Human lipoproteins have divergent neutralizing effects on *E. coli* LPS, *N. meningitidis* LPS, and complete Gram-negative bacteria

Tom Sprong,[*,†] Mihai G. Netea,[*,†] Peter van der Ley,[§] Trees J. G. Verver-Jansen,[*,†] Liesbeth E. H. Jacobs,[*,†] Anton Stalenhoef,[*] Jos W. M. van der Meer,[*,†] and Marcel van Deuren[1,*,†]

Department of General Internal Medicine* and Nijmegen University Centre for Infectious Diseases,† University Medical Centre St. Radboud, Nijmegen, The Netherlands; and National Institute of Public Health and the Environment,§ Bilthoven, The Netherlands

Abstract **The use of lipoproteins has been suggested as a treatment for Gram-negative sepsis because they inhibit lipopolysaccharide (LPS)-mediated cytokine production. However, little is known about the neutralizing effects of lipoproteins on cytokine production by meningococcal LPS or whole Gram-negative bacteria. We assessed the neutralizing effect of LDLs, HDLs, and VLDLs on LPS- or whole bacteria-induced cytokines in human mononuclear cells. A strong inhibition of *Escherichia coli* LPS-induced interleukin-1β (IL-1β), tumor necrosis factor-α, and IL-10 by LDL and HDL was seen, whereas VLDL had a less pronounced effect. In contrast, *Neisseria meningitidis* LPS, in similar concentrations, was neutralized much less effectively than *E. coli* LPS. Effective neutralization of meningococcal LPS required a longer interaction time, a lower concentration of LPS, or higher concentrations of lipoproteins. The difference in neutralization was independent of the saccharide tail, suggesting that the lipid A moiety accounted for the difference. Minimal neutralizing effects of the lipoproteins were observed on whole *E. coli* or *N. meningitidis* bacteria under all conditions tested. These results indicate that efficient neutralization of LPS depends on the type of LPS, but a sufficiently long interaction time, a low LPS concentration, or high lipoprotein concentration also inhibited cytokines by the less efficiently neutralized *N. meningitidis* LPS. Irrespective of these differences, whole bacteria showed no neutralization by lipoproteins.**—Sprong, T., M. G. Netea, P. van der Ley, T. J. G. Verver-Jansen, L. E. H. Jacobs, A. Stalenhoef, J. W. M. van der Meer, and M. van Deuren. **Human lipoproteins have divergent neutralizing effects on *E. coli* LPS, *N. meningitidis* LPS, and complete Gram-negative bacteria. *J. Lipid Res.* 2004.** 45: **742–749.**

Supplementary key words Gram-negative sepsis • lipopolysaccharide • low density lipoprotein • high density lipoprotein • very low density lipoprotein • cytokines • mononuclear cells • *Escherichia coli* • *Neisseria meningitidis*

Lipopolysaccharide (LPS), a major structural and functional component of the outer membrane of Gram-negative bacteria, is considered to be a major pathogenic element in Gram-negative septicemia (1). LPS interacts with a variety of cells to induce a wide array of inflammatory mediators. Among these, the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and the antiinflammatory cytokine IL-10 are key factors in the development of shock and disseminated intravascular coagulation (2).

Fulminant meningococcal sepsis (FMS) is an important cause of morbidity and mortality worldwide, especially in children and young adults. In addition, it is the prototypical Gram-negative sepsis syndrome, in which LPS concentrations may become extremely high and correlate with cytokine levels, morbidity, and mortality (3, 4).

LPS is composed of a polysaccharide tail to which the lipid portion (lipid A) is attached via 2-keto-3-deoxyoctanate (KDO). Lipid A, a glucosamine-derived phospholipid, is considered to be the toxic moiety of LPS and is responsible for many of the biological effects of LPS (5).

In the past, various strategies have been explored to combat the deleterious effects of LPS during sepsis. However, none of these anti-LPS strategies has shown a benefit on outcome (6–9); thus, the search for a LPS-neutralizing treatment modality continues. Lipoproteins have been shown to bind LPS and to neutralize LPS-induced cytokine production (10). Also, in animal models of sepsis, the administration of lipoproteins was found to be beneficial (11, 12), and LPS infusion experiments in humans showed that the inflammatory response is inhibited by infusion of lipoproteins (13, 14). For these reasons, lipoprotein infusion during septicemia has been advocated as an

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[1] To whom correspondence should be addressed.
e-mail: m.vandeuren@aig.umcn.nl

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adjunctive therapy. FMS especially, being the prototypical LPS-mediated disease, has been suggested as a candidate disease for treatment with lipoproteins.

To date, most investigators assessing the effect of lipoproteins have used LPS derived from Enterobacteriaceae species (*Escherichia coli* or *Salmonella typhimurium*). However, the LPSs are a heterogeneous group of molecules with interspecies differences in the length and position of the acyl chains in the lipid A portion of the LPS, the length and polarity of the polysaccharide tail, and the formation of supramolecular structures (5, 15, 16). These differences in LPS have been shown to modulate the capacity to induce cytokines (17–19), and it is likely that these differences also affect the interaction with lipoproteins. In addition, LPS is not the only component of Gram-negative bacteria that induces cytokines (20–22). Therefore, the question is to what extent an adjunctive therapy that targets only LPS, such as lipoproteins, will inhibit cytokine production by complete bacteria.

In the present study, we addressed these questions by comparing the inhibitory effect of lipoproteins on cytokine induction by LPS of *E. coli* and *Neisseria meningitidis* in human mononuclear cells. In addition, we assessed the cytokine-inhibiting capacity of lipoproteins on whole *E. coli* and *N. meningitidis* bacteria.

MATERIALS AND METHODS

LPSs and meningococci

E. coli 055:B5 TCA and phenol-extracted LPS were obtained from Sigma Chemical Co. (St. Louis, MO). *E. coli* K12, D31m4 rough (Re) LPS consisting of lipid A$(KDO)_2$ and lacking the polysaccharide tail was purchased from List Biological Laboratories (Campbell, CA). The lipid A of *E. coli* LPS is a hexaacyl phospholipid with the fatty acyl acids asymmetrically distributed (23). Meningococcal LPS was isolated by the phenol/water extraction method as described by Westphal and Jann (24). H44/76 LPS was isolated from strain H44/76 (25). Meningococcal H44/76*rfaC* LPS [lipid A$(KDO)_2$] was isolated from the isogenic mutant *rfaC* of strain H44/76, as described by Stojiljkovic et al. (26). Similar to *E. coli* LPS, meningococcal LPS has a hexaacyl lipid A; however, in the lipid A of the meningococcal LPS, the fatty acids are symmetrically distributed (27). Whereas *E. coli* LPS has a polysaccharide tail, meningococcal LPS has an oligosaccharide tail (**Table 1**).

The commercial *E. coli* LPS was contaminated with protein (8.5%, by Lowry assay) and nucleic acid (6.1%, by spectrophotometry). The meningococcal LPS was not contaminated with protein, and the nucleic acid content was 4.4%. LPS was suspended in culture medium before use.

TABLE 1. Characteristics of the different lipopolysaccharide types

Lipopolysaccharide	Lipid A, Acyl Chains	Lipid A, Molecular Conformation	Saccharide Tail
E. coli 055:B5	Hexaacyl	Asymmetrical	Polysaccharide
E. coli K12, D31m4	Hexaacyl	Asymmetrical	Absent
N. meningitidis H44/76	Hexaacyl	Symmetrical	Oligosaccharide
N. meningitidis H44/76*rfaC*	Hexaacyl	Symmetrical	Absent

Modified from refs. (19, 23, 27).

E. coli ATCC 35218 is an international reference strain. *N. meningitidis* H44/76 was isolated from a patient with invasive disease (25). Stationary-phase bacteria grown in liquid medium were used, and bacteria were heat-inactivated (1 h, 56°C).

Molarity of the LPS solutions

Measurement of KDO was used to estimate the molarity of the LPS solutions. KDO was measured by spectrophotometry, as described by Weissbach and Hurwitz (28).

Lipoproteins and lipoprotein-depleted plasma

Lipoproteins (VLDL, LDL, and HDL) and lipoprotein-depleted plasma (LPDP) were prepared from fresh EDTA plasma by sequential ultracentrifugation as described previously (29) under pyrogen-free conditions using pyrogen-free materials. Lipoproteins prepared in this manner were previously shown to be endotoxin-free by limulus amebocyte lysate (LAL) assay. Lipoproteins were dialyzed for 24 h against 0.05 M phosphate buffer, pH 7.4, containing 5 μM EDTA, with one exchange of the buffer (LPDP is the fraction of plasma that is obtained after removing the lipid fractions). Lipoproteins were isolated before each experiment from different donors. The concentration of total lipid in the lipoprotein fractions was determined based on total cholesterol as a quantitating unit. Cholesterol in the different lipoprotein fractions was measured by spectrophotometry using a commercially available kit and a Hitachi 747 apparatus (Roche Diagnostics, Almere, The Netherlands). Before use, the lipoproteins were diluted 1:1 in LPDP (unless otherwise stated). Average concentrations of lipoprotein in which the LPS or bacteria were preincubated were 376 mg/l (0.60 mmol/l cholesterol) for LDL, 130 mg/l (0.22 mmol/l cholesterol) for HDL, and 378 mg/l (0.20 mmol/l cholesterol) for VLDL. For experiments using a higher concentration of lipoproteins, the lipoproteins were concentrated approximately five times by additional ultracentrifugation of the separate lipoprotein fractions.

Human peripheral blood mononuclear cells

Blood for the isolation of peripheral blood mononuclear cells (PBMCs) was drawn in 10 ml EDTA anti-coagulated tubes (Vacutainer System; Becton Dickinson, Rutherford, NJ) from healthy human volunteers. Informed consent was obtained before each experiment, and the guidelines of the local ethics committee were followed in the conduct of these experiments. PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden). The cells from the interphase were aspirated, washed three times in sterile PBS, and resuspended in culture medium RPMI 1640 (Dutch modification; Flow Labs, Irvine, UK) supplemented with L-glutamine (2 mmol), pyruvate (1 mmol), and gentamicin (50 mg/ml).

Assay procedure

Fifty microliters of the lipoprotein diluted in LPDP or LPDP alone was preincubated with the stimuli (50 μl) for a certain time period. Then, the preincubated mixture was added to 100 μl of 5×10^6 PBMCs on 200 μl 96-well plates at 37°C and 5% CO_2 and incubated for 24 h. The supernatant was obtained by centrifugation and stored at −20°C until required for the cytokine assays. LPDP alone significantly enhanced LPS or whole bacteria-induced cytokine production in PBMCs compared with culture medium.

Cytokine assays

Levels of IL-1β and TNF-α were determined by radioimmunoassay as described by Drenth et al. (30). The lower limit of de-

tection was 80 pg/ml for both cytokines. IL-10 was determined by a commercially available ELISA kit (Pelikine compact; Sanquin, Amsterdam, The Netherlands).

Statistics and calculations

Statistical analysis was performed using an unpaired two-sided *t*-test; $P < 0.05$ was considered significant. Two-way ANOVA was performed to compare concentration curves (GraphPad Prism; GraphPad Software, Inc.). To calculate the inhibition of cytokines by the lipoproteins, cytokine production in the presence of the lipoprotein diluted in LPDP was expressed as a percentage of production in the presence of LPDP alone.

RESULTS

Cytokine induction by *N. meningitidis* LPS and *E. coli* LPS

A molarity-based comparison of cytokine induction by *N. meningitidis* and *E. coli* LPS (TCA-extracted) in PBMCs, based on assay for KDO, showed that meningococcal LPS is more potent in the induction of IL-1β and TNF-α than is *E. coli* LPS, especially at concentrations of less than 1 pmol/ml (**Fig. 1**). The minimal concentrations to induce significant IL-1β and TNF-α production were 0.0001 pmol/ml for meningococcal LPS and 0.001 pmol/ml for *E. coli* LPS. Phenol-extracted *E. coli* LPS was approximately as potent in the induction of IL-1β and TNF-α as TCA-extracted LPS (data not shown). Based on these data, in subsequent experiments LPS was used at concentrations of 0.14 pmol/ml (equivalent to ~0.6 ng/ml meningococcal LPS and 1 ng/ml *E. coli* LPS) unless otherwise stated.

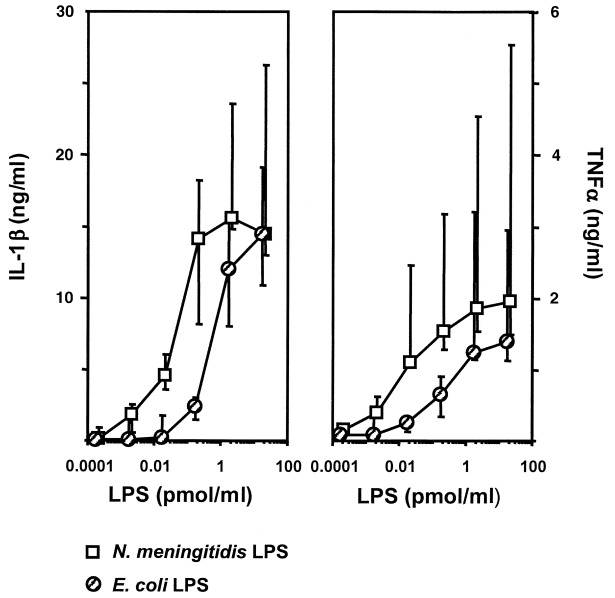

Fig. 1. Cytokines induced by *E. coli* and *N. meningitidis* lipopolysaccharide (LPS). Dose-response curves for interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) induced by *E. coli* and *N. meningitidis* LPS in human mononuclear cells based on molar concentrations of LPS (2-keto-3-deoxyoctanate assay). *N. meningitidis* LPS was more potent in the induction of IL-1β and TNF-α than was *E. coli* LPS. Medians and interquartile ranges are presented (n = 5).

Effect of lipoproteins on cytokines by *E. coli* and *N. meningitidis* LPS

The effect of the preincubation of equimolar concentrations of *E. coli* or *N. meningitidis* LPS with the lipoproteins LDL, HDL, and VLDL for 8 h on IL-1β, TNF-α, and IL-10 production in PBMCs is shown in **Fig. 2**. Lipoproteins did not induce cytokine production in the absence of stimulus, suggesting minimal contamination of the lipoprotein fractions with LPS. *E. coli* LPS-induced cytokine production was diminished by LDL to values of ~20% of production in LPDP; HDL was slightly less effective in the inhibition of cytokine production, whereas VLDL had only a minimal effect. Notably, meningococcal LPS-induced cytokine production was inhibited to a significantly lesser extent by LDL and HDL than cytokine production by *E. coli* LPS. LDL reduced the *N. meningitidis* LPS-induced cytokine production to only 50–60% of control production for IL-1β and IL-10, whereas TNF-α production was not inhibited at all. HDL inhibited *N. meningitidis*-induced cytokines less efficiently than LDL, and the effect of VLDL was minimal.

Next, we investigated the kinetics of LPS neutralization by LDL or HDL using different preincubation times of lipoprotein with LPS and different lipoprotein concentrations (**Fig. 3**). We found that inhibition of *E. coli* LPS-induced IL-1β by LDL was dependent on the preincubation time and that for maximal inhibition, 4–8 h of preincubation was required. Of importance, the inhibition of *N. meningitidis* LPS-induced IL-1β production by LDL, compared with *E. coli* LPS, required much longer preincubation times to achieve noticeable inhibition, and maximal inhibition was seen only after 24 h of preincubation (Fig. 3). The results for TNF-α and HDL showed a similar pattern, although inhibition by HDL was less efficient.

For LDL, we investigated the importance of LPS concentration on lipoprotein neutralization (**Fig. 4**). The experiments showed that TNF-α production was inhibited significantly better by *E. coli* LPS than by *N. meningitidis* LPS ($P < 0.05$ by two-way ANOVA). However, the effectiveness of inhibition by LDL is dependent on the concentration of LPS; at high concentrations, both LPS types showed minimal inhibition, whereas at the lowest concentration tested, both LPS types were inhibited to a large extent. Results for IL-1β showed a similar pattern.

In addition, the influence of preincubation with lipoproteins at concentrations encountered in normal physiological, nonseptic conditions was investigated. By additional ultracentrifugation, lipid concentrations of 1.1 mmol/l for VLDL, 3.43 mmol/l for LDL, and 1.18 mmol/l for HDL were achieved. Concentrated LDL and HDL inhibited TNF-α by both LPS types effectively and to the same extent, whereas concentrated VLDL also inhibited TNF-α but to a lesser extent than LDL or HDL (**Fig. 5**). Results for IL-1β showed a similar pattern.

Influence of lipid A composition and the saccharide tail

To investigate whether the observed differences in lipoprotein-dependent inhibition of *E. coli* and *N. meningitidis* LPS-induced cytokines were dependent on the lipid A part or the saccharide tail of the LPS molecule, variants of

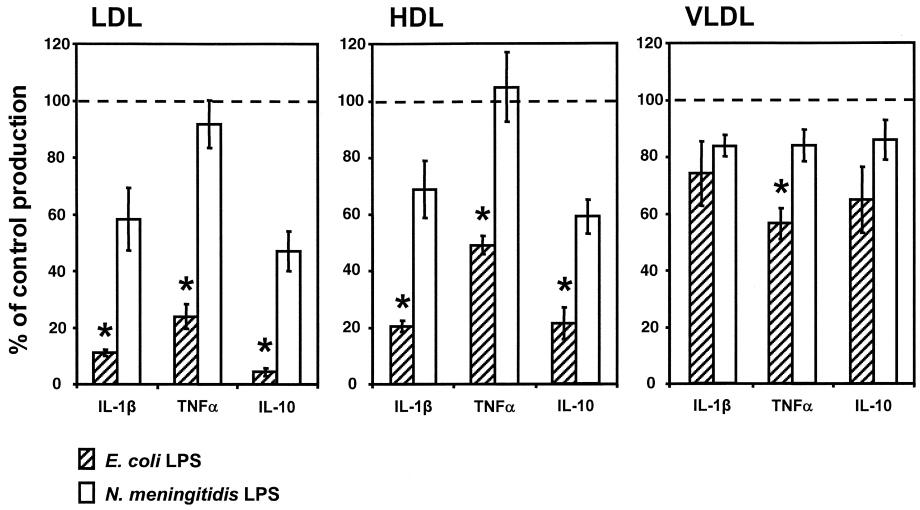

Fig. 2. Inhibition of LPS-induced cytokines by lipoproteins. Effect of preincubation of LPS (0.14 pmol/ml) with LDL, HDL, or VLDL for 8 h on IL-1β, TNF-α, and IL-10 induction by human mononuclear cells. LDL and HDL effectively inhibited *E. coli* LPS but not *N. meningitidis* LPS. Results are expressed as percentages of production in lipoprotein-depleted plasma (LPDP). Means and SEM are presented (n = 5). * $P < 0.05$ for the comparison of *E. coli* LPS with *N. meningitidis* LPS by unpaired *t*-test.

the LPS lacking the polysaccharide tail were used. *E. coli* Re K12, D31m4 LPS was approximately equipotent to *E. coli* 055:B5 LPS, and *N. meningitidis* H44/76*rfaC* LPS was approximately equipotent to *N. meningitidis* H44/76 LPS. IL-1β, TNF-α, and IL-10 produced by *E. coli* Re K12, D31m4 LPS were inhibited by LDL (**Fig. 6**) and HDL (data not shown) to a similar extent as that produced by *E. coli* 055:B5 LPS. No difference was found in the weak inhibitory effect of LDL on cytokine production between *N. meningitidis* H44/76 and *N. meningitidis* H44/76*rfaC* LPS. Thus, these data suggest that the differences in lipoprotein-dependent inhibition of cytokine production between *E. coli* LPS and meningococcal LPS are not caused by the different lengths of the saccharide tails of the LPS but by differences in the lipid A part of the LPS molecule.

Effect of lipoproteins on cytokines by *E. coli* or *N. meningitidis* whole bacteria

Because LPS is not the only cytokine-inducing moiety of Gram-negative bacteria, we assessed whether the lipoprotein fractions were capable of inhibiting the cytokine response by PBMCs to complete Gram-negative bacteria. In these experiments, the *E. coli* LPS-induced cytokine response was inhibited by LDL or HDL. In contrast, IL-1β, TNF-α, and IL-10 induced by *E. coli* and *N. meningitidis* complete bacteria were not, or were only minimally, inhibited by the lipoproteins LDL, HDL, or VLDL (**Fig. 7**). Because the amount of cytokines induced by these whole bacteria was significantly higher than the amount induced after stimulation with LPS, we tested the capacity of lipoproteins to inhibit a lower concentration of bacteria or the capacity of a higher concentration of LDL to inhibit cytokine production. Even at the lowest concentration of bacteria tested (10^4/ml), no effect of VLDL, LDL, or HDL was seen on TNF-α, IL-1β, or IL-10 production (**Fig. 8** shows the results for LDL and TNF-α). In addition, concentrated LDL (3.43 mmol/l) did not lead to more inhibition of bacteria-induced cytokines (**Fig. 9**).

DISCUSSION

In the present study, we showed that at equimolar concentrations of *E. coli* or *N. meningitidis* LPS, the in vitro neutralizing effect of lipoproteins on cytokine production

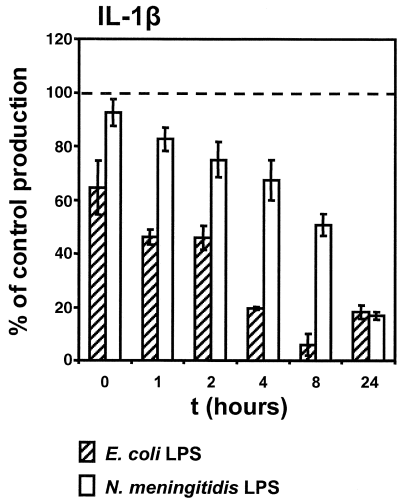

Fig. 3. Influence of preincubation time (t) on LPS neutralization. Effect of preincubation of LPS (0.14 pmol/ml) with LDL (0.6 mmol/l) for various time periods on IL-1β production in human mononuclear cells. Inhibition of LPS-induced cytokine by LDL was time-dependent, the interaction of *N. meningitidis* LPS with LDL was slower than that of *E. coli* LPS, and relatively high LDL concentrations were necessary to inhibit IL-1β production. Results are expressed as percentages of production in LPDP. Means and SEM are presented (n = 3).

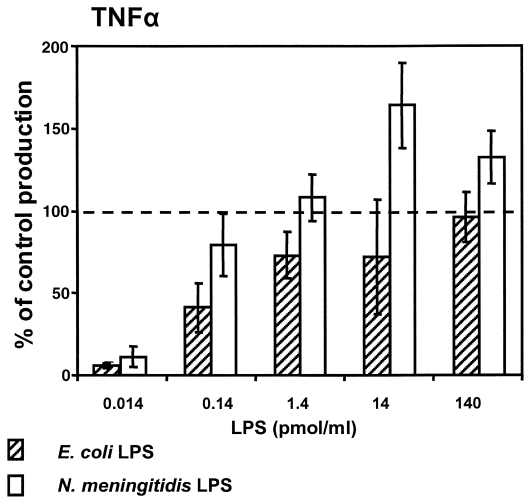

Fig. 4. Inhibition of TNF-α at various LPS concentrations. Inhibition of LPS-induced TNF-α production in human mononuclear cells by LDL (0.6 mmol/l) after stimulation with a concentration range of *E. coli* or *N. meningitidis* LPS (0.014–140 pmol/ml). LDL was preincubated with the different LPS types for 8 h. Results are expressed as percentages of production in LPDP. *N. meningitidis* LPS was significantly ($P < 0.05$) better inhibited by LDL than was *E. coli* LPS (two-way ANOVA). Means and SEM are presented (n = 3).

by *N. meningitidis* LPS was reduced in comparison with that by *E. coli* LPS. The neutralizing effect on *E. coli* LPS was shown to be time-dependent and saturable. By increasing the interaction time between the lipoproteins and LPS, increasing the lipoprotein concentration, or increasing the LPS concentration, the dissimilarity in the neutralizing effect of the lipoproteins disappeared. The

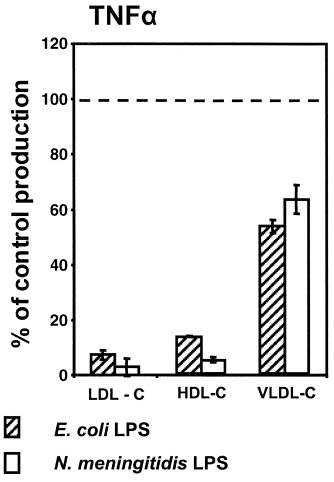

Fig. 5. Influence of concentrated lipoproteins on LPS-induced cytokine production. Effect of preincubation of *E. coli* or *N. meningitidis* LPS for 8 h with concentrated lipoprotein fractions on cytokine induction in human mononuclear cells. Lipoproteins were concentrated approximately five times, and the lipid concentrations in which the LPS was preincubated after concentration were 1.1 mmol/l for VLDL (VLDL-C), 3.43 mmol/l for LDL (LDL-C), and 1.18 mmol/l for HDL (HDL-C). Means and SEM are presented (n = 3).

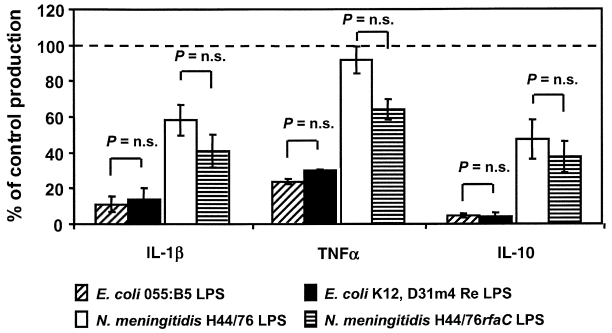

Fig. 6. Effect of the saccharide tail of LPS on inhibition by lipoproteins. Effect of preincubation of various types of LPS (~0.14 pmol/ml) with LDL (0.6 mmol/l) for 8 h on IL-1β, TNF-α, and IL-10 by human mononuclear cells. *E. coli* 055:B5 and *E. coli* K12, D31m4 Re LPS types were inhibited equally by LDL, whereas both meningococcal LPS types were less effectively inhibited. Results are expressed as percentages of production in LPDP. Means and SEM are presented (n = 5). Differences were tested for significance using an unpaired *t*-test. n.s., not significant.

difference in the extent of inhibition of LDL and HDL on both types of LPS is likely to be caused by the difference in the lipid A portion of the LPS molecule. Of importance, cytokine induction by whole *E. coli* or *N. meningitidis* bacteria was not influenced by lipoproteins.

A quantitative comparison of the biological effects of different LPS types on a weight basis may yield incorrect results because LPS from different bacteria have different molecular weights, largely because of the highly variable length of the saccharide tail. To avoid this pitfall, we assessed the molar concentration of the *E. coli* LPS and meningococcal LPS batches by determination of KDO, a highly conserved part of LPS (molecular mass 238 Da) (5, 28), and used equimolar concentrations of LPS in all comparative experiments. In accordance with previously reported data on the bioactivity of LPS (31, 32), the molarity-based dose-response curve showed that at concentrations of less than 1 pmol/ml, meningococcal LPS is ~10 times more potent in the induction of IL-1β and TNF-α than is *E. coli* LPS.

The protective effect of the lipoproteins LDL and HDL in models of Gram-negative infection and lethal endotoxemia is well documented, and the inhibition of LPS-induced proinflammatory cytokine production, through binding of LPS by the lipoproteins, is thought to be a key element in this process (10–14). In the present study, we tested whether cytokine induction by the LPS isolated from *N. meningitidis*, the bacterium that causes the prototypical Gram-negative sepsis syndrome, is inhibited by lipoproteins in a similar manner as the prototypical LPS isolated from *E. coli*.

The concentrations of LPS used in the current in vitro experiments (0.14 pmol or 0.6 ng/ml) were chosen to be in the range of concentrations of LPS (as determined by LAL assay) seen in the bloodstream during meningococcal sepsis, which range from 0.05 ng/ml to more than 10 ng/ml (4). In *E. coli* sepsis, LPS levels are generally lower (33, 34). As earlier determinations showed that meningo-

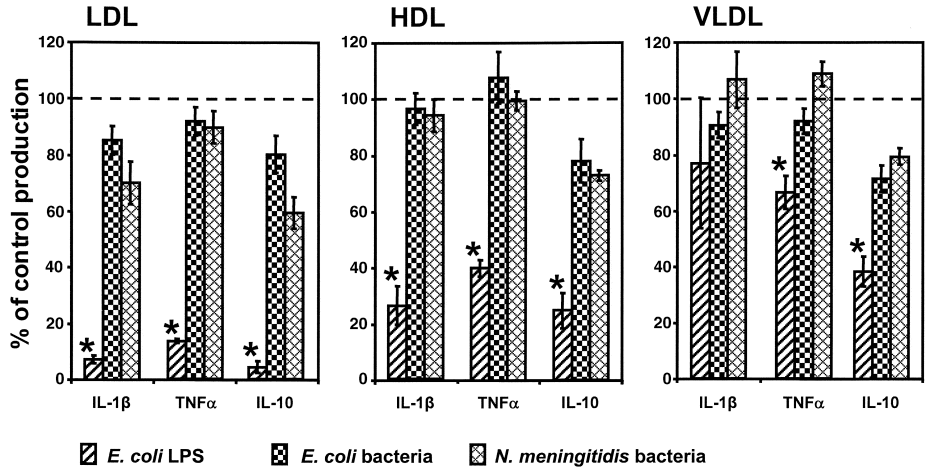

Fig. 7. Inhibition of whole bacteria-induced cytokines by lipoproteins. Effect of preincubation of *E. coli* LPS (0.14 pmol/ml) or *E. coli* and *N. meningitidis* complete bacteria (0.6×10^6) with LDL, HDL, or VLDL for 4 h on IL-1β, TNF-α, and IL-10 by human mononuclear cells. LDL and HDL effectively inhibited *E. coli* LPS but not *E. coli* or *N. meningitidis* whole bacteria. Results are expressed as percentages of production in LPDP. Means and SEM are presented (n = 5). * $P < 0.05$ for the comparison of *E. coli* LPS with the complete bacteria by unpaired *t*-test.

cocci contain ~1 ng LPS/10^6 bacteria in the outer membrane (20), the concentration of bacteria (0.6×10^6) was chosen to match the concentrations of LPS used. This bacterial concentration is in agreement with the amount of bacteria found during meningococcal sepsis (2.2×10^4/ml to 1.6×10^8/ml) (35).

Lipoprotein plasma concentrations are altered during sepsis or meningitis: LDL and HDL cholesterol decreased (to ~0.8–1.4 mmol/l for LDL and 0.2–0.4 mmol/ml for HDL), whereas VLDL cholesterol was increased to a median concentration of 2.3 mmol/ml (36, 37). Thus, the concentrations of LDL and HDL used for this study are in range with the concentrations of these lipoproteins as they occur in septic conditions. For some experiments, the lipoproteins were further concentrated approximately five times to also investigate their detoxifying capacity at concentrations that are seen under normal physiological, nonseptic conditions. As VLDL increases during sepsis, we could only study this lipoprotein at concentrations that are seen in healthy individuals.

Confirming earlier observations (38), we found that preincubation of *E. coli* LPS with LDL as well as HDL inhibited the cytokine response, whereas VLDL had a less pronounced effect. However, at identical molar concen-

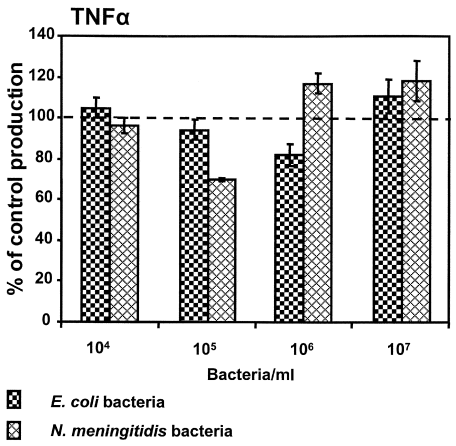

Fig. 8. Inhibition of LPS-induced TNF-α production in human mononuclear cells by LDL (0.6 mmol/l) after stimulation with a concentration range (10^4 to 10^7 bacteria/ml) of whole *E. coli* or *N. meningitidis* bacteria. LDL was preincubated with the different bacteria for 8 h. Results are expressed as percentages of production in LPDP. No effect of LDL on TNF-α production by *E. coli* or *N. meningitidis* bacteria was seen. Means and SEM are presented (n = 3).

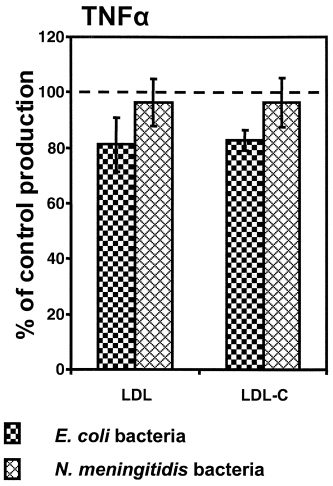

Fig. 9. Influence of concentrated lipoproteins on whole bacteria-induced cytokine production. Effect of preincubation of *E. coli* or *N. meningitidis* (0.6×10^6 bacteria/ml) LPS for 8 h with concentrated LDL on cytokine induction in human mononuclear cells. LDL was concentrated approximately five times; the LDL concentration at which the LPS was preincubated was 3.43 mmol/l. LDL indicates unconcentrated LDL (0.6 mmol/l), and LDL-C indicates concentrated LDL (3.43 mmol/l). Means and SEM are presented (n = 3).

trations and incubation times, *N. meningitidis* LPS was neutralized much less efficiently than *E. coli* LPS.

The neutralizing effect of LDL and HDL was dependent on the preincubation time of the LPS with the lipoproteins and the concentration of LPS or lipoproteins. Whereas *E. coli* was inhibited significantly after relatively short preincubation times of 1–4 h, for meningococcal LPS, maximal inhibition was only seen after 24 h of preincubation. Using a fixed preincubation time (8 h) and a fixed concentration of LDL, the extent of inhibition was influenced by the concentration of LPS. Over the complete range of concentrations tested, *E. coli* LPS was significantly better neutralized by LDL than was *N. meningitidis* LPS (as assessed by two-way ANOVA). However, at the highest concentration tested, *E. coli* LPS also showed minimal inhibition, whereas at the lowest concentration tested, both LPS types were neutralized to a large extent. To investigate the impact of higher concentrations of lipoproteins on LPS detoxification, we maximally concentrated the lipoprotein fractions by additional ultracentrifugation. In this way, lipoprotein fractions approximately five times more concentrated were obtained, and the final concentration of lipoprotein in the PBMC system now equaled concentrations seen under physiological, nonseptic conditions. At these higher concentrations of lipoprotein, both LPS types were inhibited equally and to a large extent by LDL and HDL after 8 h of preincubation. Thus, irrespective of the difference in neutralization seen at low concentrations of lipoproteins, this suggests that at physiological concentrations both *E. coli* and *N. meningitidis* LPSs are neutralized by the lipoproteins effectively. VLDL had a less pronounced neutralizing effect on cytokines, although it cannot be excluded that at even higher concentrations, as seen during sepsis, the effect of VLDL is also increased. Taken together, these results indicate that the efficient neutralization of LPS depends on the type of LPS, but a sufficiently long interaction time, a low concentration of LPS, and a high concentration of lipoproteins will also inhibit cytokines by a less efficiently neutralized LPS. Our results emphasize the concept that lipoproteins might be important for *E. coli* LPS-mediated disease. However, because during severe meningococcal sepsis, LPS concentrations are much higher, the time period from the onset of disease to admission is very short (4), and concentrations of LDL and HDL are decreased, it is unlikely that lipoproteins play an important role in neutralizing LPS during this type of infection.

The main molecular differences between *E. coli* LPS and *N. meningitidis* LPS are the size and structure of the saccharide tail and the position and distribution of the six acyl chains on the glucosamine backbone. To investigate which of these differences determine the neutralization by lipoproteins, we measured the effect of lipoproteins on cytokine induction by two types of *E. coli* and *N. meningitidis* LPS that are completely devoid of a saccharide tail, i.e., *E. coli* Re K12, D31m4 LPS and *N. meningitidis* H44/76*rfaC* LPS. This showed that the neutralizing action of lipoproteins is independent of the presence of the saccharide tail. Thus, the lipid A part, with its asymmetrical hexaacylated conformation in *E. coli* LPS and its symmetrical distribution of the six acyl chains in *N. meningitidis* LPS, accounts for the divergent effect. The bioactivity and receptor affinity of an LPS molecule is suggested to be determined by the three-dimensional conformation of the lipid A, possibly altering the bioactivity and receptor affinity of the LPS molecule (19). For LPS-lipoprotein interactions, Levine and colleagues (12) have proposed the "leaflet insertion" model, in which the fatty acyl acids of the lipid A portion of LPS are inserted into the lipid monolayer of the lipoproteins, inactivating the "toxic moiety" of LPS. We presume that the lower degree of neutralization of meningococcal LPS by lipoproteins is caused by the symmetrical lipid A structure interacting more slowly with the lipoprotein lipid monolayers.

LPS is generally considered to be the pathogenic component of Gram-negative bacteria (1). However, recently, it has been shown that non-LPS components of Gram-negative bacteria also highly influence cytokine production (20–22). Therefore, we studied whether lipoproteins affect cytokine production after stimulation with whole bacteria, even at relatively low bacterial concentrations or high (physiological, nonseptic) lipoprotein concentrations. This was not the case. This finding indicates that whole bacteria, in contrast to isolated LPS, in which neutralization by lipoproteins depends on the interaction time and concentration of LPS or lipoprotein, are impervious to neutralization by lipoproteins. We assume that LPS, anchored tightly in the outer membrane of the Gram-negative bacterium, interacts less easily with the lipoproteins than isolated LPS. In addition, it can be speculated that bacterial components, other than LPS, of *E. coli* or *N. meningitidis* that are not inhibited by lipoproteins may account for the absent cytokine-inhibitory effect of lipoproteins on whole Gram-negative bacteria. The data presented seemingly contradict the findings in animal models, in which lipoprotein administration did reduce cytokines and outcome after the administration of whole *E. coli* or after cecal ligation and puncture (39, 40). Therefore, further investigation is needed on the immune-modulating mechanisms of lipoproteins during Gram-negative sepsis.

In conclusion, LDL and HDL inhibit cytokines by *E. coli* LPS, but *N. meningitidis* LPS shows reduced neutralization by human lipoproteins. However, a sufficiently long interaction time, a low LPS concentration, or higher lipoprotein concentration also inhibits cytokines by this less efficiently neutralized LPS. In contrast, lipoproteins do not have a neutralizing effect on whole *E. coli* or *N. meningitidis* bacteria. This raises questions about the rationale for adjuvant treatment of meningococcal and other types of Gram-negative sepsis with lipoproteins.

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