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Differential Cytokine Production in Stimulated Blood Cultures from Intensive Care Patients with Bacterial Infections

Summary: Mice infected with bacteria develop an interferon-γ (IFN-γ) dependent hypersensitivity to lipopolysaccharide (LPS) and other bacterial components. The broader aim of this study is to find out whether such hypersensitivity also occurs in patients suffering from bacterial infections. The capacity of stimulated peripheral blood cells from infected, intensive-care patients to produce cytokines (IFN-γ, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)) was compared to that of healthy donors. Culturing of the cells was carried out preferentially in whole blood diluted 1:3. Whole blood cultures (WBC) were stimulated with lipopolysaccharide (LPS), whole killed *Salmonella typhimurium* and *Staphylococcus aureus* and concanavalin A (ConA), and the cytokine production was determined. Two main findings emerged from this study: The IFN-γ production by WBC of patients was, compared to healthy donors, markedly suppressed, regardless of stimulus used. Further, patients' WBC exhibited a suppressed TNF-α production after stimulation with LPS. Surprisingly, following stimulation with bacteria (*S. typhimurium* and *S. aureus*) an elevated TNF-α and IL-6 response was obtained. Thus, in severely infected patients the cytokine responses of peripheral blood cells to LPS may be suppressed, while the response to other bacterial components is enhanced.

Introduction

The release of specific microbial components, such as lipopolysaccharides (endotoxins, LPS) from gram-negative or superantigens from gram-positive bacteria, may, under not yet understood circumstances, lead to septic shock in infected patients. In experimental animals, administration of appropriate doses of isolated, purified LPS induces lethal shock that resembles septic shock in patients. The development of shock is preceded and accompanied by the production of a broad spectrum of cytokines. Of these, tumor necrosis factor-alpha (TNF-α) [1–3] and interferon-gamma (IFN-γ) [4–7], were conclusively shown to participate directly or indirectly in the development of LPS shock.

TNF-α is produced mainly by monocyte/macrophages and T cells, the main source being dependent on the stimulus employed. TNF-α is transiently present (1–2 h after injection) in the circulation of LPS-injected animals and of human healthy volunteers or cancer patients administered LPS [8, 9]. It was also found in plasma of septic patients [10]. In various experimental lethality models, the neutralization of TNF-α by specific anti-TNF antibodies mitigates or abrogates, the shock-inducing property of LPS [1, 3]. Further, recombinant TNF-α, injected into mice, induces endotoxin-like shock [11]. Dose-dependent toxic effects were also observed during infusion of recombinant TNF-α in humans [9]. Finally, TNF-α is the sole mediator of LPS-induced lethality in D-galactosamine-sensitized mice [12–14]. TNF has also been shown to mediate the lethal toxicity induced by gram-positive bacteria [2, 15].

Recently IFN-γ, a cytokine produced by T cells and NK cells, was claimed in several reports to be a mediator of experimental endotoxin shock [5, 7, 16]. Our studies in mice showed that IFN-γ mediates the endotoxin hypersensitivity induced by infection by rendering monocyte macrophages and possibly other cells hyperreactive to LPS [2, 6, 17]. Consequently, infected mice overproduce TNF-α, IFN-γ and other cytokines, after stimulation with LPS and are hypersensitive to the toxic effects of TNF-α [2, 18].

The development of LPS-hypersensitivity following infection is well documented in experimental animal models. LPS hypersensitivity in humans has been much less investigated. A publication on the development of hypersensitivity in infected volunteers dates back more than 30 years [22]. Interleukin-6 (IL-6) in combination with interleukin-1 (IL-1) was also associated with mortality of patients with septic shock [19]. In septic patients IL-6 levels are elevated and correlate with the degree of disease severity. Using IL-6 deficient mice it could be shown, however, that IL-6 is not an essential mediator of LPS toxicity [20]. Nevertheless, the measurement of IL-6 plasma levels has been recommended as an indicator for synthesis of proinflamma-

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tory cytokines rather than measurement of IL-1 and TNF since these appear only transiently during the acute phase of infection [21].

The purpose of this study was to investigate the possible presence of hyperreactivity to LPS in patients suffering from severe bacterial infections, by testing the height of cytokine production (IFN-γ, TNF-α, and IL-6) by their peripheral blood cells (PBC) in response to LPS. Since LPS is not the only biologically active component of bacteria that can cause lethal shock [2], we also investigated the possible presence of hypersensitivity to other bacterial components, by using whole killed bacteria as stimulus. It will be shown that patient blood cells exhibit an irregular pattern of responsiveness which differs depending on the stimulus used and type of cytokine measured.

Patients and Methods

Blood donors: Fourteen patients with severe infection (both sexes, 18 to 62 years old), and 15 healthy volunteers (both sexes, 22 to 48 years old) serving as controls were included in the study. Nine patients were stationary at the medical and five at the surgical intensive care units of the Freiburg University Hospital. In all of these, evidence for bacterial infection was provided by microbiological investigations. Gram-negative bacteria, including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and gram-positive *Staphylococcus aureus*, *Streptococcus* spp. and *Listeria monocytogenes* were isolated in cultures. In six patients sepsis was proven by positive blood culture and clinical criteria [23]. Eight additional patients had focal infections, either peritonitis or pneumonia, or a combination of both. In none of these patients was sepsis proven by positive blood cultures, but in four of them sepsis was diagnosed clinically. Criteria for the diagnosis of sepsis were: body temperature > 38°C or < 36°C; white blood cell count >12,000/ml or < 4,000/ml, heart rate >90 beats/min (x) and thrombocyte count < 100,000/ml or a decrease of thrombocyte count of > 30% within 24 h. All patients received antibiotics at the time of blood sampling. Systemic steroids were not administered.

Blood collection: Peripheral venous blood samples were drawn in syringes containing 75 U/ml heparin (Liquemin Hoffmann-La-Roche, Basel) and used within 2 h of sampling. The leucocyte count was determined in a cell counting chamber. Leucocytosis (14.6 ± 6.7 cells x 10^3/ml) was present in the group of patients compared to the physiological values of the healthy donors, (4.5 ± 0.9 cells x 10^3/ml). Plasma was obtained after centrifugation of a portion of each blood sample at 300 x g and stored in aliquots at −80°C.

Stimuli: LPS of *Salmonella abortus equi* was prepared as described previously [24]. Killed bacteria of *Salmonella typhimurium* strain C5 and *S. aureus* were prepared as described previously: [25]. Concanavalin A (ConA) was purchased from Pharmacia, Sweden.

Stimulation of whole blood (WB) cultures: In preliminary experiments it was found that the cytokine response of whole blood cultures to all stimuli used was highest if the heparinized blood had been diluted to 1:2 or 1:4 with culture medium (RPMI 1640 supplemented with 5.10^{-5} M 2-mercaptoethanol, 20 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/l streptomycin). At still higher dilutions, or in cultures of undiluted blood, cytokine production was suboptimal. For this reason the blood samples were diluted 1:3 with medium prior to culturing in all experiments. Portions (1.25 ml) of diluted blood were placed in 24-well tissue culture plates (Nunclon, Nunc, Denmark) and the stimulatory agent (30 µl) was added. Unless otherwise specified, culture supernatants were collected after incubation for 24 h at 37°C in a 5% CO_2 atmosphere. Aliquots were stored at −80°C until cytokine measurement.

Measurement of IFN-γ: The IFN-γ levels in plasma and supernatants were measured by an enzyme-linked immunosorbent assay, using the method of *H. Gallati* et al. [26] with the following modifications: Mouse monoclonal antibody (mAb) γ69B to human IFN-γ was used as a capture and detection (biotinylated) antibody and phosphate buffered saline, pH 7.2, as coating and washing buffer.

TNF-α bioassay: The content of TNF-α in plasma and culture supernatants was measured in a cytotoxicity test using a TNF-α sensitive L929 cell clone (C5F6) in the presence of actinomycin D, as described previously [2]. Recombinant murine TNF-α kindly donated by Dr. *G. R. Adolph*, Bender & Co. GmbH, Vienna, Austria, served as standard. Rabbit antibody, prepared against human recombinant TNF-α was used as an inhibitor to test the specificity of the TNF bioassay.

IL-6 bioassay: IL-6 levels were determined in a bioassay using clone B9 of the murine hybridoma B13.29 which is dependent on IL-6 for growth [27]. B9 cells were cultured in the presence of se-

Table 1: Cytokine response of optimally stimulated WBC from healthy donors.

	Stimuli (µg/ml)	IFNγ (ng/ml)	TNF (pg/ml)	IL-6 (U/ml x10^3)
LPS	1	9.1 ± 7.5	194.3 ± 139.5	53.45 ± 26.37
S. typhimurium	150	37.3 ± 14.4	488.2 ± 163.5	45.12 ± 14.79
S. aureus	150	6.3 ± 4.5	275.4 ± 116.9	34.16 ± 13.71
ConA	50	21.3 ± 12.2	227.4 ± 123.9	37.35 ± 14.78
Control		< 0.075*	< 10*	< 0.03*

Blood collected at 9 a.m. was diluted 1:3 with RPMI and stimulated for 24 h with the indicated agents. Cytokines in supernatants were measured as described in Patients and Methods. The results represent mean values of five donors with standard deviation.
*detection limit.

rially diluted supernatants of stimulated WBC or heated (56°C/30 min) blood plasma. In each plate, dilutions of human recombinant IL-6 (Boehringer, Mannheim) incubated with B9 cells served as standard. After 72 h of culture, growth was measured by colorimetric assay [28] after staining the cells with MTT (Thiasolyl blue, Sigma).

Measurement of cortisol in plasma: The plasma levels of cortisol were measured by a radioimmunoassay (ICN Biomedicals Inc., Carson, CA).

Statistics: Results are expressed as mean values with standard deviation ($x \pm \sigma n\text{-}1$). Data were compared by using the Student's t-test. Values of $p < 0.05$ were considered significantly different.

Results

Cytokine Production of WB Cultures of Healthy Donors

In preliminary experiments we determined the dose of the stimuli used in this study that lead to maximum cytokine responses in WB cultures. Blood samples from five healthy donors were collected at 9.00 p.m. and stimulated individually with different doses of LPS (0.00001–1.0 μg/ml), killed bacteria *S. typhimurium* and *S. aureus* (5–150 μg/ml) and ConA (5–150 μg/ml) for 24 h. IFN-γ, TNF-α and IL-6 were estimated, as described in "Patients and Methods." Unstimulated control cultures produced virtually no detectable amounts of cytokines. It was found that doses of 1 μg/ml LPS, 150 μg/ml *S. typhimurium* or *S. aureus* and 50 μg/ml ConA induced maximum production of the above cytokines and were adopted in the following experiments. The cytokine levels induced by these doses are shown in Table 1. All stimuli used were potent inducers of all three cytokines tested.

The Kinetics of Cytokine Response in WB Cultures

In further experiments WB cultures of healthy donors stimulated with LPS, *S. typhimurium* and ConA and levels of IFN-γ, TNF-α and IL-6 in supernatants after different periods of time (up to 48 h) were measured (Figure 1). With all stimuli IFN-γ was not detectable during the first 6 h. IFN-γ level was highest at 24 h of culture and remained at this level for the following 24 h. TNF-α was already detected after 1 h of incubation and increased rapidly during the following 5 h of culture. Thereafter, no profound changes in TNF-α levels were observed except for a slight, but not significant, decrease during the last 24 h of incubation. IL-6 appeared in LPS-stimulated WBC already after 1 h and in *S. typhimurium* and ConA-stimulated cultures after 4 h. Its level increased further in LPS and *S. typhimurium*-stimulated WBC up to 24 h and in ConA culture up to 6 h. No further change in IL-6 levels was observed up to 48 h of culture.

Circadian Rhythm of Plasma Cortisol Levels and IFN-γ Response in WBC

The IFN-γ responses to LPS, *S. typhimurium*, *S. aureus* and ConA of WBC obtained from the same donors at dif-

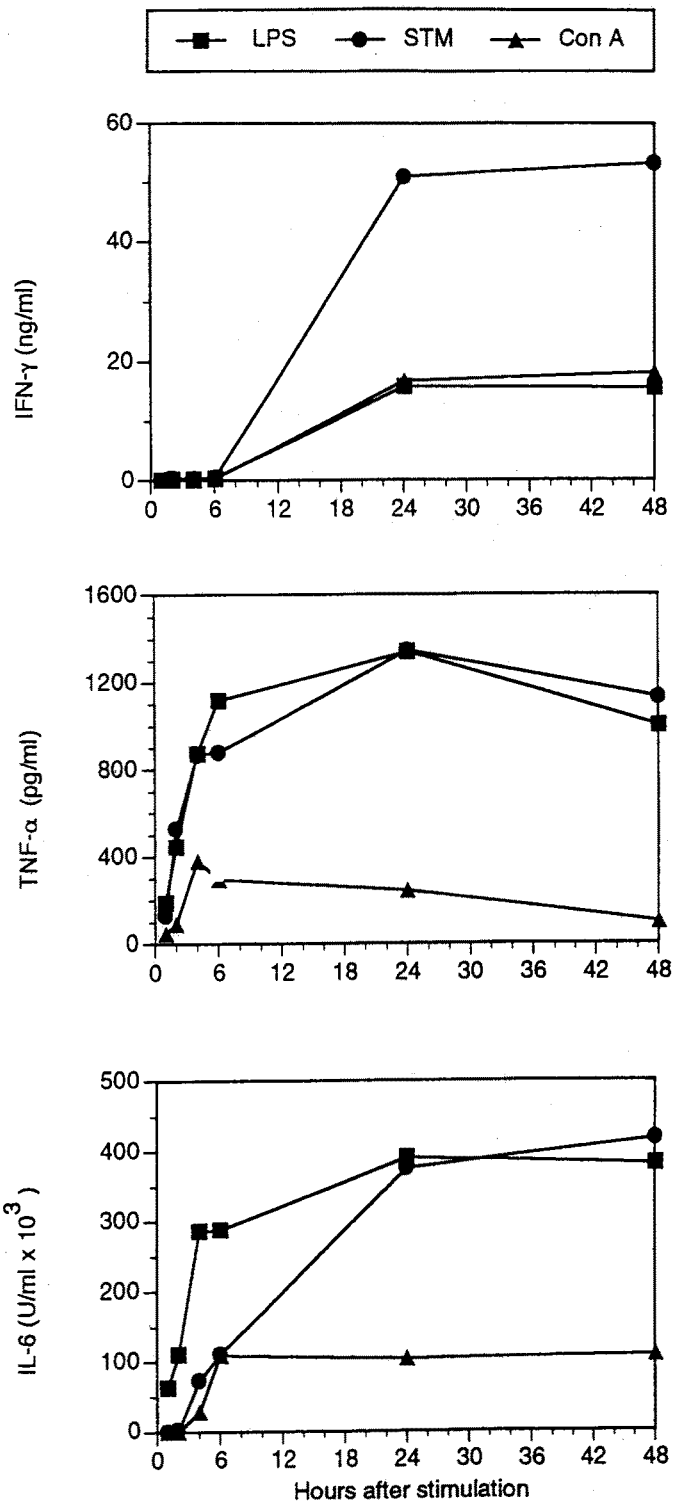


Figure 1: Kinetics of cytokine production by human WB cells stimulated with different agents. Blood sample of a healthy donor was diluted 1:3 with culture medium, divided into 1.25 ml portions and cultured in duplicate with LPS (1 μg/ml), *S. typhimurium* (150 μg/ml) or ConA (50 μg/ml) for the indicated periods of time. Pooled supernatants of duplicate cultures were assayed for IFN-γ, TNF-α and IL-6. Control cultures (without stimuli) did not produce detectable levels of cytokines. The results are representative for three different experiments.

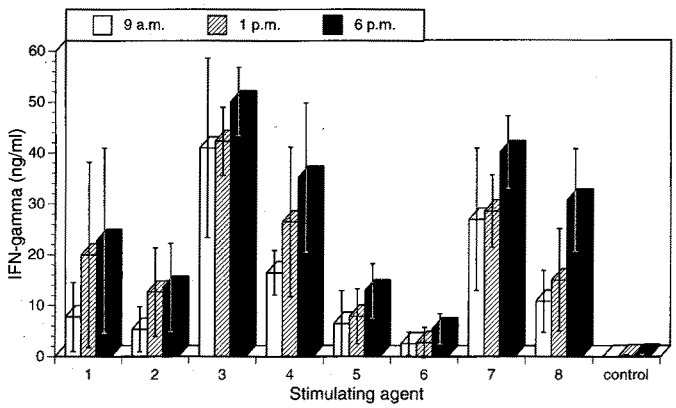
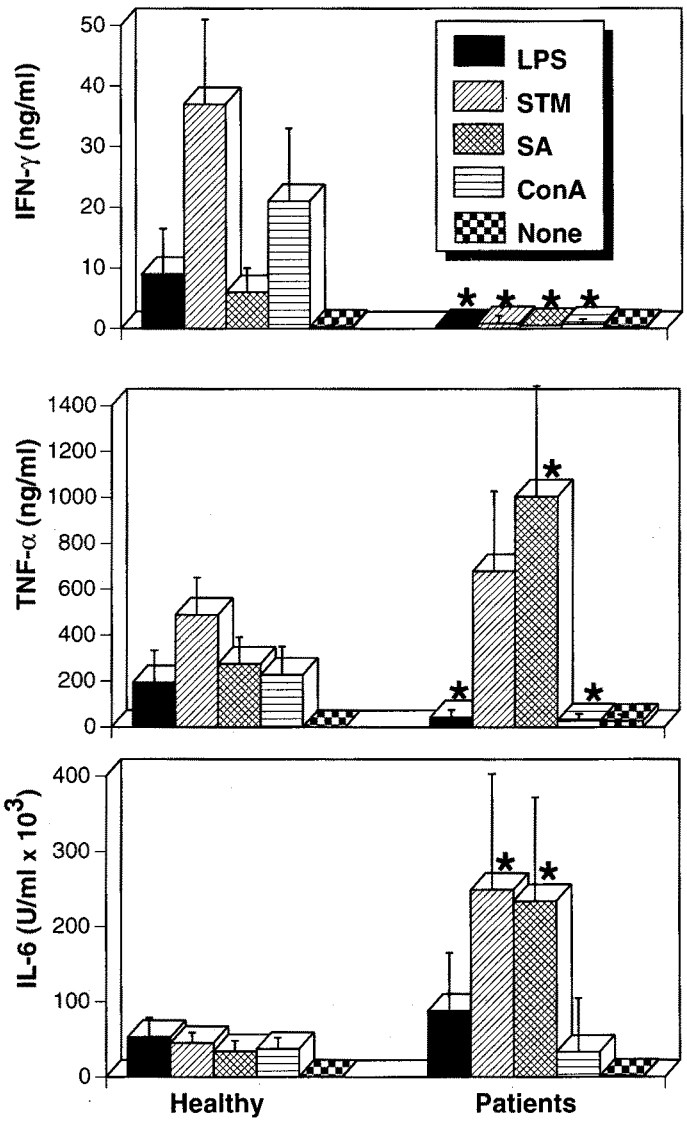


Figure 2: IFN-γ inducibility in whole blood collected at different times of the day. Blood from four healthy volunteers was collected at times indicated and WBC were prepared. The cultures were stimulated for 24 h and cytokine levels in supernatants measured. 1: 1 μg LPS; 2: 0.01 μg LPS; 3: 150 μg *S. typhimurium*; 4: 5 μg *S. typhimurium*; 5: 150 μg *S. aureus*; 6: 5 μg *S. aureus*; 7: 50 μg ConA; 8: 12.5 μg ConA.

ferent times of the day (9 a.m., 1 and 6 p.m.) were compared. As shown in Figure 2, the IFN-γ response was dependent on the time of blood collection. The lowest levels were elicited in WBC obtained at 9 a.m. and the highest in those obtained at 6 p.m. Cortisol levels in the blood samples showed inverse patterns. The average values of cortisol in the 9 a.m., 1 and 6 p.m. samples were 628 ± 366, 418 ± 133 and 241 ± 130 nmol/l, respectively. Thus, the height of the IFN-γ response was inversely correlated to the levels of cortisol present in circulation at the time of blood collection. In order to minimize circadian rhythm-dependent differences of the cytokine response, the blood samples used in this study were always drawn between 9 and 10 a.m.

Plasma Cytokine Levels and Cytokine Production by WBC from Infected Patients and Healthy Donors

Blood samples from 14 patients with severe infection were analyzed for IFN-γ, TNF-α and IL-6 and cortisol levels in plasma, and compared to those of 15 healthy donors. The results are summarized in Table 2. On average, the cortisol levels in patients were higher than in healthy



Mean values (bars) with standard deviations (vertical lines) are shown. * = $p < 0.05$ vs healthy group.

Figure 3: Comparison of cytokine response by WB cells between patients suffering from severe bacterial infections and healthy donors. WB cells of patients (17) and donors (23) were stimulated in duplicate with LPS (1 μg/ml), *S. typhimurium* (150 μg/ml), *S. aureus* (150 μg/ml) or ConA (50 μg/ml) for 24 h. Supernatants of cultures were assayed for IFN-γ, TNF-α and IL-6.

Table 2: Plasma levels of IFN-γ, TNF-α, IL-6 and cortisol.

Subjects	IFN-γ (ng/ml)	TNF-α (pg/ml)	IL-6 (U/ml)	Cortisol (nmol/l)
Healthy donors	2.7 ± 3.9	22.6 ± 13.9	72 ± 47	474 ± 270
Patients	3.5 ± 8.0	26.2 ± 25.2	739 ± 602	621 ± 364
p-value	> 0.05	> 0.05	< 0.001*	> 0.05

Blood samples of patients (14) and healthy donors (15) were collected between 9 and 10 a.m. and plasma levels of IFN-γ, TNF-α, IL-6 and cortisol measured as indicated in Patients and Methods.

subjects, the difference between the two groups, however, was not significant. IFN-γ and TNF-α plasma levels did not differ between the two groups. IL-6, however, was significantly higher in the infected patients than in the healthy volunteers. In the patients, elevated IL-6 levels, ranging from 200 to 30,011 U/ml, were found in 89% of the cases.

Blood samples from patients and healthy volunteers were cultured with the different agents under test for 24 h. Unstimulated control cultures produced no detectable IFN-γ, TNF-α or IL-6. After stimulation, differences in production of cytokines were observed between healthy and infected subjects (Figure 3). The most striking difference concerned the IFN-γ response. While high responses were obtained in stimulated WBC of healthy controls, only marginal IFN-γ responses to all stimuli were measured in the cultures of all patients independent of the underlying infection. Thus, the IFN-γ response of patients' WB cultures did not differ significantly from that of the unstimulated control cultures. Stimulus-dependent differences in the production of TNF and IL-6 were observed between healthy and infected subjects. In patients, TNF-α responses to LPS and ConA were significantly reduced ($p < 0.001$), while the IL-6 response did not differ from that of healthy donors. On the contrary, the TNF-α response to whole bacteria (*S. typhimurium* and *S. aureus*) of patients' WBC cultures was enhanced. Also, IL-6 response of patients to bacteria was higher than that of healthy controls. This result shows that while the response to LPS might be suppressed, the response to other bacterial components may even be enhanced.

Discussion

Plasma levels of IFN-γ, TNF-α and IL-6 in patients suffering from severe bacterial infections and capability of their peripheral blood cells to produce these cytokines upon stimulation with LPS, *S. typhimurium*, *S. aureus* and ConA were compared to that of healthy donors. There was no significant difference in IFN-γ, and TNF-α levels in plasma between infected and healthy subjects. This is not surprising, since it has been shown that in patients undergoing sepsis or other serious infections any elevation in the levels of TNF-α, IL-1 and IFN-γ in the circulation is only transient [29, 10]. Therefore a single measurement may not reflect the actual situation of cytokine synthesis. However, approximately 90% of the patients showed elevated IL-6 levels which is in agreement with previous studies [21, 30], showing that IL-6 may serve as an indicator for ongoing severe infectious disease.

In healthy volunteers, using the IFN-γ production as a measure, it is shown that the time of day of blood sampling influences the height of cytokine response. Cells from morning collection, upon stimulation with all agents, always produced lower levels of IFN-γ than cells collected in the evening. There was always an inverse correlation between IFN-γ levels and cortisol concentrations in plasma. This finding is in agreement with previous reports in which time-of-day-dependent variations of IFN-γ and IL-1 inducibility [31] were described and attributed to the circadian rhythm of plasma cortisol levels. The negative influence of glucocorticoids on the production of several cytokines including TNF and IL-1 is well documented [32, 33]. In the present study, all blood samples were always collected at a determined time of day (9–10 a.m.) and cytokine responses may therefore be compared more directly with one another.

The most striking difference between patients and healthy donors identified in this study concerns the IFN-γ response of their WBC which, independent of stimulus, was markedly suppressed in the patient group. The suppression is not related to glucocorticoids since the cortisol levels of patients and healthy volunteers were not significantly different. The suppressed IFN-γ production is surprising because in animal infection models, intravenous or intraperitoneal administration of LPS or other bacterial components results in enhanced cytokine production, including IFN-γ [34]. Further, spleen cells isolated from *S. typhimurium*-infected mice exhibit an enhanced production of IFN-γ when stimulated with LPS, different killed bacteria or ConA [35]. In the present study, the IFN-γ production was measured in WB cultures, since blood is the only easily accessible patient material. The response of blood cells, however, may not be representative for the whole organism. It is important therefore to clarify this question in the future, especially because PBC are widely used for the evaluation of patients' reactivity.

Patient WBC also exhibited significantly reduced TNF-α responses to LPS and ConA. These results are in agreement with previous findings showing impaired TNF-α, IL-1 and IL-6 responses of blood cells from patients during infection [36–41]. Interestingly, in the present study, the TNF-α and IL-6 response of patient WBC to killed *S. typhimurium* and *S. aureus* bacteria was considerably higher than the response of WBC of healthy donors. Considering the suppressed responses to purified LPS, it appears unlikely that the enhanced responses to *S. typhimurium* can be attributed to the LPS component. Evidence that also non-LPS components of gram-negative bacteria are responsible for TNF-α production and lethal shock was presented earlier [2]. In addition, gram-positive bacteria are endowed with numerous cytokine-inducing components such as teichoic and lipoteichoic acids, murein and exotoxins [42–44]. The finding that the cytokine (TNF-α and IL-6) response of patients' peripheral blood to whole bacteria is enhanced, while the response to LPS is suppressed, indicates that the infected patients are, at least with regard to the response of their blood cells, hypersensitive to certain bacterial components that are distinct from LPS. Whole killed bacteria therefore seem to be a more suitable tool for assessing the overall cytokine response potential of infected individuals than isolated LPS.

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Book Review

S. H. E. Kaufmann (ed.)
Concepts in Vaccine Development
580 pages, 40 figures and tables
Walter De Gruyter & Co., Berlin, New York 1996
Price: DM 178,–, $119.00

The academic field of vaccine development has been an extremely active and successful one since the introduction of immunization against smallpox by *Edward Jenner* some 200 years ago. New developments as well as improvements of established strategies appear in numerous publications practically on a daily basis and even those specialized in this area find it more and more difficult to stay well informed and up-to-date.

It is therefore very gratifying to find summaries of these advancements in journal supplements and books such as this one. "The objective of this book is to provide the reader with a state-of-the-art overview on modern vaccine design" is stated on the cover of the book and the editor and 53 contributors by and large accomplished this. In 22 important chapters topics such as the need for new vaccines (no doubt about that!), general principles of immunology (covering both humoral and cellular aspects), general principles of vaccination (including most recent advances using "naked DNA" vaccines but also the frequently underestimated role of mucosal immunity) and specific vaccination strategies (such current topics as efforts in vaccine development against malaria, AIDS and *Helicobacter pylori*) are discussed.

Despite the impressive and comprehensive collection of data, the diversity of objectives addressed and the useful references, some major points of criticism should be mentioned. In general, the editor could have avoided including subjective statements in several areas. Some examples: Most experts are disappointed by the results with the malaria vaccine Spf66 in recent efficacy trials, while the developers of the vaccine, for understandable reasons, still remain highly optimistic in their discussion in the respective chapter. The first chapter of the book on virulence-antigen strategy is eloquent and contains interesting hypotheses. However, it is highly speculative in many respects. For example, there is no scientific basis for assuming the occurrence of infections with "mild" as compared to "virulent" strains of *Bordetella pertussis*. Phase modulation of this organism can be induced *in vitro*, but there is no convincing data for the same happening *in vivo*. It also can no longer be accepted that "damaging side effects" are attributed to the whole cell pertussis vaccine after all the careful studies that have been performed which rehabilitated this type of vaccine. However, the recently introduced acellular pertussis vaccines certainly offer major advantages, as is well summarized in another chapter of the book.

Specifically, the following topics would have deserved more space in the light of their clinical and economical importance: Pneumococcal and meningococcal conjugate vaccines for use in infants; vaccines against rotavirus (and other enteropathic organisms); developments regarding new vaccines against tuberculosis; and recent advances in vaccination against Lyme borreliosis. It is to be hoped that in a second edition of the book these important aspects of modern vaccine development will be added. In conclusion, this book can be recommended to anyone with a specific interest in vaccines.

U. Heininger
Erlangen

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