Characterization of the Cytokine Immune Response in Children Who Have Experienced an Episode of Typical Hemolytic-Uremic Syndrome

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Received 19 June 2002/Returned for modification 18 September 2002/Accepted 4 August 2003

The lipopolysaccharide (LPS) of enterohemorrhagic Escherichia coli (EHEC) and Shiga toxin together substantially contribute to the pathophysiology of typical hemolytic-uremic syndrome (HUS). Both factors have been shown to be immune stimulators and could play a key role in the individual innate immune response, characterized by proinflammatory and anti-inflammatory cytokines. By use of a whole blood stimulation model, we therefore compared the LPS- and superantigen-induced cytokine responses in children who had been having recovering from an acute episode of typical HUS for at least 6 months (group 1) with those in controls, who consisted of patients seen in the pediatric neurology outpatient department for routine examination (group 2).

Samples were analyzed for cytokine protein levels and the levels of mRNA production. LPS stimulation revealed lower levels of interleukin 10 (IL-10) (P < 0.05) and increased levels of gamma interferon (P < 0.05) and increased ratios of pro- and anti-inflammatory cytokines (P < 0.05 for the IL-1/IL-10 ratio; P < 0.05 for the tumor necrosis factor alpha/IL-10 ratio) in group 1. In addition superantigen stimulation showed decreased IL-2 levels in group 1 (P < 0.01). Our results suggest an alteration of the cytokine response characterized by high proinflammatory cytokine levels and low anti-inflammatory cytokine levels as well as low levels of IL-2 production in children who have experienced an episode of typical HUS. We hypothesize that this altered immune response is not a residual effect of the infection but a preexisting characteristic of the patient. This could be one reason why individuals infected with EHEC are potentially predisposed to a systemic disease (HUS).

In recent years our understanding of the pathophysiology of hemolytic-uremic syndrome (HUS) has further improved. However, the exact mechanisms leading to typical HUS (D+ HUS, where D+ is gastroenteritis positive) secondary to infection with enterohemorrhagic Escherichia coli (EHEC) are still not completely understood. As HUS is a disease of young children and elderly adults, a substantial role of the immune system has been discussed (6, 12, 13, 19). It is well known that tumor necrosis factor alpha (TNF-α) and Shiga toxin (Stx) potentiate the toxicity of each other for endothelial cells (9, 20) and that elevated levels of antibodies to EHEC lipopolysaccharide (LPS) can be found in HUS patients (1, 3, 22). Therefore, a crucial role of the inflammation system has been suggested in HUS. Nevertheless, attempts to prove that patients with an acute episode of HUS have elevated systemic proinflammatory cytokine levels in comparison to those in children with infectious diarrhea have failed (8). Consequently, we and other groups recently added evaluation of anti-inflammatory cytokine profiles to the study design in order to monitor the balance between pro- and anti-inflammatory immune activation (10, 15, 24). We and others observed alterations in anti-inflammatory cytokine levels, even though the results are conflicting: in our D+ HUS patient group, interleukin 10 (IL-10) levels were found to be decreased in patients with EHEC infections in comparison to those in patients with bacterial gastroenteritis of other origins. Consequently, the TNF-α/IL-10 ratio for the HUS group was increased when the levels of the two cytokines were compared, reflecting an imbalance toward inflammation. This may result in the expression of an altered immunity in patients with this rare disease. Nevertheless, measurement of systemic cytokine levels is not likely to reflect the whole picture of immune activation, as cytokines mainly act in a paracrine fashion and have short half-lives in plasma (11). Furthermore, we raised the question whether the cytokine profiles observed in vivo can be reproduced by ex vivo LPS stimulation of whole blood from patients who had experienced and who were recovering from an acute HUS episode. As the results of studies with animal models suggest compromised immune activity secondary to Stx exposure (17), we decided to study the immune response to superantigen in order to characterize T-cell immunity. Finally, the results were correlated with clinical parameters like the glomerular filtration rate (GFR), blood pressure, protein and erythrocyte levels in urine, and others.

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VOL. 10, 2003

VTEC 2000, abstr. 429, 2000; S. Westerhold, A.-K. Pieper, B. Klare, P. Emmrich, and R. Oberhofer, Abstr. VTEC 2000, abstr. 96, 2000] and at the Jahrestagung der Deutschen Gesellschaft für Kinderheilkunde und Jugendmedizin, Stuttgart, Germany, September 2000 [A.-K. Pieper, S. Westerhold, M. Griebel, P. Emmrich, and R. Oberhofer, Abstr. Jahr. Dtsch. Gesellschaft Kinderheilkd. Jungmed., abstr. V121, 2000]. This study contains parts of the M.D. thesis of Anne-Kathrin Pieper.)

MATERIALS AND METHODS

Patients. This study was conducted in accordance with the precepts of the Helsinki Declaration and was approved by the Institutional Review Board of the Technical University of Munich, Munich, Germany. All children volunteered to take part in this study. Written informed consent was collected from the patients' parents and from the patients themselves, when appropriate, before entry into the study. This study was conducted at the Institute of Pediatrics of the Technical University of Munich. All subjects underwent a thorough physical examination to exclude the presence of an acute infection. In addition, blood samples were examined by routine clinical chemistry analyses. We recruited children more than 6 months after an episode of typical HUS (group 1) and controls (group 2), who consisted of patients seen in the pediatric neurology outpatient department for routine examination. Group 2 consisted of patients who had previously had complicated febrile seizures and patients with successfully treated epilepsy. Results were analyzed by linear regression by age and sex. Patients were matched where appropriate. Subjects from group 2 who were using immunomoductive drugs, such as adrenocorticotropic hormone, or who were suspected of having a systemic or genetic disease were excluded. Blood was collected from the patients between 9 and 11 a.m. Along with routine blood withdrawal, a 0.5-ml sample that was placed in EDTA (Sarstedt, Numbrecht, Germany) and a 2-ml sample that was placed in lithium-heparin (Sarstedt) were collected. Clinical data were collected from all patients, including length of illness, blood pressure, use of anti-hypertensive drugs, protein and erythrocyte levels in urine, and creatinine levels. GFR was estimated by the Schwartz formula. Patients with clinical or laboratory signs of acute inflammatory disease were excluded. Typical HUS was defined according to the present definition of the Centers for Disease Control and Prevention: hemolytic anemia with microangiopathic changes, thrombocytopenia, acute renal injury or failure, and acute symptoms or a history of HEC-positive gastroenteritis, as proved by fecal culture (n = 31) or a positive result by serological testing (n = 1). All HEC isolates had to be positive for Stx1 or Stx2, or both. In the case of a negative fecal culture, the titers of antibodies against Stx1 or Stx2, or both, had to be positive; in addition, the titers of antibodies against HEC LPS had to be positive. None of the patients in group 1 where Stx1 or Stx2, or both, had to be positive; in addition, the titers of antibodies against EHEC LPS was added, and incubation was for 72 h instead of 24 h. All incubations were performed in duplicate to exclude handling errors. Therefore, the results are averages of two identical experimental settings. Cytokine measurements and gene expression were measured from unstimulated cells to exclude false-positive results. Blood samples that showed measurable cytokine levels under these circumstances were excluded from further interpretation.

Cytokine measurement. A sandwich enzyme-linked immunosorbent assay (ELISA) based on commercial antibody pairs was used to determine the cytokine levels in the plasma samples. Antibodies against IL-10 were purchased from Pharmingen (Hamburg, Germany). Antibodies against IL-2 and IL-6 were from R&D Systems (Wiesbaden, Germany), while antibodies against TNF-α, IL-1β, IL-4, IL-8, and IFN-γ (Biozol; Endogen, Eching, Germany) were purchased. Appropriate biotinylated secondary antibodies were used throughout. Peroxidase-coupled streptavidin was obtained from Jackson Immuno (Dianova, Hamburg, Germany), and the tetramethylbenzidine (TMB) substrate was from Sigma. Recombinant human cytokines calibrated against standards, like those used in commercial ELISA kits, served as standards. Each well of the ELISA plates (Greiner) was coated overnight at 4°C with 50 μl of antibody in 0.1 mol of NaHCO3 (pH 8.2) per liter. After each well of the plates was blocked with 200 μl of phosphate-buffered saline (PBS) supplemented with 3% bovine serum albumin (BSA; pH 7.0; Serva, Heidelberg, Germany) for 2 h at room temperature, the plates were washed twice with PBS-0.05% Tween 20. Sample (50 μl/well) and tracer antibody (50 μl/well) in PBS-3% BSA were added, and the plates were incubated for 2 h. After six wash cycles, each well of the plates was incubated for 30 min with 100 μl of streptavidin-peroxidase (Dianova, Hamburg, Germany) at 1 μg/ml in PBS-3% BSA. After eight washes, 100 μl of TMB liquid substrate solution (Sigma) was added to each well, and the plates were incubated at room temperature for 5 to 30 min. After the addition of 50 μl of stop solution (1 mol of H2SO4, per liter) to each well, the absorption was measured at 450 nm by using a reference wavelength of 630 nm.

RNA preparation. RNA from whole blood was prepared with an RNeasy Blood Mini kit and by additional DNase digestion (Qiagen, Hilden, Germany), according to the instructions of the manufacturer.

Reverse transcription and competitive PCR. Reverse transcription and competitive PCR were performed as described previously (14). The Gel Doc 2000 system and Quantity One software (both from Bio-Rad, Hercules, Calif.) was used for quantification of the PCR products.

Statistical analysis. All data are given as means and standard deviations (SDs). The level of cytokine release was calculated per milliliter of blood or per leukocyte cell number. RNA products were expressed as the ratio of the amount of RNA in the sample and the amount of the control fragment. Statistical analyses were performed with SPSS software (version 10.0; SPSS GmbH, Munich, Germany). IL-10 levels and the TNF-α/IL-10 ratio were analyzed prospectively. All other parameters were analyzed in a post hoc fashion. Due to the exploratory nature of the majority of results, multiple testing was not performed. Correlations between parameters were analyzed by the Spearman rank test; partial correlations were calculated where appropriate. The threshold of significance was a P value <0.05.

RESULTS

LPS stimulation. Thirty-two patients with a history of typical HUS (group 1) and 33 controls (group 2) were enrolled for LPS stimulation. Thirteen patients from group 1 showed proteinuria at the time of blood sampling; elevated numbers of erythrocytes were seen in the urine of two children. No patient was hypertensive or showed other residual signs from the acute episode of HUS. The groups were different according to their age distributions (mean ages, 91 months for group 1 and 125 months for group 2; P < 0.01) but not with respect to sex (group 1, 17 females and 15 males; group 2, 16 females and 16 males). Consequently, a linear regression model was used to calculate the influence of age on cytokine levels. According to the ELISA results, different ages of patients had no significant influence to most cytokines on protein and mRNA levels; the exceptions were TNF-α, IL-8, and IL-10. Therefore, 23 patient pairs were matched by age and sex and evaluated for differences in the levels of these cytokine proteins. All patients were included for calculation of the other parameters. The median of the time interval between the acute episode of HUS and the time point of stimulation (Δt in group 1 was 47.8 months (SD, 6.7 months).

Stimulation of whole blood with LPS was used to analyze protein (ELISA; Table 1) and mRNA (PCR; Table 2) levels.
Significant differences were detected between the groups, in that the levels of the proinflammatory cytokines IL-1β (which affected only the mRNA level) and IL-8 were elevated in group 1. In addition, the levels of all other proinflammatory cytokines evaluated (TNF-α, IL-6, and IL-1β protein levels) turned out to be increased in these patients as well, but the differences were not significant. However, the level of IL-10, the only anti-inflammatory cytokine whose levels were measured, was markedly decreased in children with a former history of HUS in comparison to those in controls, as shown in Fig. 1. The IFN-γ level was significantly increased. As a result, the ratios of pro- and anti-inflammatory proteins (IL-1β/IL-10 and TNF-α/IL-10) were elevated in group 1. Figure 2 demonstrates these results as box plots.

Analysis of the correlation between cytokine protein levels and the clinical data for the group 1 patients showed no significant coherence. The results for cytokine RNA, however, revealed a relevant correlation between the TNF-α/IL-10 ratio, proteinuria \((R = 0.464; P = 0.01)\), and GFR \((R = -0.563; P < 0.01)\). In addition, this could be observed for GFR and IL-10 \((R = 0.373; P < 0.05)\) (Table 3). Finally, we found a weak coherence between \(\Delta t\) and IL-10 (for protein, \(R = -0.15\) and \(P = 0.42\); for mRNA, \(R = 0.06\) and \(P = 0.77\)). The same result revealed the correlation between \(\Delta t\) and the TNF-α/IL-10 ratio (for protein, \(R = 0.25\) and \(P = 0.15\); for mRNA, \(R = 0.18\) and \(P = 0.38\)).

**SEB stimulation.** Samples of whole blood from 22 patients in group 1, 13 controls (group 2), and 5 children with an acute episode of D+ HUS (group 3) were stimulated with SEB. Groups 1 and 2 were analyzed statistically; group 3 was included for illustration. Due to the “early report” character of this study, group 2 was much smaller than group 1. Groups were different according to their age distributions (mean for group 1, 46.6 months; SD, 6.9 months; mean for group 2, 143 months; \(P < 0.05\)) but not according to sex (group 1, 13 females and 9 males; group 2, 8 females and 5 males). Consequently, a linear regression model was used to calculate the influence of age and sex on lymphokine levels. No significant bias of age or sex was found. The mean \(\Delta t\) for group 1 was 46.6 months (SD, 6.9 months).

**TABLE 2.** Quantification of cytokine mRNA levels in leukocytes of whole blood from patients with a history of D+ HUS and controls determined by stimulation assays

| Cytokine        | Group 1 | Group 2 | Significance |
|-----------------|---------|---------|-------------|
|                 | Conc. (pg/ml) or ratio at the following percentile: | Conc. (pg/ml) or ratio at the following percentile: | |
|                 | 25 | 50 | 75 | 25 | 50 | 75 |  
| TNF-α*          | 26 | 0.94 ± 2 | 2.43 ± 2 | 5.07 ± 2 | 26 | 0.2 ± 2 | 0.4 ± 2 | 5.2 ± 2 | NS |
| IL-1β            | 29 | 4.2 | 8.0 | 14.1 | 27 | 1.1 | 1.5 | 9.8 | <0.05 |
| IL-6             | 31 | 0.33 | 1.02 | 1.42 | 28 | 0.23 | 0.68 | 1.65 | NS |
| IL-8             | 30 | 1.06 | 6.43 | 11.54 | 30 | 0.59 | 1.24 | 12.34 | <0.05 |
| IL-10            | 30 | 0.49 | 0.96 | 1.82 | 25 | 0.5 | 2.3 | 5.6 | <0.05 |
| γ-IFN            | 30 | 2.24 | 7.09 | 17.24 | 31 | 0.84 | 3.42 | 7.35 | <0.05 |
| IL-1β/IL-10     | 31 | 0.94 | 800 | 1,250 | 24 | 106 | 282 | 1,000 | <0.05 |
| TNF-α/IL-10     | 28 | 0.85 | 1.60 | 3.97 | 25 | 0.04 | 0.20 | 4.50 | <0.05 |

* Stimulation was performed with LPS from EHEC O111 for 24 h. All results except those for IFN-γ were corrected for monocyte cell count (per 10⁶ cells). The results for IFN-γ were corrected for lymphocyte cell count (per 10⁶ cells).

\(b\) number of patients.

\(c\) not significant.
Comparison of SEB stimulation results showed decreased levels of IFN-\(\gamma\)/H9253, IL-4, and IL-2 protein production, but only the result for IL-2 was significant, as outlined in Fig. 3. It is noteworthy that the median results for patients in group 3 were even lower more than those observed previously for the other study groups (Table 4).

Correlation analysis between the clinical results for patients in group 1 and lymphokine protein levels revealed a significant negative correlation between IL-2 levels and the degree of proteinuria at the time of stimulation. In addition, IFN-\(\gamma\)/H9253 levels excellently correlated with the period of time between the time of the acute D+ HUS episode and the time of SEB stimulation (Table 5).

**DISCUSSION**

While other groups have reported in vitro cytokine and chemokine data for HUS patients (8, 10, 15), this is the first study that focuses on the possible contribution of the immune system to the occurrence and severity of typical HUS in children by ex vivo stimulation. Our data demonstrated an imbalance between proinflammatory and anti-inflammatory cytokine levels in patients who previously had HUS when whole blood was stimulated with EHEC LPS. This result supports the in vivo observation of lower IL-10 levels and increased TNF-\(\alpha\)/IL-10 ratios during acute HUS, as published recently (24).

Our data cannot be explained by the diversity of the specificities of the antibodies used in the ELISA because we measured not only cytokine protein levels (ELISA) but also cytokine mRNA levels. This is important, because in contrast to our observations, a very well designed study by Proulx et al. (15) found increased levels of IL-10 in HUS patients in vivo. In addition, the clinical relevance of IL-10 mRNA levels and the TNF-\(\alpha\)/IL-10 ratio is suggested by the significant correlation with the patients’ GFRs and levels of proteinuria, respectively, at the time of stimulation. Because the severity of acute disease in patients with HUS is of high prognostic value for the long-term outcome (2, 5, 16), we hypothesize that an increased IL-10 response with a consequent lower TNF-\(\alpha\)/IL-10 ratio could have a protective effect in patients with acute EHEC infections. We cannot rule out the possibility that the alterations in the LPS stimulation profiles observed are the result of the HUS disease that had occurred previously and do not
preexist as an individual risk factor for a severe EHEC infection. Therefore, we studied children with a history of D+ HUS and compared the results to those for healthy, matched controls, where appropriate. The lack of a positive correlation between Δt and IL-10 levels, as well as the absence of a negative correlation between Δt and the TNF-α/IL-10 ratio, however, does support our hypothesis of a characteristic immune status that predisposes individuals to HUS.

The results of the superantigen stimulation assay (Table 4) revealed an impressively decreased IL-2 response in patients who had previously had HUS, without any hint for a T-helper-cell 1 to T-helper-cell 2 shift. Lower IL-4 and IFN-γ levels were also noted but were not significantly different. This is probably due to the small sample size. In accordance with the results of experiments with animals that received intravenous Stx injections (17), these data suggest an impaired capability of lymphocyte proliferation. By taking the SEB stimulation results for patients with an acute episode of HUS into consideration, it is likely that the alteration of lymphocyte proliferation occurs during the acute phase of HUS and restoration takes place over months or even years. The latter is underlined by the strong positive correlation between IFN-γ production and Δt (Table 5).

Like the TNF-α/IL-10 mRNA ratio, the amount of IL-2 protein secretion secondary to SEB stimulation significantly correlates with the patients’ levels of proteinuria (Table 5). Therefore, a protective effect of IL-2 secretion can be postulated.

No other clinical parameter, like erythraemia or blood pressure, seemed to be linked to cytokine production in response to either SEB or LPS stimulation. We assume that proteinuria and GFR are the most sensitive parameters for impaired health status in our patients who had previously experienced typical HUS. This hypothesis is supported by the fact that other clinical parameters also tended to correlate but missed statistical significance (data not shown).

The ongoing study to test this includes patients who had previously had EHEC infections without signs of HUS in order to support our hypothesis that there is an individual inflammatory response that predisposes an individual to typical HUS when EHEC infection occurs. In addition, experiments that will evaluate other immune-stimulating pathogenic factors, like Stx1, Stx2c, Stx2d, hemolysin, and other factors, are planned.

It is noteworthy that even in children several immunological risk factors that predispose an individual to a decreased or increased severity of a certain illness have been described for other forms of infectious diseases (7, 18, 21, 23). Nevertheless, this is the first study investigating a possible individual immunologically determined risk for D+ HUS secondary to an EHEC infection. Even though the differences between the groups were small and therefore cannot represent the exclusive key to the understanding of the pathophysiology of HUS, the variations in individual cytokine profiles that were observed could be of definite importance. Studies reproducing these results in an animal model must be undertaken to support these conclusions.

ACKNOWLEDGMENTS

We thank all nurses and physicians of the Department of Pediatrics, Technical University of Munich, for permanent support and critical discussions. Special thanks go to our routine laboratory for technical assistance and to K. Ulm for support with statistics. We are grateful for the excellent technical assistance of M. Klein, A. Biedermann, and G. Pinski. Special thanks go to Martin Bitzan for critically reviewing the manuscript.

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TABLE 4. Cytokine levels in plasma of whole blood from patients with a history of D+ HUS and controls and during an acute episode of HUS determined by stimulation assays

| Cytokine | Group 1 | Group 2 | Group 3 |
|----------|---------|---------|---------|
|          | n       | Conc (pg/ml) at the following percentile | n       | Conc (pg/ml) at the following percentile | n       | Conc (pg/ml) at the following percentile |
|          |         | 25 50 75 |         | 25 50 75 |         | 25 50 75 |
| IL-2     | 22      | 0.80 1.23 2.19 | 13      | 1.51 2.43 2.85 | <0.01 6 | 0.20 0.38 1.10 |
| IL-4     | 22      | 1.0 2.2 3.0 | 13      | 1.0 2.0 2.0 | NS 6 | 0.0 1.0 2.8 |
| IFN-γ    | 22      | 194 182 257.6 | 13      | 192.7 226.6 340.8 | NS 6 | 16.0 64.6 79.3 |

* Stimulation with SEB was performed for 72 h. The results are corrected for lymphocyte cell count (per 10^6 cells).

** n, number of patients.

** NS, not significant.

TABLE 5. Correlation between clinical results and lymphokine protein levels from patients with a history of D+ HUS

| Protein | Δt | Proteinuria |
|---------|----|-------------|
|         | P  | R  | n  | P  | R  | n  |
| IL-2    | NS |     |    | <0.05 | −0.449 | 22 |
| γ-IFN   | <0.001 | 0.734 | 22 | NS |     |    |

* Stimulation with SEB was performed for 72 h. The results are corrected for lymphocyte cell count (per 10^6 cells).
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