Production of tumor necrosis factor-α and interleukin-6 in whole blood stimulated by live Gram-negative and Gram-positive bacteria

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Objective: To investigate the production of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) induced by live Gram-negative and Gram-positive bacteria in whole blood in vitro.

Methods: In all, 49 different isolates were studied. Each of the 49 different isolates was incubated for 4 h with whole blood at a ratio of one monocyte per 1–5 bacteria. Plasma was then separated and frozen, and the concentrations of TNF-α and IL-6 were measured by enzyme immunoassays.

Results: There was a positive correlation between TNF-α and IL-6 values, $r=0.9$. Gram-negative bacteria induced higher levels of both TNF-α and IL-6 than Gram-positive bacteria. Group G streptococci (GGS) induced higher levels of TNF-α than *Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis* and group A streptococci (GAS). *Klebsiella pneumoniae* induced higher levels of TNF-α than *Haemophilus influenzae, Escherichia coli* and *Neisseria meningitidis.* GGS induced higher levels of IL-6 than *Staphylococcus epidermidis, Staphylococcus aureus* and GAS. When the relative amounts of cytokine induced by the strains were compared to serum concentrations measured on admission in patients with bacteremia caused by the same bacterial isolates there was no significant correlation.

Conclusion: Species- and strain-related differences in cytokine-inducing properties were found which may have significance in clinical infections.

Key words: In vitro, whole blood, cytokine production, live bacteria

INTRODUCTION

Cytokines are important mediators in the inflammatory host response to bacterial infection. High levels of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) have been shown to correlate with severity in patients with bacteremic infections or sepsis syndrome [1,2], and in some clinical studies blood concentrations of TNF-α and IL-6 have been shown to be higher in patients with infections caused by Gram-negative bacteria than in those with infections caused by Gram-positive bacteria [1–3]. The bacterial components able to induce cytokine production have been shown to be lipopolysaccharide (LPS) from Gram-negative organisms, and exotoxins, enterotoxins and cell wall constituents from Gram-positive bacteria [4–7]. However, previous investigations have examined either bacterial components or heat- or antibiotic-killed bacteria and usually examined single bacterial species [4,6-13].

In this paper we investigate the ability of different live Gram-negative and Gram-positive bacteria to induce the production of TNF-α and IL-6 in whole blood in vitro using a method previously published [10,12,13]. The aim was to study differences in cytokine-inducing abilities between different bacterial

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species and to compare the relative values with the concentrations in blood obtained from patients with bacteremia caused by the same isolates.

MATERIAL AND METHODS

Bacteria

Forty-four bacterial strains were clinical isolates from blood, and five (all *Streptococcus pneumoniae*) were isolates from the upper respiratory tract, in all representing eight different species which were obtained from the collection of frozen bacterial strains at the Department of Clinical Microbiology, Örebro Medical Center Hospital. The strains and their characteristics are listed in Figure 1: the pneumococci were of serotypes 3 (two isolates), 6A, 9N, 18C, 19A, 19F, 22F, 33F and 35F; the meningococci were of serogroups A, BT2, BT4, BT15, C and 29E. Included in the 44 isolates from blood were the 25 isolates from the patients described below.

The bacteria were thawed and grown on appropriate media overnight and then two colonies were added to broth suitable for each bacterial species and grown on a shaker overnight. On the day of the experiment 5 mL of the bacterial suspension was diluted with phosphate-buffered saline (PBS, cat. no. 10010-015, Gibco BRL, Life Technologies, UK) and centrifuged, the supernatant discarded and the pellet resuspended in PBS. This procedure was performed two times, and then the bacterial number was adjusted to a monocyte/bacterium ratio of 1:1–5 by counting in a Bürker chamber. The bacterial viability was checked by culture on appropriate agar plates immediately before the experiments were started.

Whole blood

Venous blood was obtained from blood donors with compatible blood groups. For each experiment, six donors were used. The blood was sampled into tubes containing endotoxin-free heparin (Endotube ET, Chromogenix AB, Sweden) and the blood was then pooled in endotoxin-free tubes. Neutrophil, monocyte and lymphocyte counts were made using a CellDyn hematocytometer immediately after pooling and gentle mixing of the blood.

In vitro stimulation experiments

A method described previously [10,12,13] was used with minor modifications. Nine hundred microliters of whole blood with the addition of 100 μL of the bacterial suspension was incubated for 4 h at 37°C in 5% CO_2 in sterile tubes (Kimble 12 × 75 mm sterile glass tubes, Labora, Sweden). The tubes were then put into iced water and immediately centrifuged; the plasma was separated and stored at −20°C until cytokine analysis was performed. Two negative controls (PBS and whole blood), and four positive controls (whole blood + 100 pg LPS and whole blood + 1 ng LPS) were included in each experiment. In total, nine experiments were performed. As different donor pools were used, the results are presented as a ratio of the amount of cytokine induced by the bacterial suspension and the amount induced by LPS in each experiment. For TNF-α the amount induced by the bacteria is divided by that induced by 1 ng LPS, and for IL-6 the amount of cytokine induced by the bacteria is divided by that induced by 100 pg LPS. The amount of cytokine produced by whole blood incubated with PBS was negligible in each experiment.

Determination of TNF-α and IL-6

Plasma was analyzed using TNF-α and IL-6 ELISA kits from R&D Systems Europe, UK. Fetal calf serum inactivated by heating at 56°C for 1 h from Invitro, Sweden (cat. no. 29-101-54) was used for dilution of the plasma samples. All samples were assayed once undiluted, and then samples with high values were diluted and retested.

Endotoxin measurements

The amount of endotoxin in each sample as well as in the reagents used in all the experiments was measured using a Limulus Amebocyte Lysate (LAL) test (COATEST, Chromogenix AB, Sweden). Samples with high values were diluted and retested. No endotoxin was detected in samples stimulated with Gram-positive bacteria. The endotoxin concentrations found in the samples stimulated with Gram-negative bacteria were variable but there were only weak correlations between the concentrations of cytokines and endotoxin.

LPS

LPS from *Escherichia coli* O111:B4 was obtained from Sigma (St Louis, Mo.) and was used as positive control in the cytokine and endotoxin assays.

Patients

Ten patients with *Staphylococcus aureus* bacteremia selected from 64 described previously [14] and 15 with *Streptococcus pneumoniae*, *Escherichia coli* or group A ($n=2$) or G ($n=3$) hemolytic streptococcal bacteremia also previously reported [2] were included.

Statistical methods

Data are expressed as mean + 1 SD. When levels of cytokines in more than two groups were compared, one-way analysis of variance was used. Post hoc multiple comparisons were tested using the Bonferroni

test at $p<0.05$. When two groups were compared, the unpaired Student's t-test was used. Correlation was studied using the Pearson correlation test. The Statview 4.01 (Abacus, Ca) software was used for the statistical calculations.

Ethical considerations

The study was approved by the local ethics committee.

RESULTS

The results are shown in Figure 1. There was a positive correlation between values obtained for TNF-α and IL-6 ($r=0.91$).

Production of TNF-α

Gram-negative bacteria induced higher levels of TNF-α than Gram-positive bacteria ($p<0.0001$). Among the Gram-positive bacteria, group G hemolytic streptococci (GGS) induced higher levels than all other Gram-positive isolates. In the group of Gram-negative bacteria, *Klebsiella pneumoniae* induced higher levels than *Haemophilus influenzae* (non-typeable or group b), *E. coli* and *Neisseria meningitidis*. *E. coli* induced higher levels than *H. influenzae* (non-typeable or group b) and *N. meningitidis*.

Production of IL-6

Gram-negative bacteria induced higher levels of IL-6 than Gram-positive bacteria, ($p<0.0001$). Among the Gram-positive bacteria, GGS induced higher levels than *Staphylococcus epidermidis*, enterotoxin-producing *Staphylococcus aureus* and group A hemolytic streptococci (GAS). There were no significant differences among IL-6 levels induced by the different Gram-negative bacteria.

Comparison of in vitro and in vivo cytokine concentrations

There was no significant correlation between cytokine concentrations produced in vitro and admission cytokine concentrations in patients with bacteremia with the same isolates. The mean values + 1 SD obtained in vitro are shown in Figure 2a, and the corresponding serum concentrations (mean + 1 SD) measured in patients with bacteremia with the same isolates are shown in Figure 2b.

DISCUSSION

Recent clinical studies [1–3] have demonstrated significant differences in blood cytokine levels in patients with different bacterial infections. In the present study, significant differences were also found in the capacity of different bacterial strains to induce TNF-α and IL-6 production in whole blood in vitro. Significant differences were found between Gram-negative and Gram-positive bacteria but also between different species.

As the ratios between monocytes and bacteria were the same during all the experiments, we believe that the differences found reflect differences between the

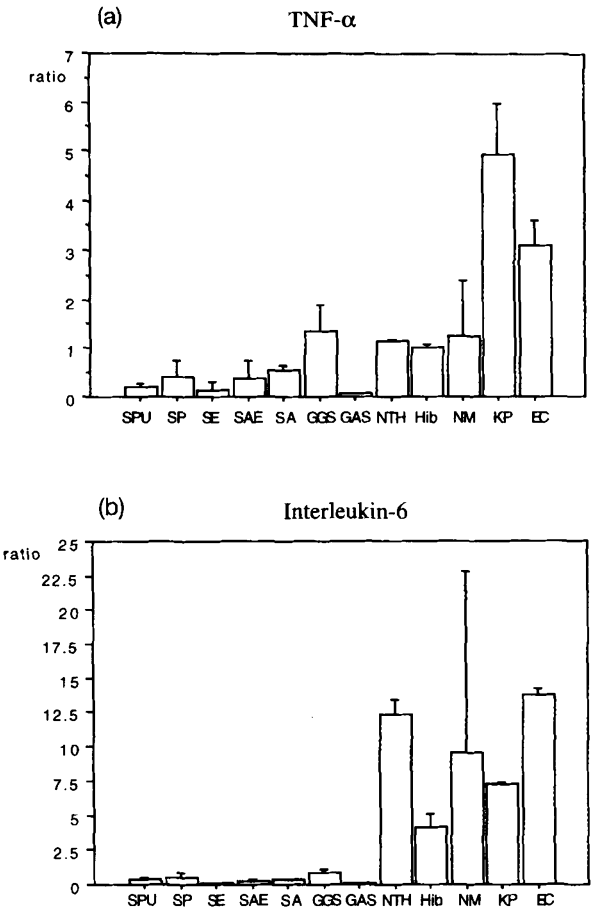

Figure 1 (a) Histogram showing mean values (+1 SD) of the ratios of production of TNF-α induced by the bacterial strain divided by that induced by 1 ng LPS. (b) Histogram showing mean values (+1 SD) of the ratios of production of IL-6 induced by the bacterial strain and that induced by 100 pg LPS. SPU, *Streptococcus pneumoniae*, upper respiratory tract isolates, $n=5$; SP, *Streptococcus pneumoniae*, $n=5$; SE: *Staphylococcus epidermidis*, $n=4$; SAE, *Staphylococcus aureus*, enterotoxin-producing, $n=5$; SA, *Staphylococcus aureus*, non-enterotoxin-producing, $n=5$; GGS, group G streptococci, $n=3$; GAS, group A streptococci, $n=2$; NTH, non-typeable *Haemophilus influenzae*, $n=3$; Hib, *H. influenzae* type b, $n=3$; NM, *Neisseria meningitidis*, $n=6$; KP, *Klebsiella pneumoniae*, $n=3$; EC, *E. coli*, $n=5$.
All strains are blood isolates except where indicated.

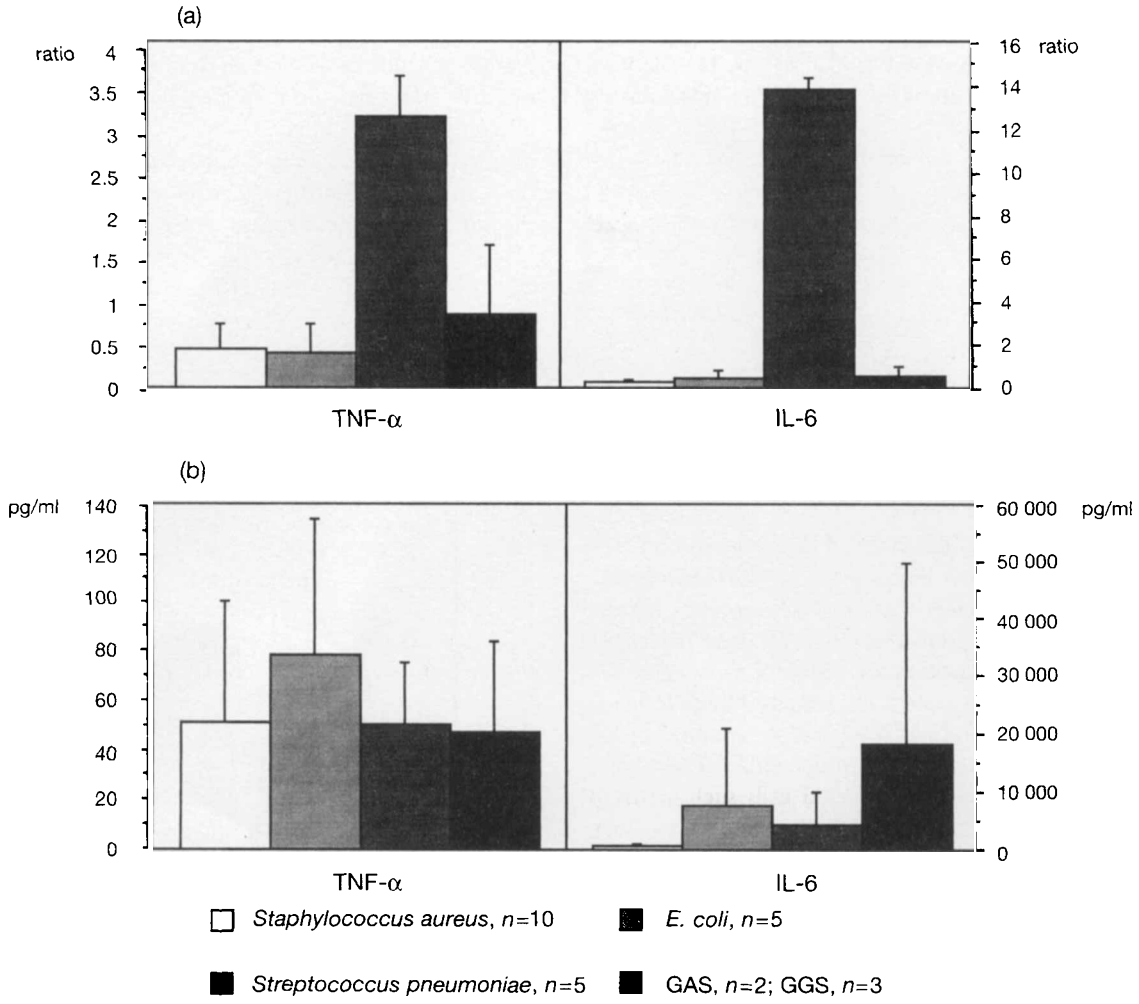

Figure 2 (a) Histogram showing mean values (+1 SD) of the ratios of the amount of cytokine induced by the bacterial strains and that induced by LPS. (b) Mean (+1 SD) serum concentrations of TNF-α and IL-6 measured on admission in patients with bacteremia caused by the same isolates as in (a).

various species' abilities to induce cytokine production. LPS is a powerful stimulator of cytokine production [5,10,13] and is probably the main cause of the high ratios found for the Gram-negative bacteria. The differences found between the different Gram-negative strains may be due to differences in LPS content. Although there was a strong correlation between the levels of the two cytokines induced in vitro, relative differences were found between species of Gram-negative bacteria. Non-typeable (NT) *H. influenzae* and *H. influenzae* type b induced similar TNF-α concentrations, but NT *H. influenzae* was a far more potent inducer of IL-6 than *H. influenzae* type b. Similarly, *K. pneumoniae* induced high levels of TNF-α compared to *E. coli*, but *E. coli* was a more potent inducer of IL-6 production than *K. pneumoniae*. Large intraspecies variation was found within the group of *N. meningitidis* isolates, as indicated by the size of the standard deviation. These results suggest that species as well as strain-specific differences in cytokine-inducing properties do exist.

Various toxins produced by Gram-positive bacteria may induce cytokine production but we found no differences between enterotoxin-producing *Staphylococcus aureus* and non-producing strains. GAS produce powerful toxins [15,16] but the two isolates studied in the present experiments induced lower levels of cytokines than did the three isolates of GGS. Whether these strains were toxin producers or not was not investigated. In addition, components of Gram-positive

bacteria such as cell wall parts may also induce inflammatory responses, as was previously shown [4,6]. The possibility of LPS contamination was ruled out, as all samples with Gram-positive bacteria were negative for LPS in the LAL test.

We found no differences in the cytokine levels induced by blood isolates of *Streptococcus pneumoniae* and isolates from the upper respiratory tract and similarly there was no difference between the levels induced by *Staphylococcus epidermidis* and *Staphylococcus aureus*, suggesting that usually non-pathogenic bacterial species have the potential to trigger an inflammatory response once they have reached the bloodstream.

Comparing the levels induced in vitro with the concentrations found in vivo caused by the same bacterial isolates, we found no significant correlation. The largest variation was seen for *Streptococcus pneumoniae* and the other streptococci. The five *E. coli* strains induced similar responses in vitro but larger variation was seen in vivo. Variation in the components of the immune response in cells and soluble mediators in the blood donor pool and in the individual patients are some possible explanations. The timing of the blood sampling in relation to the onset of symptoms in vivo may also influence the values obtained. Furthermore, other cells than circulating blood cells such as tissue macrophages, endothelial cells and epithelial cells may contribute to cytokine production in vivo [17–19], as may differences in the sensitivity of various cell lines to bacterial toxins.

The in vitro experiments were terminated after 4 h of incubation, as has been done in previous investigations [9,10,20]. Extending the period of incubation has been shown to give higher concentrations. We did not investigate whether the relative differences found between different bacterial species would disappear if the period of incubation was extended to 24 or 48 h.

In conclusion, we found significant differences between Gram-positive and Gram-negative bacterial species in their capacity to induce production of TNF-α and IL-6 in whole blood in vitro. Although we found a significant correlation between TNF-α and IL-6 production in vitro, some Gram-negative strains induced more TNF-α than IL-6 and other Gram-negative strains relatively more IL-6. We found no correlation between the cytokine levels induced in vitro and those found in vivo, which may be due to variability in the individual host inflammatory response to infection.

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