Reduced ex vivo chemokine production by polymorphonuclear cells after in vivo exposure of normal humans to endotoxin

Published in:
The Journal of Infectious Diseases

DOI:
10.1086/315840

Citation for published version (APA):
Reduced Ex Vivo Chemokine Production by Polymorphonuclear Cells after In Vivo Exposure of Normal Humans to Endotoxin

Marc J. Schultz,1,2 Dariusz P. Olszyna,2 Evert de Jonge,1 Annelies Verbon,1 Sander J. H. van Deventer,2 and Tom van der Poll1,3

Monocytes from patients with sepsis have a reduced capacity to produce cytokines, a state referred to as immunoparalysis. To determine whether polymorphonuclear leukocytes (PMNL) can be rendered hyporesponsive, PMNL from 6 healthy volunteers intravenously challenged with lipopolysaccharide (LPS; 4 ng/kg) were stimulated ex vivo with heat-killed bacteria or LPS, and the release of the CXC chemokines interleukin-8, epithelial-derived neutrophil attractant–78, and growth-related oncogen–α was measured. At 1 and 2 h after LPS administration in vivo, PMNL produced fewer CXC chemokines after stimulation with bacteria or LPS (all P < .05). Serum obtained 2 h after in vivo administration of LPS did not influence chemokine production by PMNL from 6 healthy volunteers not previously exposed to LPS. Thus, intravenous injection of LPS induces a refractory state of PMNL that is not caused by soluble factors produced in response to in vivo exposure to LPS.

Polymorphonuclear leukocytes (PMNL) play a key role in the pathogenesis of the sepsis syndrome [1]. Chemokines, an expanding family of small cytokines, can influence multiple leukocyte functions [2]. The so-called CXC chemokines specifically act on neutrophils and have been implicated as important factors in neutrophil-mediated inflammation. The prototypic CXC chemokine in humans is interleukin (IL)–8; related CXC chemokines are epithelial-derived neutrophil attractant (ENA)–78 and growth-related oncogen (GRO)–α. Of interest, PMNL are capable of producing these CXC chemokines, enabling them to perpetuate their own activation.

Severe sepsis is associated with a refractory state characterized by a reduced capacity of monocytes to produce proinflammatory cytokines after restimulation with endotoxin (lipopolysaccharide [LPS]) ex vivo [3–5]. This refractory state, also termed “LPS tolerance” or immunoparalysis, can be reproduced by administration of low-dose LPS to healthy humans [6–8]. Thus, whole blood or peripheral blood mononuclear cells obtained several hours after an in vivo injection of LPS produce fewer proinflammatory cytokines after restimulation with LPS ex vivo than do whole blood or mononuclear cells drawn before in vivo LPS exposure [6–8]. This hyporesponsiveness is at least partly mediated by soluble factors produced shortly after LPS injection, because serum obtained 2 h after in vivo administration of LPS inhibited tumor necrosis factor (TNF)–α and IL-1β production by LPS-stimulated whole blood from 6 healthy donors not previously exposed to LPS [8]. Septic serum also inhibits Escherichia coli–induced TNF release in normal whole blood [9, 10]. Recent studies suggest that not only monocytes but also PMNL may become refractory after a severe bacterial insult. Indeed, PMNL from patients with sepsis produced less IL-1β and IL-8 after stimulation with LPS or heat-killed streptococci [11, 12]. In the present study, we sought to determine the capacity of PMNL isolated from healthy humans before and after a single dose of LPS to produce IL-8, GRO–α, and ENA-78 after restimulation with heat-killed bacteria (Streptococcus pneumoniae and Pseudomonas aeruginosa) or LPS. Because a large amount of IL-8 can be found as a cell-associated form after activation of PMNL [12, 13], we further determined cell-associated IL-8 in these experiments. Finally, to determine whether soluble mediators produced in response to injection of LPS are involved in the induction of anergy of PMNL, we assessed the capacity of serum obtained after in vivo administration of LPS to reproduce the anergic state when this serum was added to PMNL from healthy subjects not challenged with LPS.

Methods

Subjects and design. Six men, 22 ± 1 years old (mean ± SE), were admitted to the Clinical Research Unit of the Academic Med-
Figure 1. Mean ± SE concentrations of interleukin (IL)-8 (ng/10^6 cells) and epithelium-derived neutrophil attractant (ENA)-78 and growth-related oncogen (GRO)-α (both in pg/10^6 cells) in supernatants of stimulated polymorphonuclear leukocytes (PMNL) isolated before and 1, 2, or 24 h after intravenous challenge with lipopolysaccharide (LPS) in 6 healthy volunteers. Isolated PMNL (0.5 × 10^6 cells in 500 μL of RPMI) were stimulated ex vivo for 24 h at 37°C in 5% CO2 with or without heat-killed Streptococcus pneumoniae (HKSP), heat-killed Pseudomonas aeruginosa (HKPA) (both at 10^7 cfu/mL), or LPS (10 ng/mL). *P < 0.05 vs. T = 0.

Stimulation of PMNL. Blood samples were drawn in sterile Vacutainer tubes containing lithium heparin (Becton Dickinson, Rutherford, NJ). PMNL were isolated by using Polymorphprep (Nycomed, Torshov, Norway), as recommended in the manufacturer’s manual. Contamination with mononuclear cells was <1% on each occasion. Cells were resuspended in culture medium (RPMI 1640; Gibco BRL Life Technologies, Paisley, UK) containing 5% heat-inactivated pooled plasma. Sterile tubes (Becton Dickinson) were filled with 500 μL of this cell suspension, and we added ~10^6 PMNL/mL and 10 μL of RPMI containing heat-killed S. pneumoniae, P. aeruginosa (both in final concentrations equivalent to 10^7 cfu), or LPS (from E. coli 0111:B4; Sigma, St. Louis; 10 ng/mL). All samples were incubated for 24 h at 37°C in 5% CO2, after which cells were spun down, and the supernatant was stored at −20°C until assays were performed. The cell pellet was resuspended in 1 mL of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl2, and 1 mM MgCl2 (pH 7.40), which was subsequently subjected to 3 freeze-thaw cycles to release IL-8 from the cells. The solution containing the cell fractions was stored at −20°C until assays were performed.

In a separate series of experiments, serum obtained from the 6 volunteers before (pre-LPS serum) and 2 h after in vivo administration of LPS (post-LPS serum) was pooled and subsequently incubated for 24 h at 37°C with PMNL from 6 other healthy volunteers (who did not receive LPS) in the presence or absence of heat-killed bacteria or LPS. In these experiments, 500 μL of the PMNL solution containing 10^6 PMNL/mL, with different concentrations of pre- and post-LPS serum (final concentrations 10% or 20%), was incubated with bacteria or LPS, as described above. Samples were handled identically to those of the first series of experiments.

Bacteria. Heat-killed S. pneumoniae (HKSP) was obtained from a clinical isolate (serotype D9). The bacteria were cultured overnight in 1 L of Todd-Hewitt broth (20 h) in 5% CO2 at 37°C, harvested by centrifugation, washed twice in pyrogen-free 0.9% NaCl, resuspended in 10 mL of 0.9% NaCl, and heat inactivated for 60 min at 80°C. Heat-killed P. aeruginosa (HKPA; serotype PA01) was obtained from a clinical isolate. Bacteria were cultured, shaken in 1 L of Luria broth (20 h) at 37°C, and heat inactivated for 60 min at 80°C. A 500-μL sample on a blood agar plate did not show bacterial growth.

Assays. IL-8, ENA-78, and GRO-α concentrations were measured by using specific ELISAs, according to the instructions of the manufacturers (IL-8: Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, detection limit 1 pg/mL; ENA-78: R&D Systems, Minneapolis, detection limit 15.6 pg/mL; GRO-α: R&D Systems, detection limit 10 pg/mL). Leukocyte counts and differentials were determined by flow cytometry in EDTA-anticoagulated blood.

Statistical analysis. All values are given as mean ± SE. Com-
parisons were done by using the Wilcoxon rank sum test. $P < .05$ was considered to represent a statistically significant difference.

**Results**

*Responses to intravenous injection of LPS.* Infusion of LPS induced an influenza-like syndrome with headache, muscle pain, and a rise in body temperature that peaked after 3–4 h (peak temperature, 38.8 ± 0.2°C). Infusion of LPS caused early declines in total leukocytes and neutrophils, which then increased. Neutrophil numbers at the time points when PMNL were isolated for ex vivo stimulation were $3.1 \times 10^5/\mu$L (0 h), $9 \times 10^5/\mu$L (1 h; $P < .05$ vs. 0 h), $4.6 \times 10^5/\mu$L (2 h), and $5.5 \times 10^5/\mu$L (24 h; $P < .05$ vs. 0 h).

*Ex vivo IL-8, ENA-78, and GRO-α production by PMNL after in vivo LPS administration.* Incubation of PMNL without LPS or bacteria resulted in low concentrations of IL-8, ENA-78, and GRO-α in supernatant plasma (<0.1, <0.2, and <0.3 ng/mL, respectively; figure 1). The capacity of PMNL to release these chemokines in supernatant after stimulation with HKSP, HKPA, and LPS strongly decreased after in vivo exposure to LPS and reached a nadir after 2 h. IL-8, ENA-78, and GRO-α production induced by incubation with bacteria or LPS ex vivo returned to baseline 24 h after the in vivo administration of LPS.

*Effect of in vivo LPS administration on cell-associated IL-8.* In accordance with the decreased levels of IL-8 in supernatant, concentrations of cell-associated IL-8 after stimulation ex vivo with HKSP, HKPA, and LPS were lower 1 and 2 h after LPS administration (figure 2).

*Effect of pre- and post-LPS serum on chemokine production by normal PMNL.* Neither addition of pre-LPS serum (obtained before LPS injection) nor addition of post-LPS serum (obtained 2 h after LPS injection) influenced the capacity of PMNL isolated from healthy subjects not previously exposed to LPS to produce IL-8, ENA-78, or GRO-α after stimulation with HKSP, HKPA, or LPS (data not shown).

**Discussion**

The phenomenon of immunoparalysis has been studied in investigations with monocytes or whole blood. Monocytes or blood obtained from patients with sepsis or other systemic inflammatory conditions or from healthy subjects exposed to low-dose LPS have a reduced capacity to produce proinflammatory cytokines after restimulation with LPS [3, 5–8]. Not only monocytes but also PMNL from patients with sepsis display a refractory phenotype (i.e., they produce less IL-1β and IL-8 than do normal PMNL after stimulation with heat-killed streptococci or LPS) [11, 12]. The present study extends these findings by demonstrating that PMNL from healthy volunteers are rendered anergic after an intravenous challenge with LPS. PMNL produced less IL-8, GRO-α, and ENA-78 after stimulation with heat-killed bacteria or LPS ex vivo after an intravenous challenge with LPS. Chemokine production was not influenced by serum obtained from subjects after exposure to LPS, indicating that PMNL anergy is not induced by soluble mediators.

Of note, PMNL isolated from peripheral blood 1 and 2 h after LPS injection likely differed from the population present in blood before LPS administration, because endotoxemia results in adhesion of PMNL to the vascular endothelium and in subsequent release of PMNL from bone marrow. In addition, although PMNL preparations were >99% pure, a theoretical possibility remains that contaminating monocytes influenced our results. Hence, the present data should be interpreted taking these considerations into account.

Marie et al. [12] demonstrated that ex vivo IL-8 production by circulating PMNL was reduced in patients with sepsis or during a cardiopulmonary bypass procedure. Of considerable interest, these authors found that PMNL cannot be rendered anergic to LPS by previous exposure to LPS in vitro. On the contrary, PMNL pretreated with LPS in vitro had enhanced IL-8 production after reexposure to LPS [12]. These findings suggest that anergy of PMNL is mediated via mechanisms that differ from those underlying anergy of monocytes. Indeed, monocytes can be made hyporesponsive by preexposure to LPS in vitro [14, 15]. Our study demonstrates that in vivo exposure to LPS does render PMNL anergic for further stimulation with heat-killed bacteria or LPS.
LPS (and heat-killed bacteria). PMNL used in our investigation were first challenged with LPS in their natural (in vivo) environment—that is, in close contact with other blood components and the endothelium before being stimulated ex vivo with bacteria or LPS. Because preincubation of PMNL with LPS in vitro does not result in PMNL hyporesponsiveness [12], these data suggest that LPS does not induce an anergic state in PMNL in the absence of other cell types or soluble mediators.

The capacity to produce PMNL-associated IL-8 also decreased after intravenous injection of LPS. Marie et al. [12] had a similar finding in patients after cardiopulmonary bypass surgery, but not in patients with sepsis. Clearly, additional studies are warranted to evaluate possible mechanisms underlying the discrepancies between IL-8 that is released and IL-8 that remains cell-associated after stimulation of isolated PMNL.

We were interested in learning whether soluble factors produced after in vivo administration of LPS are involved in the hyporesponsiveness of PMNL, considering, first, that the LPS hyporesponsiveness of monocytes can be reproduced in part by addition of serum from patients with sepsis [9, 10] or from healthy subjects injected with LPS [8], and, second, that LPS cannot induce PMNL anergy when incubated with isolated PMNL in vitro (see above) [12]. The results of our experiments, in which we stimulated PMNL from healthy volunteers not exposed to LPS, in the absence and presence of serum obtained from LPS-challenged volunteers, suggest that soluble factors do not play a role in the induction of anergy of PMNL after exposure to LPS in vivo. This finding further supports the notion that PMNL anergy is mediated via pathways that, at least in part, differ from those involved in monocyte anergy.

References