Patterns of Proinflammatory Cytokines and Inhibitors during Typhoid Fever

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Cytokines and inhibitors in plasma were measured in 44 patients with typhoid fever. Ex vivo production of the cytokines was analyzed in a whole blood culture system with and without lipopolysaccharide (LPS). Acute phase circulating concentrations of cytokines ($\pm$SD) were as follows: interleukin (IL)-1β, <140 pg/mL; tumor necrosis factor-α (TNFα), 130 $\pm$ 50 pg/mL; IL-6, 96 $\pm$ 131 pg/mL; and IL-8, 278 $\pm$ 293 pg/mL. Circulating inhibitors were elevated in the acute phase: IL-1 receptor antagonist (IL-1RA) was 2304 $\pm$ 1427 pg/mL and soluble TNF receptors 55 and 75 were 4973 $\pm$ 2644 pg/mL and 22,865 $\pm$ 15,143 pg/mL, respectively. LPS-stimulated production of cytokines was lower during the acute phase than during convalescence (mean values: IL-1β, 2547 vs. 6576 pg/mL; TNFα, 2609 vs. 6338 pg/mL; IL-6, 2416 vs. 7713 pg/mL). LPS-stimulated production of IL-1RA was higher in the acute than during the convalescent phase (5608 vs. 3977 pg/mL). Inhibited production of cytokines during the acute phase may be due to a switch from a proinflammatory to an antiinflammatory mode.

Typhoid fever is caused by the facultative intracellular gram-negative bacillus *Salmonella typhi* and occasionally by *Salmonella paratyphi*. Although salmonellae contain lipopolysaccharide (LPS; bacterial endotoxin), the clinical picture of typhoid fever differs from gram-negative sepsis, and the role of endotoxin in the pathophysiology of typhoid fever is controversial [1].

The proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor-α (TNFα; cachectin), IL-6, and IL-8 have been implicated in the pathogenesis of sepsis caused by gram-negative microorganisms [2–4]. When LPS is injected intravenously into animals or human volunteers, elevated concentrations of these cytokines can be detected, and the symptoms and signs of sepsis are mimicked [5–7]. Elevated circulating levels of TNFα have been correlated with poor prognosis in sepsis, meningococcemia, and cerebral malaria [7–10]. In contrast, in infections with intracellular pathogens, such as *Leishmania* species, *Listeria monocytogenes*, or mycobacteria, administration of TNFα inhibits the outgrowth of the microorganisms, whereas administration of an-

tibodies to this cytokine are detrimental [11–16]. In experimental *Salmonella typhimurium* infection in mice, the role of TNFα is similar to that in other intracellular infections [17–19]. However, in calves with *S. typhimurium* sepsis, the cytokine pattern appears to differ from that seen after intravenously administered LPS. Where TNFα rose 1 h after LPS administration, salmonella sepsis caused a barely detectable increase in TNFα [20].

In contrast to these animal studies, circulating cytokines (TNFα, IL-6, and IL-1β) were elevated in children with typhoid fever in Chile [21]. Butler et al. [22] studied the outcome of typhoid fever in adults in Nepal and found that higher values of IL-6 and soluble TNF receptor p55 were related to poorer outcome.

In 1989, joint research on several aspects of typhoid fever was started between Nijmegen University and Diponegoro University. To obtain more insight into the pathophysiology of typhoid fever, we measured levels of circulating pyrogenic cytokines (IL-1β, TNFα, TNFβ [lymphotoxin], and IL-6) and concentrations of IL-8, the cytokine inhibitor IL-1 receptor antagonist (IL-1RA), and the soluble TNF receptors p55 and p75 (sTNF-R). In addition, we investigated the capacity of blood cells to produce IL-1β, TNFα, IL-6, and IL-1RA ex vivo in the acute and convalescent phases of hospitalized patients with typhoid fever.

We used the whole blood cytokine test as described by van Deuren et al. [23] and Nerad et al. [24]. This assay is simple, reproducible, and especially suitable for use in laboratories that are not particularly well equipped for work with cytokines. In addition, the method may be less artificial than is isolating mononuclear cells over a gradient and probably is a more natural mirror of what happens in vivo, because plasma factors and other cells are left in situ.

Table 1. Characteristics of 44 hospitalized culture-proven typhoid fever patients.

Characteristic	Complicated disease	Uncomplicated disease
No. of patients	16	28
Mean age (range), years	20 (14–34)	24 (14–60)
Males/females	7/9	12/16
Median (range) of days with fever before admission	10.0 (4–20)	8.5 (4–30)
Leukocyte count at admission (range)	$7.3 \times 10^9/L$ (2.6–37.0)	$4.4 \times 10^9/L$ (1.6–7.4)
Complications	16	—
Pneumonia	10	
Delirium	2	
Perforation	5	
Bleeding	1	

Patients and Methods

The study was done in Dr. Kariadi Hospital, Diponegoro University, Semarang, Indonesia, beginning in December 1990. Blood and bone marrow cultures were done for all adult patients (>14 years old) hospitalized with suspected typhoid fever (defined as fever >38.5°C and at least one of the following signs: relative bradycardia, abdominal complaints, mental changes, signs of complicated typhoid fever, enlarged liver or spleen and no apparent other disease). If blood or bone marrow cultures were positive for *S. typhi* or *S. paratyphi* A or patients were found to have perforated ilea at surgical exploration, typhoid fever was considered proven.

A total of 44 patients were studied. Patient characteristics are shown in table 1. Complications of typhoid fever were defined as gastrointestinal bleeding, intestinal perforation, shock, delirium, stupor or coma, pneumonia, or diffuse intravascular coagulation.

Treatment consisted of chloramphenicol (40 mg/kg/day orally) if leukocyte counts were >2 × 10⁹/L. If fever did not subside within 6 days, treatment was changed to sulfamethoxazole (800 mg) and trimethoprim (160 mg) twice daily or ampicillin (1 g four times daily). Surgical patients received ampicillin, metronidazole, and gentamicin during and after surgery. No cyclooxygenase inhibitors were given. Only 2 patients received a single dose of 120 mg of dexamethasone, but not before blood was obtained for cytokine measurement. Most patients were discharged 7–10 days after defervescence, which we defined as convalescence. No patients died.

Cytokine measurements. On admission and during convalescence, blood was drawn for cytokine measurement. Venous blood samples were aseptically collected into sterile 4-mL tubes (Vacutainer; Becton Dickinson, Rutherford, NJ) containing EDTA. Unless stated otherwise, a total of 3 tubes of blood was drawn from each patient [23]. To each tube, 250 μL of aprotinin (Trasylol 2500 kallikreine inactivating units [KIU]; Bayer, Leverkusen, Germany; final concentration 625 KIU/mL) was added through the stopper by a tuberculin needle and syringe. One tube was centrifuged directly (1250 *g*, 10 min), platelets from the supernatant plasma were removed by a second centrifu-

gation (15,000 *g*, 1 min), and plasma was collected and stored at −20°C until assayed for cytokines. To one of the two remaining tubes, 50 μL of LPS (*Escherichia coli* serotype O55:B5; Sigma, St. Louis; final concentration 10 μg/mL) was added to stimulate cytokine production. Unstimulated samples contained aprotinin only (no LPS). Both tubes were incubated at 37°C for 24 h.

For 17 (random) patients, a fourth 4-mL tube of blood was obtained in the acute phase into which indomethacin was added (0.5 μg/mL final concentration). For 26 (random) patients, we obtained a total of six tubes of blood. From two of these, we removed the plasma, replacing it with a like amount of PBS.

TNFα was determined by an RIA (detection level 100 pg/mL), described in [25]. Normal TNFα values for our laboratory (circulating concentrations and ex vivo production without LPS below the detection limit and ex vivo production after 24 h of stimulation with LPS) are 3780 ± 950 pg/mL.

IL-1β was measured by RIA according to the method of Lisi et al. [26] but without chloroform extraction (detection level 140 pg/mL). Normal values for our laboratory (circulating concentrations and ex vivo production without LPS below the detection limit and ex vivo production after 24 h of stimulation with LPS) are 6930 ± 3160 pg/mL.

IL-6 was measured by an ELISA as described (detection level, 20 pg/mL) [27]. Normal values for our laboratory (circulating concentrations and ex vivo production without LPS) were below the detection limit.

IL-8 was measured by ELISA quantikine (R & D Systems, Europe, Abingdon, UK). The detection limit was 45 pg/mL, and normal values were below the detection limit. We sought TNFβ (lymphotoxin) by ELISA quantikine (R & D Systems, Europe) but failed to detect any. IL-1RA was determined by an RIA according to the method of Poutsiaka et al. [28] (detection level 300 pg/mL). Normal values for our laboratory (circulating concentrations and ex vivo production without LPS below the detection limit, ex vivo production after 24 h stimulation with LPS) were 5757 ± 1060 pg/mL.

sTNF-R was measured by an enzyme-linked immunobinding assay (Hoffman-La Roche, Basel, Switzerland; detection level, 80 and 300 pg/mL for p55 and p75, respectively). Normal values for circulating concentrations are 1.50 ng/mL (p55) and 2.51 ng/mL (p75). All samples from the same patient were analyzed in the same run in duplicate to minimize analytical errors.

Statistics. When frequency distribution was parametric, we used paired and unpaired Student's *t* test. When not parametric, Wilcoxon signed-rank test or Mann-Whitney *U* test was used. $P < .05$ was considered significant.

Results

Circulating cytokines and inhibitors during acute and convalescent phases of typhoid fever. Concentrations of pyrogenic cytokines during the acute phase (IL-1β, IL-6, TNFα, lymphotoxin) are shown in figure 1. IL-1β concentrations were below the detection limit in both acute and convalescent phases. IL-6 concentrations ranged from undetectable (<20 pg/mL) to 600 pg/mL (median, 73). TNFα concentrations ranged from below the detection limit to 310 pg/mL (me-
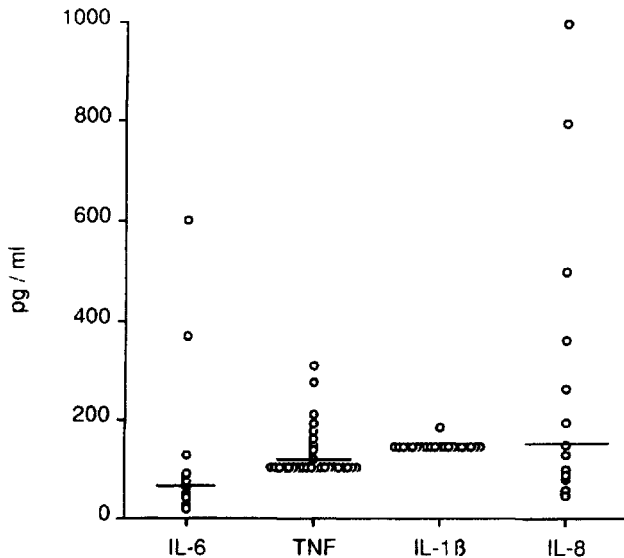
Figure 1. Circulating concentrations of pyrogenic cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8 in patients during acute phase of typhoid fever. Patients had been ill >1 week. Horizontal continuous circles = detection limit; horizontal bars = median values. In comparison with normal values, IL-6, IL-8, and TNFα are slightly elevated.

Circulating cytokines and inhibitors in complicated and uncomplicated disease. No differences were found in circulating cytokines or inhibitors between the 16 patients with complicated and the 28 patients with uncomplicated disease courses.

Ex vivo production of cytokines and inhibitors during acute and convalescent phases. Unstimulated whole blood cultures did not have detectable IL-1β, TNFα, or IL-6 (not shown). After incubation with LPS for 24 h, the supernatants contained detectable amounts of these cytokines, which were significantly lower in the acute phase than in convalescence (IL-1β: 2547 ± 3319 vs. 6576 ± 6275 pg/mL, $P <$ .001; TNFα: 2609 ± 2443 vs. 6338 ± 4366 pg/mL, $P <$ .001; IL-6: 2416 ± 1531 vs. 7713 ± 3809 pg/mL, $P =$ .01; figure 3).

In the acute phase, there was a correlation between the LPS-stimulated production of IL-1β and TNFα ($r =$ .664), IL-1β and IL-6 ($r =$ .531), and TNFα and IL-6 ($r =$ .434). Such correlations were not found during convalescence.

When indomethacin was added to the stimulated samples, the concentrations of TNFα and IL-1β in the acute phase were not different from those without indomethacin (2859 ± 2630 vs. 2609 ± 2443 and 2782 ± 2821 vs. 2547 ± 3319 pg/mL for TNFα and IL-1β, with and without indomethacin, respectively). Also, removal of plasma and replacement with PBS did not change the stimulated production of TNFα and IL-1β in the acute phase (TNFα: 3307 ± 3920 pg/mL; IL-1β: 2244 ± 2512 pg/mL).

The IL-1RA concentrations found in unstimulated cultures (not shown) were similar to those during the acute phase. However, the LPS-stimulated production of IL-1RA was high and reached significantly higher concentrations in the acute phase of the disease (5608 ± 1832 pg/mL) than during convalescence (3977 ± 1974 pg/mL; $P <$ .05). sTNF-R, IL-8, and lymphotoxin were not generated in the cultures.

Ex vivo production of cytokines and inhibitors during complicated and uncomplicated courses of disease. In the acute

dian, 110) in the acute phase and to 300 pg/mL (median, below detection limit) during the convalescent phase. All lymphotoxin concentrations were below detection limits during acute and convalescent phases. IL-8 concentrations were detectable in the acute phase (median, 145; range, 47–998 pg/mL) but lower during the convalescent phase (median, 46; range, 46–180 pg/mL).

Inhibitors such as IL-1RA and sTNF-R (p55 and p75) were significantly higher in the acute phase than during the convalescent phase: IL-1RA, 2304 ± 1427 pg/mL versus 469 ± 324 pg/mL; sTNF-R55, 4973 ± 2644 pg/mL versus 1671 ± 532 pg/mL; and sTNF-R75, 22,865 ± 15,143 pg/mL versus 5971 ± 2750 pg/mL (figure 2).
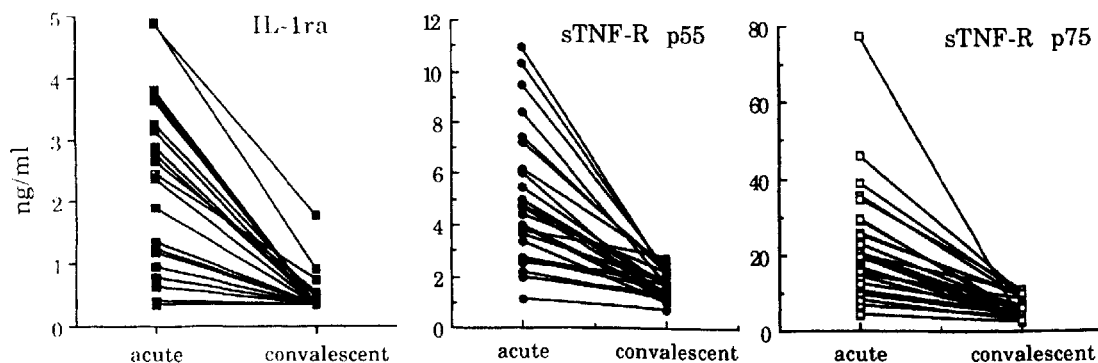

Figure 2. Individual concentrations of circulating interleukin (IL)-1 receptor antagonist (ra) and tumor necrosis factor (sTNF-R) soluble receptors 55 and 75 in patients during acute and convalescent phases of typhoid fever. Inhibitors like IL-1ra and sTNF-R (p55 and p75) were significantly higher in acute phase than during convalescent phase.
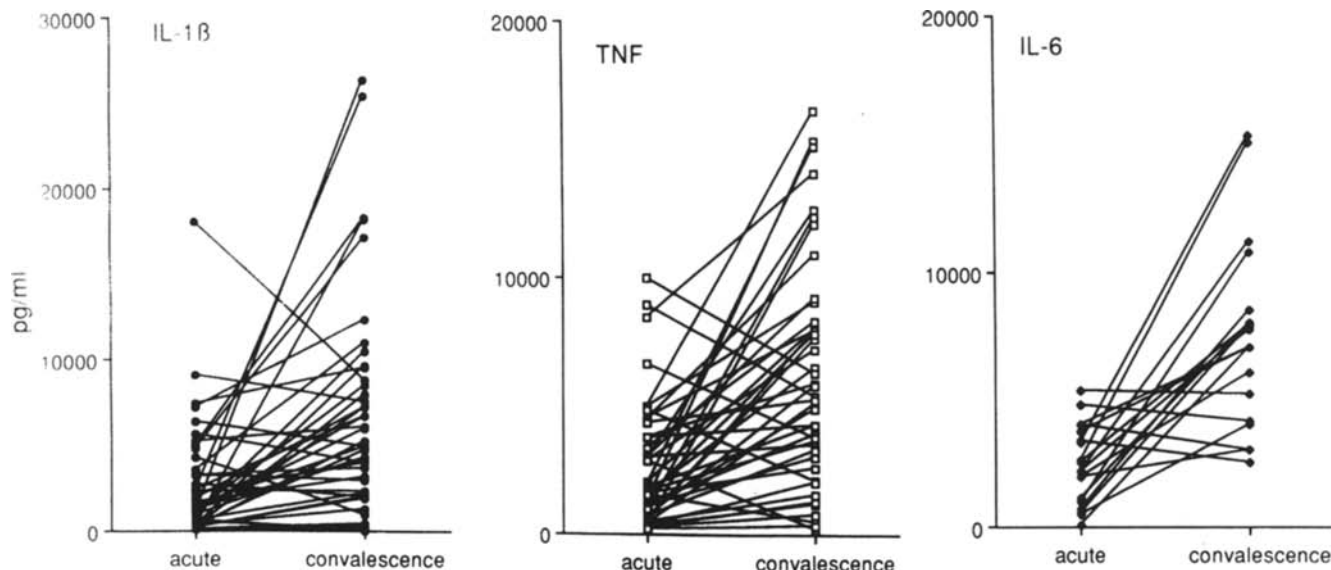
Figure 3. Individual production capacity of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 in acute and during convalescent phases of typhoid fever. After incubation with lipopolysaccharide for 24 h, supernatants contained detectable amounts of cytokines, which were significantly lower in acute phase than during convalescence.

phase, patients with complicated disease had significantly less IL-1β production after ex vivo stimulation with LPS (1341 ± 1373 vs. 6563 ± 1342 pg/mL; $P < .005$) and a trend towards lower TNFα production (1650 ± 1407 vs. 3064 ± 2770 pg/mL; $P = .06$). Such differences were not found for the production of IL-1RA or IL-6. During the convalescent phase, the ex vivo–stimulated production of IL-1β and TNFα did not differ for complicated and uncomplicated cases of disease.

Discussion

In this study we found several signs of cytokine activation during typhoid fever. The concentrations of circulating inhibitors such as IL-1RA and sTNF-R were high in the acute phase of the disease. IL-1RA is known not to be detectable in the circulation of normal subjects. Normal values for sTNF-R are 1.50 ng/mL (p55) and 2.51 ng/mL (p75) [29, 30]. We also found that the production capacity of pyrogenic cytokines in whole blood is depressed in the acute phase of typhoid fever but is restored during the convalescent phase. Although the patients in our study usually had severe typhoid fever, we found that those with complicated disease courses had significantly lower proinflammatory cytokine production capacity than did those with uncomplicated disease.

A low production capacity of cytokines has been found in other serious conditions, such as severe postoperative infection [31], sepsis [32–34], and attacks of familial Mediterranean fever [35, 36]. In these reports, all investigators used isolated peripheral blood mononuclear cells or tissue macro-

phages. We have previously found depressed cytokine production capacity in the whole blood culture system during the acute phase of meningococcal disease and during *Pneumocystis carinii* infection (unpublished data). From these studies and the work presented here, we conclude that the depressed cytokine production capacity is not a consequence of fewer white blood cells during the acute phase of the infection, since we found no correlation between leukocyte count and cytokine production (table 1).

Many investigators [31–36] have interpreted the finding of low cytokine production capacity as exhaustion of cytokine-producing cells, which could be a consequence of exposure in vivo to stimuli such as endotoxin. Our finding that IL-1RA is produced in high concentrations argues against such a hypothesis and also rules out the possibility that the decreased production of proinflammatory cytokines is due to an enhanced lysis of producing cells or to increased inactivation of LPS by lipids in the acute phase. Although we have been unable to demonstrate that the proinflammatory cytokines and IL-1RA are produced by the same kind of cells, we hypothesize that after the initial phase of infection, cytokine-producing cells switch from a balanced proinflammatory to an antiinflammatory repertoire. Our findings that patients in the acute phase of typhoid fever have high concentration of soluble sTNF-Rs in their blood is in agreement with this notion.

Since our cultures used whole blood, we investigated whether some common circulating factor could be responsible for the correlated low production capacity of the cytokines IL-1β, TNFα, and IL-6 in the acute phase. Cyclooxygenase products, such as prostaglandin E_2, which inhibit

production of IL-1 and TNFα [37], were not responsible since addition of indomethacin to the whole blood cultures did not lead to significant changes in cytokine production. Likewise, removal of plasma and addition of saline before incubation did not overcome the suppression in the acute phase of the disease.

It is possible that exposure in vivo to other inhibitory factors will explain the low cytokine production capacity. Schindler et al. [38] demonstrated that exposure of isolated mononuclear cells to IL-6 inhibits the production of IL-1 and TNFα. In the present study, we found no correlation between IL-6 concentrations in plasma and the magnitude of the production of IL-1β and TNF (r = .041 and .035, respectively). Exposure to other cytokines such as, IL-4, IL-10, and transforming growth factor-β could, however, play a role. Vannier et al. [39] have provided evidence that exposure of cells to IL-4 suppresses the IL-1 production but up-regulates the synthesis of IL-1RA.

With few exceptions, patients with typhoid fever have a continuous fever. Hence, pyrogenic cytokines would be expected to be present in the circulation during the acute phase of the disease. In our series of febrile patients with typhoid fever, we could not detect appreciable concentrations of the pyrogenic cytokines IL-1β, TNFα, and lymphotoxin. The concentrations of IL-6, generally considered a relatively weak pyrogen [40], were low compared to findings with other febrile conditions [3, 41]. We did detect elevated concentrations of IL-8, but this cytokine is considered nonpyrogenic [42].

Thus, the question of which pyrogens are responsible for the continuous fever in typhoid fever remains unanswered.

Acknowledgment

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