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A Cyanobacterial Lipopolysaccharide Antagonist Inhibits Cytokine Production Induced by *Neisseria meningitidis* in a Human Whole-Blood Model of Septicemia[▽]

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Septicemia caused by *Neisseria meningitidis* is characterized by increasing levels of meningococcal lipopolysaccharide (Nm-LPS) and cytokine production in the blood. We have used an in vitro human whole-blood model of meningococcal septicemia to investigate the potential of CyP, a selective Toll-like receptor 4 (TLR4)–MD-2 antagonist derived from the cyanobacterium *Oscillatoria planktothrix* FP1, for reducing LPS-mediated cytokine production. CyP (≥1 μg/ml) inhibited the secretion of the proinflammatory cytokines tumor necrosis factor alpha, interleukin-1β (IL-1β), and IL-6 (by >90%) and chemokines IL-8 and monocyte chemoattractant protein 1 (by ~50%) induced by the treatment of blood with pure Nm-LPS, by isolated outer membranes, and after infection with live meningococci of different serogroups. In vitro studies with human dendritic cells and TLR4-transfected Jurkat cells demonstrated that CyP competitively inhibited Nm-LPS interactions with TLR4 and subsequent NF-κB activation. These data demonstrate that CyP is a potent antagonist of meningococcal LPS and could be considered a new adjunctive therapy for treating septicemia.

Infections with *Neisseria meningitidis* (a meningococcus), characterized by meningitis and potentially fatal septicemia, continue to be major health problems worldwide (37). The World Health Organization has estimated that meningococcal infection accounts for 500,000 new cases each year, with 50,000 deaths (http://www.who.int/csr/resources/publications/meningitis/whoemcbac983.pdf). The introduction of the conjugated serogroup C polysaccharide vaccine into the infant immunization program has led to a significant decline in serogroup C infections in developed countries where such vaccines have been introduced (2), and this strategy is likely to be extended to the serogroup A, W135, and Y polysaccharides in developing countries. However, no vaccines exist for preventing infection by serogroup B meningococci, which are now the predominant strains in temperate countries.

In individuals lacking humoral immunity to meningococci, bacterial proliferation in the blood leads to bacteremia and clinical disease, ranging from mild meningococcemia to meningitis and fulminant septicemia, which is an acute compartmentalized intravascular inflammatory response that is characterized by the up-regulation of cytokine and chemokine production (5). Patients with septicemia rapidly develop life-threatening symptoms that include persistent circulatory collapse, renal and lung failure, hemorrhagic skin lesions, disseminated intravascular coagulation, and multiple-organ failure (5). Even with antibiotic treatment, the mortality rate can be as high as 20 to 50% for patients with fulminant septicemia, whereas that for patients with meningitis but without septicemia is ~5% (7).

It is generally accepted that the meningococcal lipopolysaccharide (designated Nm-LPS), which resides in the outer membrane (OM) of the bacterium, is largely responsible for inducing intravascular inflammation (7). Indeed, the progression of fulminant septicemia closely correlates with increasing levels of circulating Nm-LPS and the consequent production of cytokines, notably interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), IL-1β, and IL-8 (5, 9, 39). LPS-stimulated cytokine production by myeloid cells is believed to depend on the association of LPS and the serum LPS-binding protein, which then forms a multimer with CD14 (41), a glycophosphatidylinositol-linked cell surface glycoprotein on the myeloid cell surface (18). The association of this complex with an adjacent Toll-like receptor 4 (TLR4) molecule (3) and an accessory protein MD-2 molecule (35) (designated TLR4–MD-2) transduces the LPS signal via MyD88 (30) to eventually activate nuclear factor kappa B (NF-κB) and trigger cytokine production (31). The activation of monocytes by Nm-LPS has been shown to be dependent on CD14 and TLR4–MD-2 (44).

Recently, an LPS-related molecule derived from the cyanobacterium *Oscillatoria planktothrix* FP1 and termed CyP was reported to act as a selective TLR4–MD-2 receptor antagonist (28). The *Cyanobacteria* form a very large taxa, and the chemical analysis of cyanobacterial LPS has shown that although these molecules are basically similar to LPS from gram-nega-

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tive bacteria, there are differences between the two in chemical and biological properties (22). *Neisseria meningitidis* gram-negative LPS consists of symmetrical hexa-acyl lipid A, a core structure containing two 2-keto-deoxy-octulosonic acids (KDO) and two heptoses (L-glycero-D-manno-heptopyranoside) with short polysaccharide side chains attached. However, unlike most enteric gram-negative LPS, there are no multiple repeating units of O antigens in meningococcal LPS (21). LPS molecules derived from cyanobacteria, including various *Oscillatoria* spp., contain glucose, xylose, mannose, and rhamnose, but unlike gram-negative LPS, the presence of galactose and glucosamine is variable and both KDO and heptose either are present in low concentrations (0.15 to 2% of LPS) or are absent (22, 28). Cyanobacterial LPS molecules also contain relatively large quantities of oleic, palmitoleic, linoleic, and linolenic acids, which typically are absent from gram-negative LPS molecules. In addition, unlike lipid A from gram-negative LPS, including the Nm-LPS, lipid A from cyanobacteria does not contain phosphorus (22).

The cyanobacterial LPS-related molecule CyP acted as a competitive inhibitor of *Escherichia coli* LPS binding to the receptor complex on human dendritic cells (DC) (28). In addition, CyP was nontoxic in mice in vivo, and it was nontoxic in both human and mouse cells in vitro and did not itself stimulate DC responses (28). Significantly, CyP suppressed gene transcription and cytokine production in DC treated with *E. coli* LPS and also protected mice from endotoxic shock caused by *Salmonella enterica* serovar Abortusequi LPS (28). In the current study, we used an in vitro human whole-blood model of *Neisseria meningitidis* septicemia to evaluate the potential of CyP as an antagonist able to reduce the intravascular inflammatory response.

MATERIALS AND METHODS

Bacteria and growth conditions. *Neisseria meningitidis* strain MC58 (B:15: P1.7,16b) was isolated from an outbreak of meningococcal infections that occurred in Stroud, Gloucestershire, United Kingdom, in the mid-1980s (29). *Neisseria meningitidis* serogroup C strain MenC11 (15) was obtained from the Meningococcal Reference Unit of the Manchester Health Protection Agency, and serogroups A (strain designation MC12), X (MC27), Y (MC31), W135 (MC39), and Z (MC34) were obtained from the Scottish Meningococcal Reference Unit (Glasgow, Scotland).

All bacteria were grown at 37°C in an atmosphere containing 5% (vol/vol) CO_2 on supplemented proteose-peptone agar (43) and suspended in phosphate-buffered saline (PBS) for infection experiments.

Preparation of OM and Nm-LPS. Outer membranes (OM) were prepared by the extraction of wild-type MC58 whole cells with lithium acetate (40), and Nm-LPS was purified from MC58 by extraction with hot phenol, as described previously (12, 23). LPS from *Escherichia coli* serotype 0111:B4 was purchased from Sigma (Poole, Dorset, United Kingdom). The quantitation of both Nm-LPS and *E. coli* LPS was based on dry weight. OM and LPS preparations were suspended in sterile, endotoxin-free PBS (100 μl) prior to addition to whole blood.

CyP. CyP was purified from the freshwater cyanobacterium *Oscillatoria planktothrix* FP1 by a phenol-guanidinium thiocyanate-based method as described previously (28). The silver staining of the purified cyanobacterial extract separated by deoxycholate-polyacrylamide gel electrophoresis mainly showed the presence of an LPS-like glycolipid (28), and contamination was minimal and similar to that observed for commercially available gram-negative LPS products. Protein contamination was <3% (wt/wt), and mean nucleic acid contamination was <30% (and consisted mainly of degraded RNA). The level of KDO was low (0.15%, wt/wt), and endotoxin activity, as quantified by the *Limulus* amoebocyte lysate assay, was 4 endotoxin units (EU)/mg; that for *E. coli* LPS was 15,000 EU/mg (28). The concentration of CyP was estimated using the Pro-Q-emerald 300 LPS gel stain kit (Invitrogen), and each band was quantitated using a charge-coupled display camera. In addition, the specific activity of CyP was determined by the inhibition of TNF-α production in culture supernatants of human THP1 cells stimulated with *E. coli* LPS and are expressed as the mean inhibition per microgram of CyP.

Several biological assays were carried out to confirm the purity of CyP. The absence of stimulatory activity by CyP preparation alone was confirmed by the absence of TNF-α production in THP1 cells. In addition, when added to granulocyte-macrophage colony-stimulating factor (GM-CSF)-cultured DC derived from the bone marrow of wild-type CH3/HeN, BALB/C, or C57BL/6 mice, CyP (20 μg/ml) did not induce TNF-α or IL-6 secretion. Significantly, in experiments with DC derived from the bone marrow of TLR4-defective C3H/HeJ mice, CyP (20 μg/ml) also did not induce TNF-α or IL-6 secretion. In addition, no NF-κB activity was observed in human Jurkat cells transfected with human TLR2 and human TLR9 after treatment with CyP (data not shown). Moreover, CyP has been reported to bind to MD-2, LPS-binding protein, and polymyxin B (28), thus demonstrating strong similarities to gram-negative bacterial LPS. CyP was dissolved in sterile, endotoxin-free PBS prior to addition to whole blood.

Human whole-blood model. The whole-blood model was carried out according to the procedure described by Ison (20). Whole human venous blood was taken from a healthy volunteer with informed consent, collected into sterile Vacutainer tubes containing sodium heparin anticoagulant (Becton Dickinson Systems, New Jersey), and processed within 1 h. Whole blood (1 ml) was dispensed into sterile 24-well tissue culture plates (Greiner bio-one; Germany), and triplicate wells were treated with various concentrations of live bacteria, isolated OM, or LPS from meningococci (Nm-LPS) or *E. coli* cells (Ec-LPS) in the presence of various concentrations of CyP. In addition, control wells consisted of blood alone and blood with CyP only. The plates were incubated at 37°C, initially on a rocking table (20 rpm) for the first 3 h and then without rocking for the remainder of the experiment(s). Samples were taken at 6 and 24 h and centrifuged (13,000 × *g* for 5 min), and the plasma layer was removed and stored at −20°C for analysis.

Measurement of cytokine production in human plasma. The levels of the proinflammatory (IL-1α, IL-1β, IL-6, and TNF-α), chemoattractant (monocyte chemoattractant protein 1 [MCP-1], IL-8, and regulated upon activation, normal T cell expressed and secreted [RANTES]), anti-inflammatory (IL-10), and growth factor-related (GM-CSF) cytokine proteins were quantified by sandwich immunoassay, as described previously (12). A two-sample *t* test was used to compare the mean levels of cytokine secretion after particular treatments, with $P < 0.05$ being significant.

Isolation and stimulation of human mo-DC. Buffy coats were obtained from the Swiss Blood Center, Basel, Switzerland, and ethical permission for the use of human primary cells was obtained from the Federal Office of Public Health of Switzerland. Monocytes were purified from peripheral blood mononuclear cells by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi). Monocyte-derived DC (mo-DC) were generated as described previously by culturing monocytes in RPMI medium supplemented with 10% (vol/vol) fetal bovine serum, GM-CSF (Gentaur), and IL-4 (33). mo-DC (0.5×10^6 cells/ml) were stimulated with Nm-LPS or Ec-LPS (100 ng/ml) in the absence or presence of CyP (2.5 to 20 μg/ml). The production of human TNF-α and IL-6 cytokines was measured in mo-DC supernatant fluids after stimulation for 20 h, using DuoSet enzyme-linked immunosorbent assay development kits (R&D Systems). Cytokine data are from measurements with standard deviations below 5%.

TLR4 transfection assay. Human Jurkat cells (1×10^7; expressing human MD-2) were transfected as described previously (28) by electroporation (250 V, 975 μF; Gene Pulser II; Bio-Rad) with 1.5 μg of expression vector encoding human TLR4 together with 1 μg of 3× NF-κB luciferase reporter vector (kindly provided by G. Natoli; IFOM, Milan, Italy). After 24 h, the cells were washed, resuspended in 12 ml of fresh medium, and plated onto 12-well plates with 0.8 ml of cells for each stimulatory condition (100 ng/ml of Nm-LPS or Ec-LPS, in the absence or presence of 5 to 20 μg/ml of CyP). After 8 h of stimulation, the cells were harvested and lysed, and reporter gene activity was measured using the luciferase assay system (Promega) and Veritas luminometer (Turner BioSystems) according to the manufacturers' instructions. Jurkat cells transfected with empty vector together with 3× NF-κB luciferase reporter vector were used as a mock control. Luciferase activity in the presence of CyP is shown as a mean percentage of the activity observed from cells stimulated with LPS alone (100%), with error bars denoting the standard deviations.

RESULTS

Effect of cyanobacterial LPS antagonist CyP on cytokine production in human whole blood treated with Nm-LPS. Our first experiments were aimed at investigating the effects of CyP on cytokine production in human whole blood treated with

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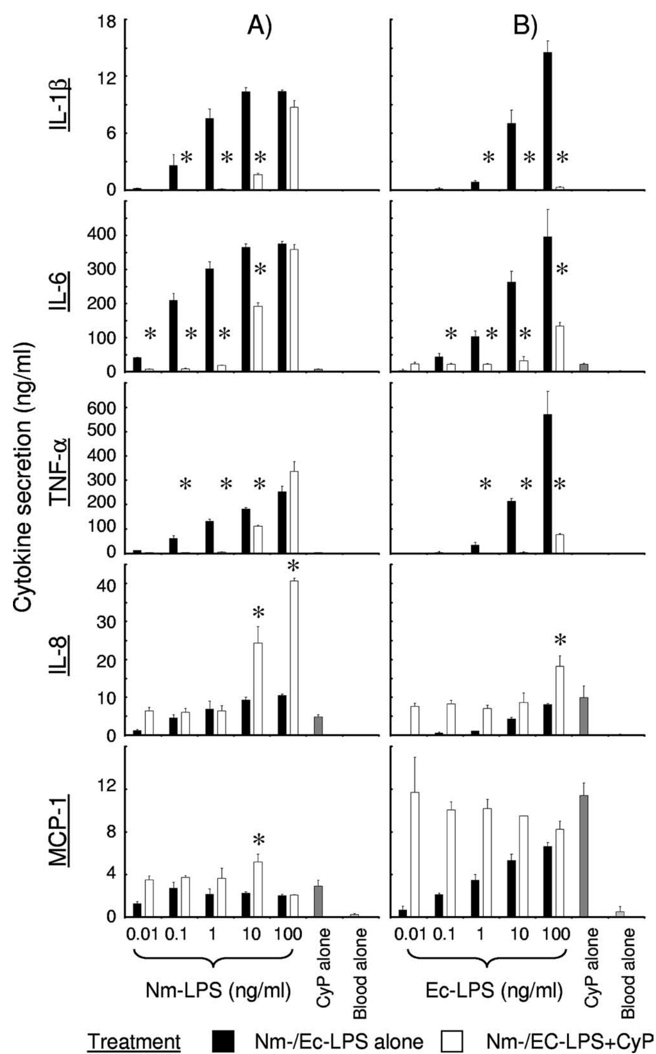

FIG. 1. Effect of CyP on proinflammatory cytokine and chemokine production in whole human blood treated with various doses of Nm-LPS or Ec-LPS. Whole blood (1 ml) either was left untreated or was treated with Nm-LPS (A) or Ec-LPS (B) in the presence or absence of CyP (20 μg/ml) or CyP alone, and cytokine production was measured after 6 h. The columns represent mean cytokine secretion for three independent wells, and the error bars represent the standard deviations. Data are shown from representative experiments ($n = 6$). *, $P < 0.05$.

various doses (1 fg/ml to 1,000 ng/ml) of pure LPS from *Neisseria meningitidis* (Nm-LPS) and *Escherichia coli* (Ec-LPS). Initially, a concentration of 20 μg/ml of CyP was used in these experiments according to the study of Macagno and colleagues, who demonstrated that this concentration effectively suppressed Ec-LPS-induced cytokine production by human mo-DC in all experimental conditions tested (28).

CyP was added to whole blood at the same time (t = 0 h) as each LPS preparation, and cytokine production was measured after 6 h. Doses of Nm-LPS alone of between 0.01 and 1,000 ng/ml induced proinflammatory cytokines IL-1β, IL-6, and TNF-α and the chemokines IL-8 and MCP-1 in whole blood (Fig. 1A), whereas doses of <0.01 ng/ml had no significant effect. CyP inhibited the secretion of IL-1β, IL-6, and TNF-α, which was induced by doses of 0.1 to 1 ng/ml Nm-LPS, by >95% ($P < 0.05$) and by between 50 and 85% ($P < 0.05$) for cytokines induced by 10 ng/ml Nm-LPS (Fig. 1A). No cytokine inhibition ($P > 0.05$) was observed with the highest doses of Nm-LPS tested (100 to 1,000 ng/ml).

By contrast, the secretion of the chemokines IL-8 and MCP-1 was not inhibited by CyP but was indeed increased (Fig. 1A). Significantly, CyP (20 μg/ml) alone induced these chemokines, and an additive effect was observed in the presence of high doses of Nm-LPS. CyP and Nm-LPS (10 to 100 ng/ml) together induced approximately three- to fourfold increases in IL-8 and MCP-1 ($P < 0.05$) levels compared to those of secretions induced by Nm-LPS alone (Fig. 1A).

We next investigated whether the effects of CyP on cytokine production in whole blood treated with Nm-LPS were a general feature of its antagonist properties toward gram-negative LPS. Therefore, similar experiments were carried out with Ec-LPS. As observed with Nm-LPS, CyP inhibited the secretion of IL-1β, IL-6, and TNF-α induced by Ec-LPS, with a reduction of between 70 and 98% ($P < 0.05$) in cytokine levels induced by doses of 1 to 100 ng/ml (Fig. 1B). In addition, a synergistic effect between CyP and the highest dose of Ec-LPS (100 ng/ml) resulted in increased IL-8 induction compared to that of induction by CyP alone, but a similar effect was not observed for MCP-1 (Fig. 1B).

During the course of these (and subsequent) experiments, there was no hemolysis or other visible signs of toxicity, such as cell clumping or floating cells in control wells containing human whole blood treated with CyP (20 μg/ml) alone. This lack of toxicity is consistent with the previous study from Macagno and colleagues (28), who reported that CyP also was nontoxic in vivo in mice and in vitro in human and mouse cell lines.

CyP inhibits Nm-LPS-induced proinflammatory cytokine production by human mo-DC and TLR4-mediated NF-κB activity. A previous study with human mo-DC demonstrated that CyP inhibited LPS from *E. coli* by interacting with the TLR4–MD-2 extracellular domain (28), and we investigated whether this mechanism was involved in the CyP inhibition of Nm-LPS activity as well. This was studied with two well-characterized systems, namely, the culture of mo-DC with the measurement of TNF-α and IL-6 secretion as biological markers of activation, and the measurement of luciferase activity as a marker of NF-κB activation in human MD-2-positive Jurkat cells transfected to ectopically express human TLR4.

Treatment with Nm-LPS and Ec-LPS induced similar levels of both TNF-α and IL-6 secretion by mo-DC (Fig. 2A). The optimal inhibition of TNF-α and IL-6 release induced by Nm-LPS required 10 to 20 μg/ml of CyP, concentrations that were four- to eightfold higher ($P < 0.05$) than the dose of CyP (2.5 μg/ml) required for inhibiting Ec-LPS-induced secretion. In experiments with Jurkat cells, both LPS molecules (100 ng/ml) stimulated NF-κB activity only in cells transfected with TLR4, and this activity was significantly inhibited (70 to 80%; $P < 0.05$) by CyP at all doses tested (Fig. 2B). When both LPS molecules were tested at a higher concentration of 400 ng/ml, 40 to 70% inhibition was observed depending on the dose of CyP (data not shown).

Effect of CyP on cytokine production in human whole blood treated with meningococcal OM. A characteristic feature of meningococcal infection is the shedding of OM vesicles, or

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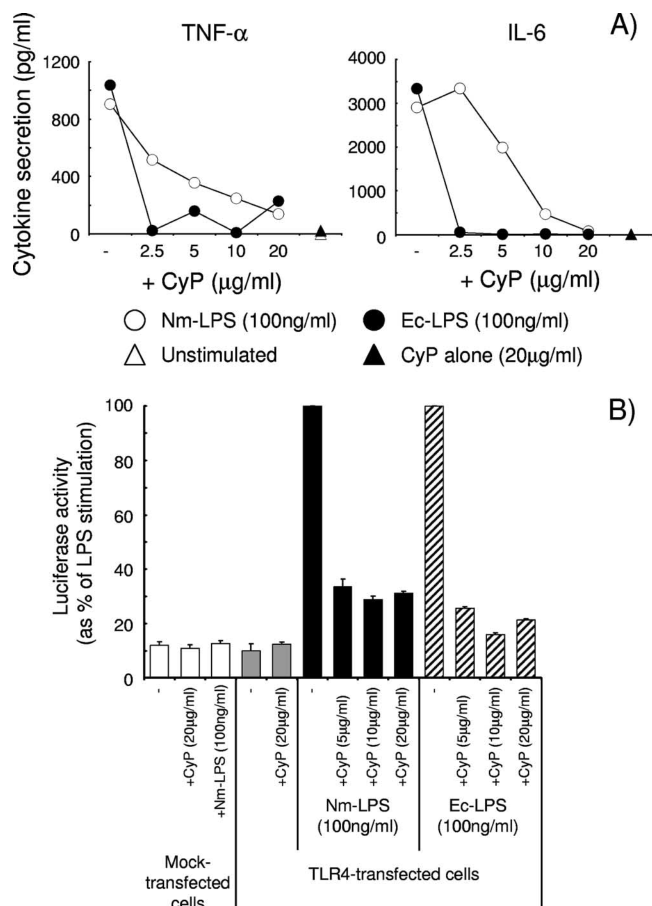

FIG. 2. Effect of CyP on LPS-induced proinflammatory cytokine production by human DC and TLR4-mediated NF-κB activity. (A) Human mo-DC were treated with Nm-LPS or Ec-LPS and various doses of CyP (0 to 20 μg/ml), and TNF-α and IL-6 cytokine secretion was measured. Data are representative of two independent experiments. (B) Human Jurkat cells (expressing human MD-2), transfected to express human TLR4, were treated with Nm-LPS or Ec-LPS and various doses of CyP (0 to 20 μg/ml), and luciferase activity measured as a marker of NF-κB activation. The columns represent the mean luciferase activity from three independent wells, and the error bars represent the standard deviations. Data are shown for a representative experiment ($n = 2$).

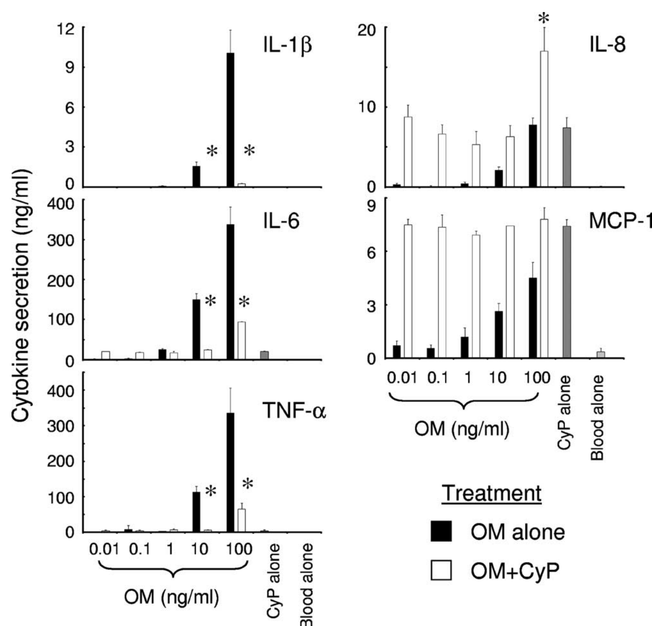

FIG. 3. Effect of CyP on proinflammatory cytokine and chemokine production in whole human blood treated with various doses of meningococcal OM. Whole blood (1 ml) either was left untreated or was treated with OM in the presence or absence of CyP (20 μg/ml) or with CyP alone, and cytokine production was measured after 6 h. The columns represent mean cytokine secretion for three independent wells, and the error bars represent the standard deviations. *, $P < 0.05$.

blebs, and these structures are believed to constitute the main mechanism by which LPS is disseminated rapidly during bacteremia (32, 36). In the current study, human whole blood was incubated with various doses of OM (0.01 to 100 ng protein/ml) in the presence or absence of CyP (20 μg/ml), and cytokine production was quantified after 6 h. CyP significantly inhibited the secretion of IL-1β, IL-6, and TNF-α induced by doses of 10 to 100 ng/ml OM by between 70 and 99% ($P < 0.05$) (Fig. 3). By contrast, a synergistic effect between CyP and the highest dose of OM (100 ng/ml) resulted in increased IL-8 induction compared that of CyP alone, but a similar effect was not observed for MCP-1 (Fig. 3). Thus, the pattern of cytokine production induced by OM was similar to that observed with pure LPS molecules and was inhibited by CyP. Similarly, no inhibitory effect was observed on chemokine production induced by OM.

CyP inhibits proinflammatory cytokine production in human whole blood infected with live *Neisseria meningitidis*. We next investigated whether CyP could inhibit cytokine secretion induced by meningococcal infection. Human whole blood was infected with various doses of live serogroup B *Neisseria meningitidis* strain MC58 (10^2 to 10^8 CFU/ml), a range that covers the concentrations observed during bacteremia (5, 7), in the presence or absence of CyP (20 μg/ml), and cytokine secretion was quantified after 6 h. Infection with doses of bacteria of $\geq 10^4$ CFU/ml resulted in the significant production of proinflammatory cytokines (Fig. 4). CyP strongly inhibited (by 80 to 93%; $P < 0.05$) the secretion of IL-1β, IL-6, and TNF-α induced by 10^4 meningococci. At the higher dose of 10^6 bacteria, the inhibitory effect of CyP was less pronounced, with a reduction ($P < 0.05$) of IL-1β and IL-6 levels by approximately 30 and 20%, respectively, but with no effect ($P > 0.05$) on TNF-α secretion (Fig. 4). CyP did not inhibit proinflammatory cytokine secretion induced by 10^8 meningococci ($P > 0.05$), and as observed with pure LPS and OM, CyP also did not inhibit IL-8 or MCP-1 secretion. At certain bacterial doses, it resulted in additive levels of chemokine production (Fig. 4).

We next sought to determine if CyP also showed inhibitory activity toward other meningococcal serogroups. Therefore, human whole blood was infected with 10^4 CFU/ml of a selection of different serogroups in the presence or absence of CyP (20 μg/ml), and cytokine production was quantified at 6 h. Serogroup A, B, C, W135, X, and Z meningococci all induced proinflammatory cytokines and chemokines. CyP significantly inhibited ($P < 0.05$) the secretion of IL-1β, IL-6, and TNF-α induced by all serogroup bacteria, with mean levels of inhibi-

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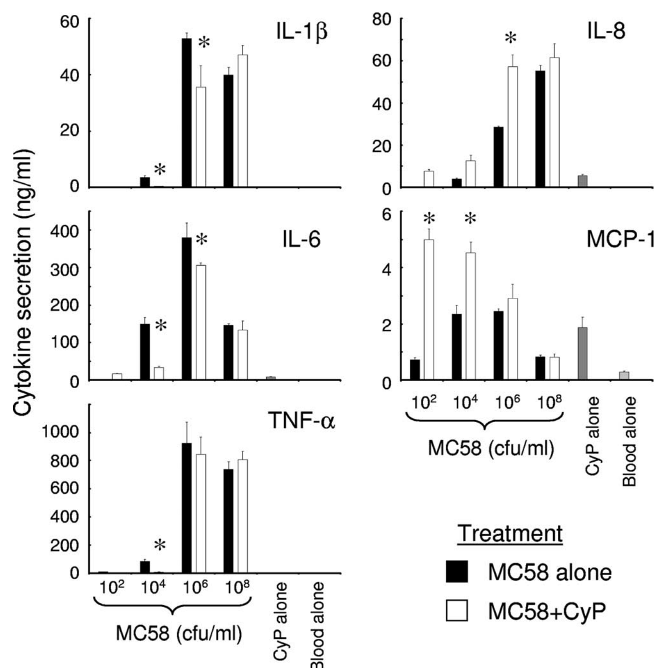

FIG. 4. Effect of CyP on proinflammatory cytokine and chemokine production in whole human blood infected with serogroup B *Neisseria meningitidis* strain MC58. Whole blood (1 ml) either was left untreated or was infected with various doses of meningococci in the presence or absence of CyP (20 μg/ml)or CyP alone, and cytokine production was measured after 6 h. The columns represent mean cytokine secretion for three independent wells, and the error bars represent the standard deviations. Data are shown for a representative experiment ($n = 2$). *, $P < 0.05$.

tion ranging from 70% for IL-6 to >97% for IL-1β and TNF-α (data not shown). As observed with serogroup B meningococcal infection at the higher dose of ~10^6 CFU, the inhibitory effect of CyP also was generally less pronounced against infection with the other serogroup bacteria. However, CyP did show a capacity to inhibit cytokine secretion induced by some serogroups more effectively than others. For example, whereas CyP did not inhibit the secretion of TNF-α induced by serogroups B and C ($P > 0.05$) and showed only low levels of inhibition (≤10%) toward W135 and X ($P < 0.05$), it inhibited cytokine secretion induced by serogroups A and Z by approximately 56% ($P < 0.05$; data not shown). In addition, CyP inhibited the serogroup C stimulation of IL-1β secretion by approximately 8% ($P < 0.05$) but showed higher activity against serogroups A, X, W135, and Z, reducing cytokine secretion by between 35 and 50% ($P < 0.05$). By contrast, CyP inhibited IL-6 secretion induced by all the serogroups similarly, with a reduction of between 10 and 20% ($P < 0.05$) (data not shown).

Effect of various doses of CyP on cytokine production in human whole blood infected with live *Neisseria meningitidis* or treated with pure Nm-LPS. Thus far, our data have demonstrated that a dose of 20 μg/ml CyP (28) inhibited proinflammatory cytokine (IL-1β, IL-6, and TNF-α) secretion in human whole blood treated with live meningococci, Nm-LPS, and native OM. However, CyP at this concentration stimulated chemokine production. Therefore, we investigated whether using lower concentrations of CyP could reduce chemokine secretion but still effectively suppress proinflammatory cytokine production induced by live bacteria and Nm-LPS.

Human whole blood was treated with 10^4 CFU/ml of live *Neisseria meningitidis* MC58 and various doses of CyP from 0.01 to 20 μg/ml, and cytokine production was quantified at 6 h (Fig. 5). The significant inhibition ($P < 0.05$) of proinflammatory cytokines was effected by CyP doses as low as 0.1 μg/ml. At this dose, the levels of IL-1β, IL-6, and TNF-α were reduced by approximately 65 to 80% (Fig. 5). Increasing the dose of CyP to ≥1 μg/ml reduced IL-1β and IL-6 secretion by >90%, and in the case of TNF-α, optimal inhibition (≥95%) was observed with doses of 1 to 5 μg/ml CyP. Treatment of blood with CyP alone resulted in a dose-dependent increase in the chemokines IL-8 and MCP-1, but minimal induction was observed with the lowest doses of CyP tested (0.01 to 1 μg/ml). Moreover, doses of 0.1 to 1 μg/ml CyP resulted in the inhibition of IL-8 and MCP-1 secretion by approximately 50% ($P < 0.05$) rather than the stimulation seen at higher concentrations (Fig. 5).

Similar results were observed with Nm-LPS. When whole blood was treated with 1 ng/ml Nm-LPS, the minimal dose of CyP required to inhibit IL-1β, IL-6, and TNF-α secretion by >95% ($P < 0.05$) was 1 μg/ml CyP. At a higher dose (10 ng/ml Nm-LPS), doses of ≥5 μg/ml CyP were required for the significant inhibition ($P < 0.05$) of cytokine secretion. In addition, a dose of 1 μg/ml CyP inhibited IL-8 and MCP-1 secretion induced by 1 to 10 ng/ml Nm-LPS by approximately 50% ($P < 0.05$) (data not shown).

Finally, during the course of all these experiments, neither IL-1α, GM-CSF, nor the anti-inflammatory cytokine IL-10 were induced by Nm-LPS, Ec-LPS, isolated OM, live bacteria, and/or CyP. In addition, RANTES was the only molecule detected in normal, untreated whole blood, but its levels remained unchanged following any of the treatments with or without CyP (data not shown).

DISCUSSION

For patients with meningococcal septicemia, there is a direct correlation between increasing levels of Nm-LPS in the blood and the production of high concentrations of cytokines, including IL-1β, TNF-α, IL-6, and IL-8 (5, 7). Although antibiotic therapy is the primary treatment for meningococcal infection and the plasma levels of both LPS and cytokines have been reported to decline immediately following administration (8), adjunctive therapies that neutralize LPS likely would improve clinical outcomes. However, the results from the clinical trials of some candidate therapies have been disappointing. Neither human serum antibodies to the rough *E. coli* J5 mutant that expresses the LPS core structure nor HA-1A, a human/mouse chimeric monoclonal antibody directed against lipid A, reduced the mortality rate in patients with meningococcal septicemia (13, 16). In addition, although treatment with the recombinant 21-kDa N-terminal fragment of bactericidal/permeability-increasing protein did reduce some complications of meningococcal sepsis, the trial was unable to detect differences in mortality (25). The current study was undertaken to investigate the use of CyP, a cyanobacterial LPS antagonist, as a potential therapy for down-regulating cytokine production in a human whole-blood model after treatment with Nm-LPS and OM and after infection with live meningococci.

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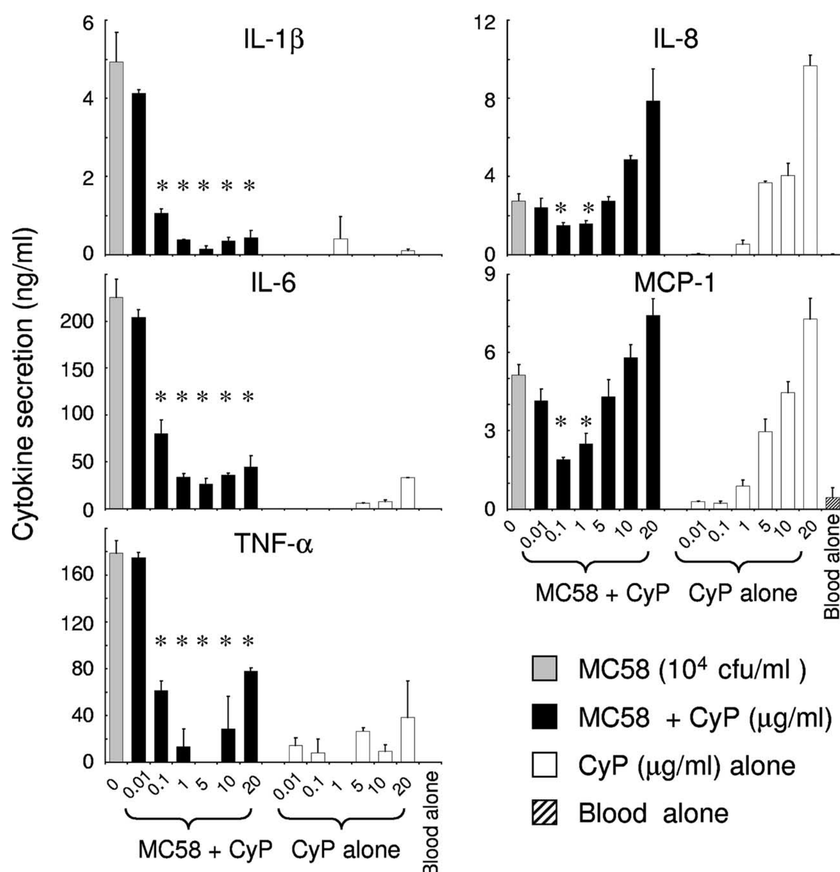

FIG. 5. Effect of various concentrations of CyP on proinflammatory cytokine and chemokine production in whole human blood treated with live meningococci. Whole blood (1 ml) either was left untreated or was infected with approximately 10^4 CFU of strain MC58 and various doses of CyP (0.01 to 20 μg/ml) or CyP alone, and cytokine production was measured after 6 h. The columns represent mean cytokine secretion for three independent wells, and the error bars represent the standard deviations. Data are shown for a representative experiment ($n = 2$). *, $P < 0.05$.

In the current study, CyP was a potent antagonist for Nm-LPS, significantly inhibiting the secretion of IL-1β, TNF-α, and IL-6. Moreover, the fact that CyP similarly inhibited cytokine secretion induced by Ec-LPS suggests that CyP acts through a common mechanism as an antagonist of the activity of gram-negative LPS. This was demonstrated by the findings that CyP efficiently inhibited cytokine production from human mo-DC treated with both Nm-LPS and Ec-LPS and, moreover, that both LPS molecules stimulated NF-κB activity in Jurkat cells expressing human MD-2 transfected with TLR4. Our data are consistent with the study of Macagno et al., which reported that CyP acted as a specific TLR4–MD-2 antagonist, directly binding to MD-2 to competitively inhibit LPS binding and the downstream activation of NF-κB (28).

In the current study, CyP also was antagonistic to meningococcal LPS in the form that it is naturally presented during infection, i.e., as a major component of OM blebs and on intact bacteria. This is particularly significant, since increasing levels of Nm-LPS in the blood reflect bacterial growth and the release of OM blebs, events that closely correlate with the development of septic shock, multiple-organ failure, and death (6). CyP potently inhibited cytokine production in whole blood that had been treated with up to 10 ng/ml Nm-LPS, 100 ng/ml OM, and 10^4 CFU/ml of live bacteria. Clinical studies have reported that levels of purified or OM-bound LPS of ≥0.7 ng/ml were associated with bacteremia in all patients, with a range from 0.6 ng/ml in individuals with mild systemic meningococcal disease (8) to 2 to 3 ng/ml for systemic meningococcal disease patients with persistent septic shock (6, 7, 14, 38). Thus, CyP could inhibit cytokine production in vitro induced by levels of bioactive LPS that are found in patients with meningococcal septicemia. Moreover, cytokine inhibition was independent of the meningococcal serogroup, demonstrating broad applicability, and CyP itself had no effect on meningococcal viability or growth.

In contrast to its effects on proinflammatory cytokine production, CyP alone, at the highest concentration tested (20 μg/ml), induced the secretion of chemokines IL-8 and MCP-1 in whole blood. Although the CyP preparation consists mainly of an LPS-like glycolipid, which most likely is responsible for the antagonistic effects (28), it is possible that minor contaminants are responsible for stimulating chemokine expression. However, several other possibilities cannot be excluded. In the complex heterogeneous cell environment of whole human blood, it is possible that CyP itself induces chemokine secretion by stimulating cells such as neutrophils and monocytes in a TLR4-independent manner and/or by up-regulating chemokine receptor expression.

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Furthermore, despite the fact that these chemokines were not transcriptionally induced in human DC by CyP (28), it is possible that in different cells the threshold levels for the induction of chemokines are lower than those for proinflammatory cytokines; thus, when CyP competitively binds to the TLR4–MD-2 receptor complex and inhibits Nm-LPS interaction, low levels of CyP activation and signaling may occur that are sufficient to induce some expression of IL-8 and MCP-1 but not of IL-1β, IL-6, and TNF-α. The current study also demonstrated that an additive effect on IL-8 and MCP-1 secretion sometimes was observed with the highest concentrations of CyP (20 μg/ml) and Nm-LPS tested. In this case, it is possible that Nm-LPS with CyP and/or minor contaminants are acting synergistically on TLR4–MD-2 and/or other, as-yet uncharacterized receptors on the surfaces of blood cells. Although these exact mechanisms remain unknown, by reducing the concentration of CyP to ≤1 μg/ml, the efficient inhibition of proinflammatory cytokine production still was observed; however, the significant, though not total, down-regulation of chemokine secretion induced by Nm-LPS and live bacteria also occurred. Notably, cytokine and chemokine inhibition still was observed after 24 h (data not shown). Therefore, it is possible to modulate the concentration of a single dose of the CyP preparation in order to maintain an immunosuppressive effect on cytokine production while permitting some chemokine production. This may be important in vivo as a mechanism for controlling the recruitment of immune effector cells necessary for pathogen removal without increasing the damage to host cells due to the release of microbicidal agents.

The efficacy of CyP in the human whole-blood model of septicemia was dependent on the time of administration. In the current study, CyP was effective when added to blood within 1 h of stimulation with meningococci or meningococcal components but not at later times (data not shown). This contrasts with the study of Macagno and colleagues, which reported that Ec-LPS-induced cytokine production by purified mo-DC could be inhibited by CyP added up to 6 h after endotoxin stimulation (28). It is likely that both the cellular heterogeneity of whole human blood and the rapid activation of different myeloid cells by meningococcal components, compared to the activation of a monoculture of mo-DC in vitro, explains in part the differences in biological effects observed.

Many studies have described different lipid A analogues and chemically modified LPS derivatives (10, 11, 19, 27, 34) as well as LPS-neutralizing molecules (1, 4, 17) as potential inhibitors of inflammatory responses induced by Ec-LPS. To date, only LPS-like molecules isolated from the bacteria *Rhodobacter capsulatus*, *Helicobacter pylori*, *Porphyromonas gingivalis*, *Capnocytophaga ochracea*, and *Oscillatoria planktothrix* FP1 have been reported to act as LPS receptor antagonists (24, 26, 28, 42). To our knowledge, the current study is the first report demonstrating that CyP, an LPS-like molecule derived from a cyanobacterium, is a potent antagonist of Nm-LPS, inhibiting cytokine production in an in vitro model of septicemia. Therefore, CyP represents a potentially new adjunctive therapy for the treatment of meningococcal septicemia and other endotoxin-mediated inflammatory responses.

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