., 13.8%). Similarly, in the supernatant of repeatedly challenged monocytes (challenge with 100 ng of LPS per ml after pretreatment with 10 ng of LPS per ml), 11.8 ± 1.6 ng of LPS per ml (i.e., 11.8%) could be detected. Thus, the variation of mCD14 expression after LPS stimulation was not associated with an alteration of LPS uptake.

DISCUSSION

The results of this investigation show a slow biphasic change in the pattern of mCD14 expression in human monocytes after in vitro incubation with LPS. At an early time point there was a reduction, and subsequently there was a strong increase, in the expression of CD14 mRNA and mCD14. In contrast, sCD14 was released at a time point when CD14 mRNA was downregulated. These early monocyte membrane changes are similar to the results of Wright et al. (34) and Bazil and Strominger (2). Bazil and Strominger (2) explained their observation as a shedding of sCD14 from the membrane. In addition, our results show a reduction in CD14 mRNA. We did not investigate whether this was due to an accelerated degradation or a diminished synthesis of CD14 mRNA. Furthermore, the signal which alters transcription is unknown. Thus, LPS simultaneously caused a reduction of its membrane binding site and an enhancement of its soluble binding site. The functional consequences of these quantitative receptor changes remain to be investigated. The decrease in mRNA, together with a reduced expression of mCD14 and a transient increase in sCD14 release, render it unlikely that LPS caused CD14 internalization. The early disappearance of mCD14 was much more evident in PBMC than in purified monocytes and was only modest in monocyte-derived macrophages. In contrast, the early enhancement of sCD14 release was absent in PBMC, weak in monocytes, and clearly evident in macrophages. This suggests that early LPS effects on CD14 are influenced by at least two factors, namely, the presence of lymphocytes and the degree of differentiation. The modulation by environmental factors may explain the variable results obtained by different authors testing for mCD14 at 3 to 18 h after LPS challenge (2, 24, 33).

The increase of CD14 after a 2-day incubation with LPS was a major finding. The LPS effect on mCD14 expression was greater than on sCD14 release in all cell preparations, and the phenomenon was slightly different according to cell type. The potency of LPS was higher in monocytes than in PBMC or macrophages, but the maximal effect was higher in macrophages. We documented that in purified monocytes, the increase in the expression of mCD14 and sCD14 protein was due to an enhanced CD14 transcription. A comparable effect was observed after 2 days of LPS treatment in the monocytic cell

line Mono-Mac 6. In these cells the change in the expression of CD14 was associated with the differentiation induced by LPS (36). Similarly, LPS was described to induce LPS receptors in human bone marrow cells, as measured by LPS-FITC binding or by anti-CD14 antibodies (27). Our findings show that LPS enhanced CD14 independently of the degree of maturation, in monocytes as well as in macrophages, and this finding was corroborated by measurement of membrane CD14 fluorescence as well as LPS-FITC binding.

The slowly appearing changes in CD14 expression may have a broader physiological significance, since they were caused not only by purified LPS. Heat-killed *E. coli*, cell wall extracts from *S. aureus*, and the cell wall component lipoteichoic acid had effects similar to those produced by LPS. Cell wall components from gram-positive organisms did not act by contaminating LPS, since they did not contain any endotoxin, as determined by a *Limulus* assay, and polymyxin B did not block their action. The fact that cell wall components from gram-positive bacteria were able to induce an effect on CD14 suggests that CD14 has a broad specificity for natural ligands. This is in agreement with earlier observations. Indeed, the W-1 antigen of *Blastomyces dermatitidis*, acylpolygalactoside from *Klebsiella pneumoniae*, and streptococcal cell wall polysaccharides were found to bind to monocyte CD14 (13, 26, 30). In addition, lipoarabinomannan, peptidoglycan, and cell wall extracts from gram-positive organisms induce a TNF-α response which can be blocked by anti-CD14 antibodies (28, 32, 35). However, in many other aspects, the LPS effect on its own receptor differed from that on cytokines. First, in contrast to the LPS-induced cytokine liberation, it was not modified by LBP, which enhances the affinity of binding of LPS to CD14. Second, although approximately 90% of the LPS rapidly disappeared from the incubation medium, cells had to be exposed for more than 30 min in order to get an increase of CD14 after 44 h. Thus, some signal had to remain on the cell for several hours. In contrast, a 5-min exposure to LPS is sufficient for an increase in TNF-α mRNA to occur (10). The different bacterial stimuli exerted their effects on CD14 not via an intermediate product such as TNF-α, IL-6, or IFN-γ, as proved by our blocking experiments with different anti-cytokine antibodies. These experiments also revealed the interesting counteractive property of IFN-γ. We previously showed that IFN-γ antagonized the LPS effect on CD14 in PBMC (18). This indicates that the LPS action on its receptor CD14 is modulated by other cytokines and may be very modest in the presence of a large LPS-induced IFN-γ release (3). The time course of the CD14 enhancement after LPS was strikingly similar to that for the late LPS-induced increase of the TNF receptors R55 and R75 and of soluble TNF-R75 (22), which is also preceded by an early downregulation of the receptors. Both CD14 and the TNF receptors play an important role in the pathophysiology during endotoxemia and sepsis. These two receptors may oppose each other in the regulation of LPS responsiveness. The enhanced CD14 could serve to antagonize LPS adaptation, and the TNF receptor could provide a means of systemic inactivation of TNF by the formation of TNF-sTNF-R complexes.

We studied the effects on CD14 of repeated LPS stimulation, i.e., under conditions of an adapted TNF response. The behaviors of TNF-α and CD14 were divergent in the adaptation experiments. This indicates that the downregulated TNF-α response to LPS was not due to decreased CD14 expression. In contrast, the CD14 response was opposite that of TNF-α. The same observation has been made previously for Mono-Mac 6 cells (37). The increased CD14 expression was not related to a reduced LPS uptake in adapted cells, since LPS disappeared from the extracellular medium with the same kinetics in fresh or adapted cells. Tolerance is a discordant phenomenon, since in peritoneal macrophages low-dose LPS pretreatment and high-dose LPS challenge were shown to abolish TNF and IL-6 production but to enhance IL-1β and granulocyte colony-stimulating factor production (14). Therefore, the upregulated CD14 may well be functional and play a role in signal transmission for cytokines other than TNF.

The enhanced CD14 expression and release, late after bacterial stimulation, may counterregulate against the adaptation phenomenon and thus maintain cell responsiveness.

ACKNOWLEDGMENTS

We thank Martin Wesp for excellent technical assistance and A. De Sola Pinto for secretarial assistance.

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Editor: S. H. E. Kaufmann