

Differential Induction of Pro- and Anti-Inflammatory Cytokines in Whole Blood by Bacteria: Effects of Antibiotic Treatment

J. T. M. FRIELING,¹ J. A. MULDER,² T. HENDRIKS,¹ J. H. A. J. CURFS,² C. J. VAN DER LINDEN,¹
AND R. W. SAUERWEIN^{2*}

Departments of Surgery¹ and Medical Microbiology,² University Hospital Nijmegen,
6500 HB Nijmegen, The Netherlands

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The in vitro production of interleukin-1 β (IL-1 β), IL-6, and the IL-1 receptor antagonist (IL-1ra) in whole blood upon stimulation with different bacterial strains was measured to study the possible relationship between disease severity and the cytokine-inducing capacities of these strains. *Escherichia coli*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Bacteroides fragilis*, *Capnocytophaga canimorsus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* induced the cytokines IL-1 β , IL-6, and IL-1ra. Gram-negative bacteria induced significantly higher levels of proinflammatory cytokine production than gram-positive bacteria. These differences were less pronounced for the anti-inflammatory cytokine IL-1ra. In addition, blood was stimulated with *E. coli* killed by different antibiotics to study the effect of the antibiotics on the cytokine-inducing capacity of the bacterial culture. *E. coli* treated with cefuroxime and gentamicin induced higher levels of IL-1 β and IL-6 production but levels of IL-1ra production similar to that of heat-killed *E. coli*. In contrast, ciprofloxacin- and imipenem-cilastatin-mediated killing showed a decreased or similar level of induction of cytokine production as compared to that by heat-killed *E. coli*; polymyxin B decreased the level of production of the cytokines.

The introduction of potent antimicrobial agents has increased survival among patients with gram-negative sepsis, but mortality still varies between 20 and 50%, especially in patients with shock (30). This high mortality may be explained by the reaction of the patient to cell wall components from bacteria, in particular, lipopolysaccharide (LPS), which induces an uncontrolled inflammatory reaction resulting in tissue damage and organ failure. LPS is released by living and growing bacteria, but killing of the bacteria by antimicrobial agents may additionally liberate LPS. This may lead to an initial deleterious effect of antibiotic treatment on the condition of the patient. Such phenomena have been described for typhoid fever (4) and for suspected gram-negative sepsis (32) and are supported by studies which show increased concentrations of LPS upon antibiotic treatment of clinical (21) or experimental (17, 25, 26, 33) infections and in in vitro models (3, 5-7, 12, 13, 24, 35).

The amount of LPS released is determined by the type and concentration of the antimicrobial agent as well as the nature and sensitivity of the bacteria. Plasma LPS concentrations usually do not correlate with clinical symptoms (31). It is the induction of cytokines through cell wall components like LPS which mediates the biological responses during bacterial infections. Cytokine levels and types of cytokines have repeatedly been shown to correlate with clinical outcome (8, 19, 36). The key role of cytokines is also demonstrated by the capability of anti-tumor necrosis factor antibodies to prevent the Jarisch-Herxheimer reaction when these antibodies are given before antibiotic treatment (14). LPS is only one of the bacterial cell wall components that can induce an inflammatory response (11). Gram-positive microorganisms, which lack LPS in their

cell wall, induce the release of cytokines by peptidoglycans and teichoic acid (23, 34).

We studied the relationship between the capacity of morphologically intact bacteria to induce cytokines in whole blood and the clinical severity of the infection. In addition we studied the effect of *Escherichia coli* treated with a panel of different antibiotics on cytokine induction.

The following mediators, which have been shown to correlate with disease severity and patient outcome, were measured: interleukin-1 β (IL-1 β), IL-6, and the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1ra).

MATERIALS AND METHODS

The following microorganisms were studied: *E. coli* ATCC 25922, *Bacteroides fragilis* ATCC 10584, *Staphylococcus aureus* ATCC 25293, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 6305, *Streptococcus pyogenes* ATCC 43202, and clinical isolates of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Capnocytophaga canimorsus*. Bacteria were cultured in brain heart infusion broth. The numbers of bacteria were adjusted to the required turbidity by measuring the optical density. After dilution the bacteria were killed by X-ray irradiation at a dose of 25 kGy and were added to whole human blood.

Whole-blood stimulation. A modification of the method described by Desch et al. (9) was used for whole-blood stimulation. Whole blood was drawn from healthy hospital employees in a sterile vacuum tube (Sherwood Medical, Ballymoney, Northern Ireland) to which sterile pyrogen-free heparin (Organon Technika, Boxtel, The Netherlands) was added as anticoagulant at a final concentration of 50 IU/ml of blood. Whole blood (0.5 ml) was added to a sterile polypropylene tube containing 10 μ l of stimulus or (as a control) dilution buffer followed by incubation at 37°C for 24 h. After incubation the tubes were centrifuged and plasma was collected and stored at -20°C until it was used.

LPS from *E. coli* serotype O55:B5 (product no. L4005; Sigma, St. Louis, Mo.) at a final concentration of 10 μ g/ml was used in each test as a reference for cytokine induction in the whole-blood assay.

An IL-6 enzyme-linked immunosorbent assay was performed as described before (18). In brief, a 96-well plate (Costar 3500 EIA/RIA plate) was coated overnight at 4°C with anti-IL-6 antibody (BE-8) and was washed with wash buffer (phosphate buffer with 0.05% Tween 20). Wells were blocked with phosphate buffer containing 5% bovine serum albumin (product no. A7030; Sigma), and after an hour at room temperature, the plate was washed and samples or a standard (recombinant IL-6) was added, followed by incubation for 1 h at 37°C. Washing was repeated, and a second biotinylated antibody to IL-6 was added. After 1 h of incubation at room temperature, the plate was washed, streptavidin-horseradish peroxidase (product no. S5512; Sigma) was added to the wells for 45

* Corresponding author. Mailing address: Department of Medical Microbiology, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Phone: 31 24 3614356. Fax: 31 24 3540216.

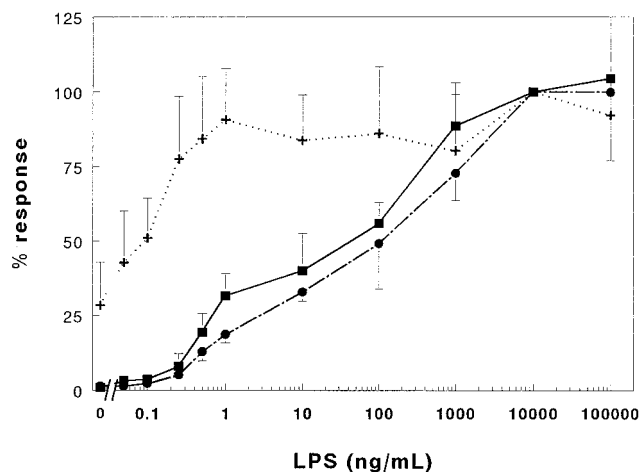


FIG. 1. Cytokine response (mean \pm SD) to different concentrations of LPS. Results are expressed as the percent response compared to that of 10 μ g LPS per ml. ●, IL-1 β ; ■, IL-6; +, IL-1ra.

min, and the plate was subsequently washed. Thereafter, *o*-phenyldiamine (0.5 mg/ml; product no. P6912; Sigma) was added, and after 15 to 20 min the color reaction was stopped with H₂SO₄ (2.5 M). The optical density was read at 492 nm with an automated plate reader (Titertek Multiskan MCC/340; Labsystems, Amsterdam, The Netherlands). The detection limit of the assay was 20 pg/ml.

IL-1 β and IL-1ra levels were determined by radioimmunoassays (20, 22). Recombinant human IL-1 β and IL-1ra were used as standards. The detection limits of the assays varied between each run and were usually between 0.02 and 0.20 ng/ml.

LPS was detected by a chromogenic *Limulus* amoebocyte lysate assay (Coatest, Chromogenix, Mölndal, Sweden) by the endpoint method according to the instructions of the manufacturer. The detection limit of the assay was 12.5 pg/ml.

To investigate the effects of antibiotic treatment on cytokine induction in whole blood, *E. coli* ATCC 25922 was cultured for 2.5 h in Trypticase soy broth medium to induce logarithmic growth. Subsequently, bacteria were cultured in broth at a concentration of 5×10^9 bacteria/ml in the presence of antibiotics for 24 h. Heat-killed *E. coli* showing morphologically intact microorganisms on Gram staining were used as controls. After incubation the antibiotic-treated *E. coli* samples were tested in the whole-blood assay. The final concentration in whole blood was 10^5 bacteria/ml.

The final concentrations of the antibiotics used were as follows: ciprofloxacin, 4 μ g/ml (Bayer, Mijdrecht, The Netherlands); cefuroxime, 80 μ g/ml (Glaxo, Zeist, The Netherlands); gentamicin, 10 μ g/ml (Schering-Plough, Weesp, The Netherlands); imipenem-cilastatin, 50 μ g/ml (Merck Sharp & Dohme, Haarlem, The Netherlands); and polymyxin B, 15 μ g/ml (Pfizer, Capelle a/d IJssel, The Netherlands). These concentrations were based on the peak levels in human plasma during treatment with a standard intravenous dose. Antibiotic concentrations exceeded the MICs by >40 times in all cases.

Statistical analysis. One-way analysis of variance was used for statistical analysis. Comparisons between bacteria and antibiotics were calculated by the Newman-Keuls test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Cytokine response to LPS in whole blood. Figure 1 shows the dose-dependent increase in IL-1 β and IL-6 concentrations upon the addition of LPS to the whole-blood system. An LPS concentration of >0.1 ng/ml was required for the induction of IL-1 β and IL-6. The level of production of IL-1 β in three donors induced by 10 μ g of LPS per ml was 55.3 ± 20.0 ng/ml, and the level of IL-6 production was 26.8 ± 12.5 ng/ml (values are means \pm standard deviations [SDS]). In the absence of LPS there was some constitutive presence of IL-1ra at 1.2 to 3.6 ng/ml, but no constitutive production of IL-1 β or IL-6 was detectable. An increase in the level of IL-1ra production could already be achieved by 20 pg of LPS per ml. The maximum level of IL-1ra production in whole blood was obtained with roughly 10,000-fold lower concentrations of LPS than were required for the maximum level of IL-1 β and IL-6 production.

The level of IL-1ra production induced by 10 μ g of LPS per ml was 12.6 ± 1.6 ng/ml (mean \pm SD).

Cytokine induction by different bacteria. In Fig. 2 the results of IL-1 β , IL-6, and IL-1ra production for seven donors from stimulation with 10 μ g of LPS per ml are given as percentages. The concentration of IL-6 induced by 10 μ g of LPS per ml was 84.2 ± 29.5 ng/ml; that of IL-1 β was 35.7 ± 11.6 ng/ml and that of IL-1ra was 11.9 ± 3.0 ng/ml (means \pm SDs for seven donors). Cytokine induction by bacteria at a concentration of 0.5×10^6 CFU/ml could be detected for each bacterial species. The production of IL-6 and IL-1 β was significantly higher in the presence of *E. coli*, *N. meningitidis*, and *N. gonorrhoeae* than in the presence of gram-positive bacteria. Differences between these gram-negative bacteria and the gram-positive bacteria were also significant for IL-1ra production, but they were less pronounced. The gram-negative bacteria *B. fragilis* and *C. canimorsus* showed capacities to induce cytokines comparable to those of the gram-positive bacteria tested.

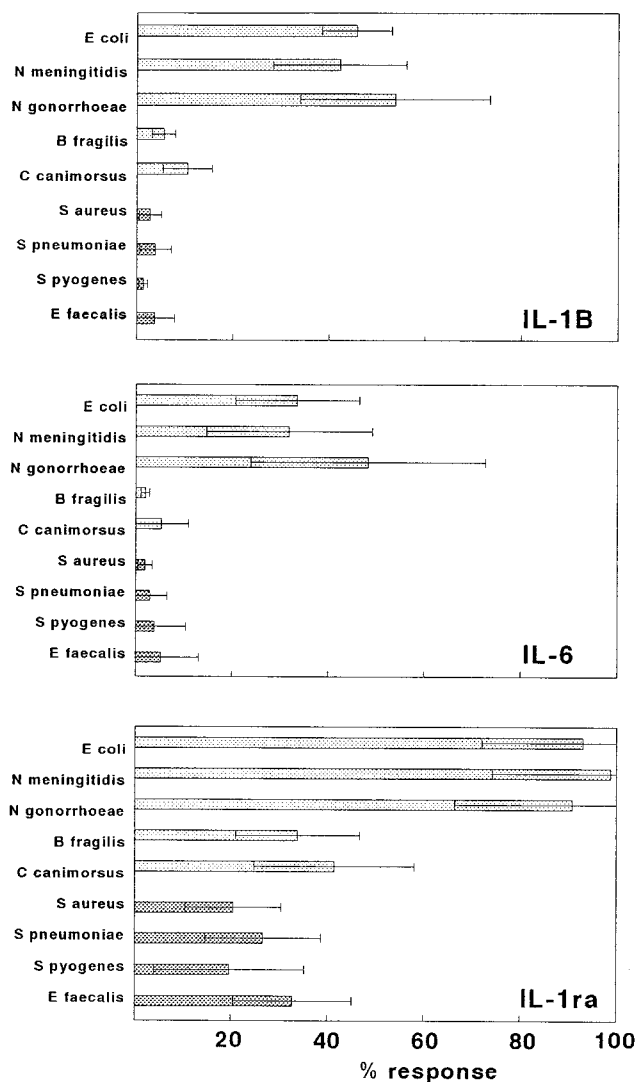


FIG. 2. IL-1 β , IL-6, and IL-1ra responses (mean \pm SD) of seven donors to gram-negative and gram-positive bacteria at a bacterial concentration of 0.5×10^6 CFU/ml. Results are expressed as the percent response compared to that of 10 μ g of LPS per ml.

TABLE 1. Cytokine induction by antibiotic-killed *E. coli*

Antibiotic	% Cytokine induction ^a		
	IL-1 β	IL-6	IL-1ra
Cefuroxime	282.6 \pm 28.4	177.4 \pm 46.3	104.4 \pm 9.2
Gentamicin	145.4 \pm 11.2	143.3 \pm 32.0	114.9 \pm 8.9
Ciprofloxacin	86.8 \pm 10.3	88.9 \pm 13.6	108.8 \pm 10.1
Imipenem	88.4 \pm 40.1	87.5 \pm 9.4	100.8 \pm 6.4
Polymyxin B	25.8 \pm 4.2	14.6 \pm 7.6	65.6 \pm 20.7
ANOVA ^b	<0.0001 ^c	0.0002 ^d	0.003 ^e

^a Values are means \pm SDs of three tests. Values are expressed as the percentage of induction compared to that by heat-killed *E. coli*.

^b NOVA, analysis of variance.

^c By the Newman-Keuls test, $P < 0.05$; comparisons were significantly different except ciprofloxacin versus imipenem.

^d By the Newman-Keuls test, $P < 0.05$ for cefuroxime versus ciprofloxacin and imipenem and polymyxin B versus all other antibiotics.

^e Newman-Keuls test, $P < 0.05$ for polymyxin B versus all other antibiotics.

Induction of cytokines by antibiotic-killed *E. coli*. The cultures of *E. coli* were killed by the tested antibiotics within 1 to 18 h. This was determined by subculturing. To ascertain that all bacteria were killed, the experiments were performed after incubation of the cultures with the antibiotics for 24 h.

At the concentrations used in this study, the antibiotics cefuroxime, gentamicin, ciprofloxacin, and imipenem did not influence the LPS-induced production of IL-1 β , IL-6, and IL-1ra in whole blood. Polymyxin B did significantly reduce the induction of cytokines by LPS (data not shown).

Table 1 presents the results of IL-6, IL-1 β , and IL-1ra induction by antibiotic-killed *E. coli*. IL-1 β production by the heat-killed *E. coli* was 22.0 \pm 13.9 ng/ml, IL-6 production was 54.5 \pm 31.6 ng/ml, and IL-1ra production was 14.8 \pm 2.0 ng/ml (means \pm SDs).

The levels of IL-6 and IL-1 β induction by antibiotic-treated *E. coli* differed significantly among the antibiotics used. Compared to heat-killed *E. coli* at 10⁵ CFU/ml, cefuroxime-treated *E. coli* induced a significant increase in IL-6 and IL-1 β production. Gentamicin treatment also led to increased IL-1 β and IL-6 induction, although the increase in IL-6 induction was not significant. Ciprofloxacin and imipenem killed the bacteria without significantly increasing the level of induction of IL-6 and IL-1 β . Treatment of *E. coli* with polymyxin B resulted in a decrease in the levels of both IL-6 and IL-1 β induction. The level of IL-1ra production was little influenced by antibiotic killing except for that by polymyxin B.

The level of production of IL-1 β induced by cefuroxime- and polymyxin B-treated *E. coli* differed significantly from the level of production induced by heat-killed *E. coli*. Differences between antibiotics in the induction of IL-1 β production were significant for all antibiotics except imipenem versus ciprofloxacin. Differences between antibiotics in IL-6 induction capacity were not significant for gentamicin versus ciprofloxacin, cefuroxime, and imipenem and were not significant for imipenem versus ciprofloxacin. For IL-1ra induction capacity, only the differences between polymyxin B versus heat-killed *E. coli* and the other antibiotics were significant.

Effect of antibiotic treatment of *E. coli* on the LPS concentration. The activity of the suspension of heat-killed *E. coli* in the *Limulus* amoebocyte lysate assay (Fig. 3) was lower than that of the suspension of antibiotic-treated *E. coli* (10⁵ CFU/ml). Cefuroxime treatment gave the highest increase in *Limulus* amoebocyte lysate assay activity among the antibiotics tested. Gentamicin resulted in less of an increase in *Limulus*

amoebocyte lysate assay activity than cefuroxime, followed by ciprofloxacin and imipenem. Polymyxin B treatment of *E. coli* resulted in hardly any increase in *Limulus* amoebocyte lysate assay activity; however, polymyxin B interfered with the *Limulus* amoebocyte lysate assay.

In order to get an impression of the fraction of LPS that is bound to the bacteria and the fraction that is free, cultures were filtered through a sterile pyrogen-free 40- μ m-pore-size filter. Figure 3 shows the increase in *Limulus* amoebocyte lysate assay activity for antibiotic-treated *E. coli* compared to the *Limulus* Amoebocyte lysate assay activity for the heat-killed *E. coli* for both total and free LPS. The LPS concentration in the heat-killed *E. coli* was 1.9 \times 10⁵ pg/ml, and after filtration it was 7.8 \times 10⁵ pg/ml. The fraction of free LPS was very low after cefuroxime treatment and came into the range of the total amount of LPS after treatment with the other antibiotics, although the total amount was still higher.

DISCUSSION

In this study we investigated the production in whole blood of the proinflammatory cytokines IL-1 β and IL-6 and the anti-inflammatory cytokine IL-1ra stimulated by a number of bacteria. Major differences were found between gram-negative and gram-positive bacteria in the induction of IL-1 β , IL-6, and IL-1ra production in whole blood. The higher levels of production of cytokines induced by gram-negative bacteria might indicate that in gram-negative infections high levels of cytokine induction lead to a more acute and fulminant clinical picture compared to that in infections with gram-positive bacteria. Although gram-positive organisms are capable of inducing cytokine production, 100- to 1,000-fold more gram-positive bacteria are needed to induce the same concentration of cytokines induced by gram-negative bacteria. Thus, gram-negative organisms are more potent inducers of IL-1 β , IL-6, and IL-1ra than gram-positive bacteria in the whole-blood assay, which confirms the studies with isolated monocytes performed by Timmerman et al. (34) and Mattson et al. (23).

The relation between the capacity to induce cytokine production in the whole-blood assay and disease severity is less

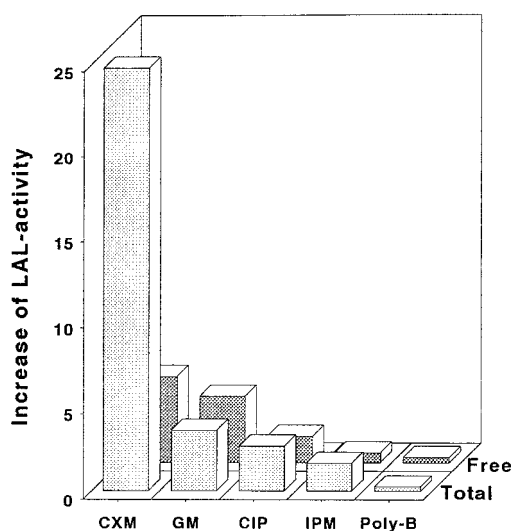


FIG. 3. *Limulus* amoebocyte lysate assay (LAL) activity increase in cultures of antibiotic-killed *E. coli* (10⁵ CFU/ml). Results are expressed as the fold increase in *Limulus* amoebocyte lysate assay activity compared to the activity of the same amount of heat-killed *E. coli*. CXM, cefuroxime; GM, gentamicin; CIP, ciprofloxacin; IPM, imipenem-cilastatin; and Poly-B, polymyxin B.

obvious for the individual bacterial strains. Gram-negative sepsis caused by *N. meningitidis* is a fulminant disease with a high mortality rate which may be reflected by the organism's capacity to induce a strong cytokine response in our in vitro system. In contrast, *N. gonorrhoeae* shows an induction capacity similar to that of *N. meningitidis* in our system, but bacteremia with *N. gonorrhoeae* presents with mild clinical symptoms. Similarly, *C. canimorsus* induces a very low level of production of cytokines but can induce a very serious clinical picture of sepsis with a high mortality rate (2). The reason for this inconsistency might be the anatomical location of the infection (e.g., in the central nervous system) or production of toxins (e.g., by *C. canimorsus* [15]).

The differences in the modes of action of cefuroxime, gentamicin, ciprofloxacin, and imipenem probably result in the differences in inducing cytokine production by antibiotic-treated *E. coli*. Imipenem makes the bacteria osmotically unstable, and cefuroxime, also a β -lactam antibiotic, inhibits the formation of intercellular septa in dividing bacteria, resulting in long filaments (28). In the latter case, one may assume that more cell wall components are formed. This assumption is confirmed by the high levels of IL-1 β and IL-6 production. Dofferhof et al. (10) also found that killing of *E. coli* by cefuroxime induced higher levels of production of tumor necrosis factor in isolated monocytes compared to that induced by *E. coli* killed with other antibiotics. In their tests, killing of *E. coli* with imipenem also resulted in the lowest level of induction of tumor necrosis factor, and the highest level of production was achieved with *E. coli* killed with cefuroxime. The LPS concentration found in the cultures with cefuroxime-killed bacteria also indicates the formation of more cell wall mass. The large difference between the amounts of total and free LPS with cefuroxime-treated *E. coli* might indicate that most of the LPS is constituted in the filaments which might not pass through the filter. Gentamicin acts on protein synthesis, while ciprofloxacin acts on DNA gyrase. This might lead to a minimal change in production of cell wall components. In the study of Prins et al. (29), gentamicin killing of *E. coli* resulted in less tumor necrosis factor production than when ciprofloxacin was used. In our study the opposite was true for IL-1 β and IL-6. The explanation Prins et al. give is the ability of aminoglycosides to bind and inactivate LPS (1, 16). The addition of LPS to whole blood in the presence of either gentamicin, ciprofloxacin, cefuroxime, or imipenem did not influence cytokine production, leaving the methodological differences as a possible explanation for this inconsistency. In our tests gentamicin might influence the structure of the cell wall in such a way that the cell wall components that are able to induce cytokine production become more available for cytokine-producing cells. This is supported by the finding that the amount of LPS measured in the cultures was almost equal to that found in cultures of bacteria killed by imipenem or ciprofloxacin, although the level of induction of cytokine production by the last two antibiotics was much lower. Polymyxin B acts in a completely different way. It kills the bacteria by changing the osmotic barrier. The major reason for the low level of induction of cytokines is the binding of polymyxin B to LPS, which results in a less biologically active form of the LPS (6), as can be seen in our tests. This well-known phenomenon not only interferes with the cytokine induction capacity but also disturbs the activity of LPS in the *Limulus* amoebocyte lysate assay. Therefore, no answer can be given to the question of how much LPS can be found after killing of *E. coli* by polymyxin B in this study.

Differences between antibiotics for the induction of the anti-inflammatory cytokine IL-1ra were not large; only polymyxin B-treated *E. coli* induced a lower level of induction of IL-1ra

production. This probably results from the fact that the IL-1ra production induced by LPS is about 10,000-fold more efficient than the IL-1 β production induced by LPS (Fig. 1). With high concentrations of bacteria, the production capacity of IL-1ra is already on a plateau and neither an increase nor a small decrease in cytokine-inducing components can result in a change in the level of IL-1ra production. At lower concentrations of treated *E. coli* (2×10^3 CFU/ml), the production of IL-1ra shows a pattern similar to those for IL-1 β and IL-6 (data not shown).

The modified ex vivo cytokine production model that is used here is a good model for comparing multiple stimuli. Other models that have been described used larger volumes of blood, which limits the amount of stimuli which can be tested in one experiment (27). Also, blood diluted with medium is used, but this changes the cellular environment. Here, we showed that a small volume of blood (0.5 ml) can sufficiently produce the cytokines IL-1 β , IL-6, and IL-1ra when the blood is stimulated. The anticoagulant that is used also influences the level of cytokine production. Maximum cytokine production was achieved by the use of endotoxin-free heparin (data not shown).

The results of this study are in agreement with findings by others with respect to LPS release by antibiotic-killed bacteria (10, 29, 37). However, this study shows that there is a discrepancy between the concentrations of LPS measured in the *Limulus* amoebocyte lysate assay and the induction of cytokines in whole blood. Components other than LPS, such as other cytokine-inducing products derived from killed bacteria or even the drugs administered, could be responsible for this discrepancy. Clinical studies with humans should be done to see whether the differences between antibiotics seen in whole blood are clinically relevant. Since the differences between the induction of cytokines by different bacteria in whole blood cannot always predict disease severity, clinical studies seem to be even more essential.

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