Elevated Levels of Lipopolysaccharide-Binding Protein and Soluble CD14 in Plasma in Neonatal Early-Onset Sepsis

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No data on lipopolysaccharide-binding protein (LBP) in newborns with sepsis have been available up to now. We therefore determined levels of LBP and soluble CD14 (sCD14) in plasma of healthy and septic neonates in order to evaluate their potential diagnostic role. The study included prospectively collected patient samples of two recently published studies on cytokine expression in neonatal sepsis. Twenty-nine septic patients were enrolled in the present analysis. Samples—either cord blood or peripheral blood—from patients admitted within the first 24 h of life for suspicion of sepsis and cord blood samples of a control group of 40 healthy mature infants delivered spontaneously were analyzed. For seven of the septic group, a second sample collected between 24 and 48 h of life was available. Levels of sCD14 and LBP in plasma were determined by an enzyme immunoassay using recombinant CD14 and LBP as standards. LBP and sCD14 were correlated to cytokine plasma levels. In septic neonates, LBP (median, 36.6 versus 7.8 μg/ml; \(P < 0.001\)) and sCD14 (median, 0.42 versus 0.28 \(\mu\)g/ml; \(P = 0.038\)) were increased in plasma levels when compared to those of healthy neonates and strongly correlated to granulocyte colony-stimulating factor (G-CSF), interleukin-1β (IL-1β), IL-6, and IL-8 levels. LBP levels in septic neonates analyzed between 24 and 48 h of life even increased when compared to samples obtained at or shortly after delivery (median, 36.6 versus 60 \(\mu\)g/ml; \(P = 0.038\)). In summary, levels of LBP in plasma of neonates with early-onset sepsis are significantly elevated; the elevated plasma levels seem to persist for more than 24 h, which could provide the clinician with a prolonged time period to identify the newborn with bacterial sepsis.

Bacterial sepsis is a leading cause of neonatal morbidity and mortality. Recent data from the United States demonstrate an incidence of 3.5 cases per 1,000 live births; 16% of these patients died (24). The increased susceptibility of the newborn for septic infections has been investigated intensively in the past with respect to developmental deficiencies of the host defense system, which include a delayed maturation of the specific humoral and cellular immune response of neonatal B and T cells (16) and a decreased capability of neonatal cells to secrete cytokines like tumor necrosis factor alpha (TNF-\(\alpha\)), gamma interferon (IFN-\(\gamma\)), interleukin-1β (IL-1β), IL-6, IL-8, and IL-12 (1, 15, 23, 26, 35). Moreover, compromised functions of the innate immune system with a defective activation of the complement cascade have been described (16), as have those with deficiencies of neonatal myelopoiesis (6).

However, for neonatal sepsis, little is known about a group of molecules playing a central role in the innate immune system. Among them are the myeloid antigen CD14, which is involved in the recognition of a wide variety of bacterial products (17, 34), and lipopolysaccharide-binding protein (LBP), which is the principal plasma protein responsible for transporting endotoxin to immune effector cells bearing CD14 on their cell surfaces (21). Endotoxins, or lipopolysaccharides (LPS), are major activating molecules in the pathophysiology of sepsis caused by gram-negative bacteria. LPS induces its strong effects by the interaction with CD14-bearing inflammatory cells, such as mononuclear blood cells (9, 31); LPS monomers from cell walls of gram-negative bacteria bind to LBP, which transfers LPS to CD14 on the phagocyte cell membrane. The current view is that this process promotes the binding of Toll-like receptor (TLR) 4 (17), which signals into the interior of the cell and induces the release of a cascade of proinflammatory cytokines, such as TNF-\(\alpha\), IL-1β, IL-6, and IL-8. These cytokines are key mediators in the pathogenesis of the systemic inflammatory response (22). In contrast, TLR2 is implicated in the recognition of gram-positive bacterial components, bacterial lipoproteins, and zymosan (29, 30). As a matter of fact, the majority of neonatal infections is caused by a gram-positive bacterium, Streptococcus agalactiae, accounting for as much as one-third to one-half of all cases of neonatal early-onset sepsis (2, 24). It has been reported recently that purified group- or type-specific carbohydrates or lipoteichoic acids of S. agalactiae activate cells of the innate immune system primarily via CD14 and complement receptors (CR) (8, 17). Other investigators have shown that CR3 but not CR4 seems to be involved in activation of human monocytes by streptococci, whereas CR4 is involved in activation by Staphylococcus aureus (7). In addition, there is evidence that TLR2 can function as a signaling receptor for Listeria monocytogenes, but not for S. agalactiae (9). Despite the fact that the signaling pathways are not yet very clear, we have shown recently that in cases of neonatal early-onset sepsis, levels of proinflammatory cytokines in plasma are extraordinarily high (3). In addition, a strongly increased cytokine gene expression in vivo in cord blood cells of neonates with sepsis was observed (4).
Considering these observations, we were interested in the concentrations of LBP and sCD14 in plasma of neonates. Since they belong to the innate immune system, we hypothesized that these mediators should also be elevated in neonatal sepsis. Plasma samples collected during two previous studies (3, 4) offered us the opportunity to determine sCD14 and LBP levels in septic and healthy mature neonates. Concentrations in plasma of neonates with early-onset sepsis at presentation were determined and correlated to the previously measured cytokine plasma concentrations.

MATERIALS AND METHODS

Study population. The study was performed at the University Children’s Hospital Freiburg, Freiburg, Germany, and included patient samples of two recently published studies on cytokine expression in neonatal sepsis (3, 4). Patients enrolled in the present analysis comprised of mature newborns from whom plasma samples used in the previous studies were available. The newborns included had been admitted to the Children’s Hospital between June 1993 and December 1996. Samples—either cord blood or peripheral blood—from patients admitted within the first 24 h of life for suspicion of sepsis and cord blood samples of a control group of healthy mature infants delivered spontaneously were analyzed.

The medical records of all patients and their mothers had been evaluated retrospectively in the two previous studies, which had been approved by the local ethics committee. In this evaluation, sepsis was defined—as described in detail previously (3, 4)—as the substantial clinical suspicion within the first 24 h of life and, in addition, (i) a blood culture turning out positive or (ii) at least three of the following criteria being met: (a) C-reactive protein (CRP) at >2 mg/100 ml within 48 h after onset of clinically suspected sepsis, (b) pneumonia as diagnosed by X ray or by microscopic or cultural evidence in tracheal aspirate, (c) gastric aspirate with microscopic or cultural evidence of bacterial infection or positive latex agglutination test for S. agalactiae in urine, (d) proportion of immature (bands and less mature forms) to total neutrophils of >0.2 documented in any complete blood count (CBC) within 48 h after clinically suspected sepsis, and (e) maternal fever or antibiotic treatment within 48 h of delivery or premature (<48 h) rupture of membranes prior to delivery or histopathology of amnionitis of the placenta or cord.

Blood samples. Cord blood samples were obtained within 15 min of delivery. Samples were collected aseptically by squeezing or puncturing the cord. Blood samples other than cord blood were obtained by venipuncture or drawn from an indwelling arterial or venous line. For patients without available cord blood, the time of taking the initial blood cultures was the time of collecting the indwelling arterial or venous line. For patients without available cord blood, samples other than cord blood were obtained by venipuncture or drawn from an indwelling arterial or venous line. Blood samples of a control group of healthy mature infants delivered spontaneously were analyzed.

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Soluble CD14. The amount of sCD14 was determined by enzyme immunoassay (EIA) as previously described (10) with three modifications: the anti-CD14 monoclonal antibodies (MAbs) biG12 and biG13 (IgG1) were coupled to the plate, and the POD-labeled MAb biG7 (IgG1) was used as detection antibody. The protein concentration of sCD14, which was used as a standard, was estimated by calculation of protein extinction coefficients at 280 nm from amino acid sequence data (11). The intra- and interassay coefficients of variance were <10%. All reagents were obtained from biometec GmbH, Greifswald, Germany.

LBP. The human LBP-cDNA was amplified using primers 5′LBP (GCA TCT AGA CCA TGG GGC CCT TGG CCA GAG CCC TGC C) and 3′LBP (His), (GCA TCT AGA CTA GTG ATG ATG ATG ATG AAT CAC TCT CAT GTA TTG GAC ATT GGC A), thereby introducing six C-terminal histidines and flanking XbaI sites (template cDNA kindly provided by R. R. Schumann, Berlin, Germany). The PCR product was cloned into the XbaI site of pPOL-DHFR (kindly provided by P. Kufer, Munich, Germany) and sequenced to exclude PCR-based errors. Culture, transfection, and methotrexate selection of CHO cells was performed as described elsewhere (25). For preparation of LBP, recombiant CHO cells were cultured in serum-free CHO-SFM II-medium (Life Technologies GmbH, Eggenstein, Germany) in the presence of 500 nM methotrexate. Culture supernatants were dialyzed against 50 mM Tris-0.1 M NaCl (pH 8.0) and affinity purified on TALON metal affinity resin (Clontech, Palo Alto, Calif.). The column was washed with Tris-NaCl buffer until the optical density at 280 nm (OD280) of the flowthrough was less than 0.04 and the bound (His)6-LBP was eluted with Tris-NaCl buffer containing 0.1 M NaCl-EDTA. The eluate was dialyzed against phosphate-buffered saline and the LBP content was measured by determining OD280. The purity of the LBP preparation obtained by this method was >95% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. LBP-deficient mice (13) were immunized with recombinant LBP (rLBP) to produce MAbs. Anti-human LBP MAbs’ clone 2D11E2 (IgG2a) and POD-labeled clone 4D6G2 (IgG1) were used in a sandwich EIA for quantification of LBP using rLBP as a standard. The protein concentration of the recombinant standard was determined by the same method as described for CD14. The intra- and interassay coefficients of variance were below 10%. The detection limit of the assay is 0.45 μg/ml. In the meantime, a commercialized assay based on this method is available (biometec GmbH).

Cytokines and C-reactive protein (CRP). Data on cytokine levels in plasma were derived from two recently published studies (3, 4). Cytokine analysis had been performed by a double-sandwich EIA technique using commercial kits specific for human cytokines granulocyte colony-stimulating factor (G-CSF), TNF-α, IL-1β, IL-6, and IL-8 (Quantikine; R&D Systems Europe, Abingdon, United Kingdom) as described in detail previously (3). CRP concentrations in plasma were measured by the nephelometric method (Array protein system; Beckman Instruments, Munich, Germany).

Statistical analysis. The differences between the median values of cytokine plasma levels of the two groups were analyzed by the Wilcoxon-Mann-Whitney U test. All values are expressed as medians, quartiles, and ranges. The correlation was calculated using the Spearman’s rank sum procedure. A two-tailed P value of <0.05 was considered significant. Analyses were performed using the SPSS software package (SPSS 8.0 for Windows).

RESULTS

Plasma samples. Samples from 29 septic neonates were available for analysis of sCD14 and LBP concentrations in plasma. Cord blood samples were available in 19 patients of the septic group, the remaining 10 samples were obtained within the first 24 h of life. Cord blood samples from 40 healthy newborns served as controls. The birth weights were similar in both groups (median, 2,750 versus 3,290 g; P = 0.82), as was the gestational age (median 40 weeks in both groups). For seven patients of the septic group, a second sample collected between 24 and 48 h of life was available. The clinical characteristics of these patients were similar to those for whom follow-up samples were not available (e.g., median birth weight, 2,530 versus 2,850 g; P = 0.97).

sCD14 and LBP plasma levels. Newborns of the septic group had significantly higher levels of sCD14 and LBP in the plasma samples collected from cord blood or shortly after delivery than those of the control group (Fig. 1a and b). Similar results were obtained when analyzing cord blood samples only; levels in cord blood from septic patients were significantly higher than levels in cord blood of healthy newborns for either sCD14 (median, 0.4, and quartiles, 0.35 to 0.62 μg/ml, versus median, 0.28, and quartiles, 0.21 to 0.36 μg/ml; P = 0.001) or LBP (median, 30.3, and quartiles, 10.65 to 50.4 μg/ml, versus median, 7.8, and quartiles, 6.15 to 10.62 μg/ml; P < 0.001). When analyzing the data separately only for those patients with blood culture-proven infection (n = 6), the median level of sCD14 was 0.5 μg/ml (quartiles, 0.38 to 0.65 μg/ml), the median LBP level 23.6 μg/ml (quartiles, 4.2 to 42.8 μg/ml). These results were comparable to the results found for septic patients without a positive blood culture (sCD14: median, 0.41, quartiles, 0.38 to 0.63 μg/ml, P = 0.71; LBP: median, 36.3, quartiles, 14.1 to 65.0 μg/ml, P = 0.25).

Plasma levels of sCD14 and LBP obtained on day 2. A second plasma sample taken between 24 and 48 h after delivery was available for seven patients of the septic group. When
comparing the LBP and sCD14 levels in plasma of these patients at delivery (cord blood) and at follow-up, a significant increase of LBP (median, 60.0 versus 42.6 μg/ml; \( P < 0.05 \)) was observed. In contrast, levels of sCD14 in plasma remained stable (median, 0.49 versus 0.48 μg/ml) (Table 1). When the median value of LBP in the follow-up samples was compared to that of the whole group of patients with day 1 samples (\( n = 29 \)), there was also a significant increase (median, 36.6 versus 60 μg/ml; \( P < 0.05 \)), whereas sCD14 levels remained constant (median, 0.42 versus 0.49 μg/ml).

**Bacteriological findings.** For the septic group, the organism detected in a majority of blood, urine, or gastric aspirates was *S. agalactiae* (\( n = 15 \)). Enterococci from four patients and *Escherichia coli* from two patients were cultured. The levels of sCD14 and LBP in plasma did not differ between the subgroup of newborns with proven *S. agalactiae* infection (medians, 0.4 and 36.6 μg/ml, respectively) and those with other etiologies. For all patients from whom samples of both time points were available, the causative organism was *S. agalactiae*.

**Correlation of sCD14 and LBP levels to cytokine levels in plasma.** Cytokine levels in plasma were derived from the data of two previous studies for correlation analysis and are given in Table 2. There was a strong correlation when comparing sCD14 and LBP levels (Spearman’s correlation coefficient [CC], 0.748, \( P < 0.001 \)). Likewise, when correlating either sCD14 or LBP levels to levels of G-CSF in plasma (CC, 0.555 and 0.577; \( P < 0.001 \) and \( P < 0.001 \), respectively), IL-1β (CC, 0.37 and 0.469; \( P = 0.037 \) and \( P = 0.007 \), respectively), IL-6 (CC, 0.45 and 0.486; \( P = 0.001 \) and \( P < 0.001 \), respectively), and IL-8 (CC, 0.41 and 0.482; \( P = 0.007 \) and \( P = 0.001 \), respectively), a less pronounced but significant correlation was observed. There was no correlation between either sCD14 or LBP and TNF-α levels in plasma (CC, 0.216 and 0.23; \( P = 0.206 \) and \( P = 0.178 \), respectively).

**DISCUSSION**

The pathophysiology of neonatal sepsis differs significantly from that of adult endotoxin shock, for which the role of sCD14 and LBP has been investigated intensively (13, 14, 21). The vast majority of neonatal sepsis, however, is caused by

**TABLE 1.** Concentrations of sCD14 and LBP in plasma of cord blood samples of seven septic newborns and blood samples obtained on the second day of life

| Protein | CB (n = 7) | Day 2 sample (n = 7) | \( P \) value |
|---------|-----------|----------------------|--------------|
| sCD14 (μg/ml) | 0.48 | 0.49 | NS |
| Quartiles | 0.35–0.87 | 0.4–0.83 | |
| Range | 0.005–1.19 | 0.34–0.88 | |
| LBP (μg/ml) | 42.6 | 60 | <0.05 |
| Quartiles | 30.3–60 | 48–74.4 | |
| Range | 0.005–73.5 | 39–78 | |
| CRP (mg/dl) | 0.6 | 7.2 | <0.05 |
| Quartiles | 0.6–2.6 | 5.9–7.2 | |
| Range | 0.6–2.8 | 3.1–10 | |

* CB, cord blood. For comparison, CRP levels are also given.

* Differences were considered significant at a \( P \) value of <0.05, NS, not significant.
**TABLE 2. Concentrations of G-CSF, TNF-α, IL-1β, IL-6, and IL-8 in plasma**

| Protein     | Concentration (pg/ml) in plasma of: | P value |
|-------------|-------------------------------------|---------|
|             | Septic newborns (n = 29) | Healthy newborns (n = 40) |
| G-CSF       | Median 5,000 | 80 | <0.001 |
|             | Quartiles 1,372.3–36,996 | 20.5–129.8 |
| IL-6        | Median 1,326.2 | 4.35 | <0.001 |
|             | Quartiles 167.6–6,205.2 | 1.15–8.61 |
| IL-8        | Median 2,811.2 | 21.41 | <0.001 |
|             | Quartiles 443.86–6,473.03 | 5.03–70.03 |
| IL-1β       | Median 48.5 | 3.9 | 0.001 |
|             | Quartiles 6.03–339.03 | 3.9–6.75 |
| TNF-α       | Median 40 | 6.8 | 0.001 |
|             | Quartiles 11.9–89.6 | 4.4–20.4 |
|             | Range 8–2,854 | 4.4–77.6 |

* Data are concentrations of G-CSF, TNF-α, IL-1β, IL-6, and IL-8 in plasma of cord blood or peripheral blood samples of septic newborns obtained within the first 24 h of life or in plasma of cord blood samples of healthy newborns, as derived from two previous studies (3, 4).

**gram-positive bacteria, S. agalactiae in particular is the single most important pathogen, accounting for as much as one-third to one-half of all cases of neonatal early-onset sepsis, which is defined as sepsis beginning within the first 4 days of life (2).**

The initial event of neonatal early-onset sepsis caused by S. agalactiae is supposed to occur prior to birth, since the majority of infected newborns present clinically as sepsis syndrome within the first 24 h of life. As a rule, early-onset sepsis results from an ascending infection of bacteria from the maternal rectovaginal flora invading the amniotic fluid and coming into contact primarily with mucosal cells of the fetal gastrointestinal and respiratory tract. During the last years, several clinical studies, including our own, have demonstrated that levels of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, in plasma are highly elevated in cord blood of neonates with early-onset sepsis (3, 4, 18, 19, 27).

In the present study, which advantageously could be based upon data on the expression of a variety of cytokines, we were able to show that levels of LBP and sCD14 in plasma also are increased during neonatal early-onset sepsis. There is only one previous study reporting elevated levels of CD14 in serum of neonates with sepsis (5). In that study, however, the measurement of sCD14 was performed at a postnatal age of about 2 weeks, with a wide time range. Up to now, no data have been published on the concentration of LBP in the blood of neonates. In the present study, we found significantly elevated levels of sCD14 and LBP in samples from septic neonates collected at or immediately after birth when compared to samples from healthy control neonates. These findings indicate that the neonate at the moment of birth is capable of releasing relevant amounts of sCD14 and LBP, but it is also important to recognize that such an excessive response, as in adults, with sepsis is lacking (10, 14, 20). Equally elevated concentrations of either sCD14 or LBP were also documented for the 15 newborns with S. agalactiae infections. These data show that gram-positive bacteria, such as S. agalactiae, which do not possess LPS as constituents of their cell wall, are capable of inducing a significant secretion of CD14 and LBP in vivo. Due to the lack of a relevant number of proven infections by gram-negative bacteria, however, we were not able to compare patients with infections by gram-positive or gram-negative bacteria. It has been described recently that both LPS and cell wall preparations of S. agalactiae induce TNF-α secretion from human monocytes in a CD14-dependent manner (7). Addition of very small amounts of sCD14 (0.1 µg/ml) and/or LBP (0.01 µg/ml) even enhanced the activation of TNF-α secretion by LPS and S. agalactiae in monocytes in vitro (17). Taken together with the in vivo findings of our study, one may speculate that there is indeed a pathophysiological role of CD14 and LBP in the activation of the neonatal immune system during the onset of S. agalactiae sepsis. CD14 and LBP seem to contribute in vivo to the signaling of monocyte activation in response to S. agalactiae.

Apparently, healthy neonates fail to mount a fully expressed sCD14 production, whereas LBP levels are comparable to those of healthy adults. Soluble CD14 is present in sera of healthy adults. The mechanisms of sCD14 release have been studied extensively (see reference 28 for review). The reason for the lower sCD14 levels in neonates remains to be evaluated. Additionally, neonates did not respond with excessive sCD14 release during infection, as it has been shown for adults with levels of up to 20 µg/ml in plasma in polytrauma (20) or sepsis (14). Nevertheless, we observed significant differences between septic and healthy newborns (Fig. 1). Whether the reduced sCD14 levels in healthy newborns depend on the gestational age and equal that of adults rapidly in early life remains to be investigated. Blanco et al. (5) reported sCD14 levels of 1.73 µg/ml with a standard deviation of 0.83 at a postnatal age of about 2 weeks, which is comparable to the values (mean ± standard deviation) of 1.4 ± 0.29 µg/ml that we measured previously with healthy adults by using the test configuration presented here. It is noteworthy that the comparison of absolute protein concentration using enzyme-linked immunoassays is difficult to interpret due to a different protein calculation procedure of the standard or different antibodies used.

LBP levels of healthy neonates in this study were at the same magnitude as those from adults. Recently, Opal et al. (21) reported elevated LBP levels in plasma of septic adults with a cutoff value of >7.4 µg/ml, which were again nearly identical to our neonatal sepsis group. Interestingly, LBP levels in the study of Opal et al. were less highly elevated in nonsurvivors than in adult survivors. Fortunately, all newborns in our study survived.

LBP is a hepatically synthesized, acute-phase protein, which...
like other acute-phase reactants, such as CRP, is supposed to be induced by IL-6 (22). When analyzing the time course of CRP in neonatal sepsis in this study and previous studies, we observed—as it is well known to neonatologists—that CRP plasma levels were not elevated within the first hours of life but increased slowly, reaching a maximum at the age of 48 h or later (3). Although the liver has been held responsible for the IL-6-induced production of LBP, there is recent evidence for the synthesis of LBP in the intestinal mucosa as well, which is not inducible by LPS or IL-6 alone but by a cocktail of cytokines or glucocorticoids (32). This might be an explanation for the independency from the gram-negative or gram-positive nature of the microorganisms mentioned above, for the early onset of the phenomenon, and for the lack of correlation to CRP or TNF-α levels in plasma.

The data of the study presented here are interesting from a pathophysiological point of view. However, they may also be relevant for clinicians. While conventional parameters such as CRP usually are not elevated in cord blood or soon after birth, we have shown recently that cytokine concentrations in plasma are extraordinarily high at birth but drop very soon thereafter, offering only for a very short period of time the advantage of a high sensitivity and specificity (3). In contrast, when analyzing concentrations of sCD14 and especially of LBP in plasma of the very small group of patients in this study, where samples from different time points were available, levels in plasma even increased during the first 24 to 48 h of life. Whereas LBP levels were measured by enzyme-linked immunosorbent assay (ELISA) in our study, there is, in the meantime, a commercialized assay of LBP in a chemiluminescence analyzer, a random access fully automated immunoassay system (IMMULITE; DPC Biemann, Bad Nauheim, Germany), which allows assessment of LBP within 60 to 90 min. Together with the advantage of this new assay system, the findings of our study might encourage more research in this field. We hope that measuring LBP levels in plasma might in the future provide the clinician with a prolonged period of time to identify the newborn with bacterial sepsis. We believe that although data were obtained in a study design that was not established to follow these questions and although the number of samples analyzed in this study was rather small, these data should form the basis for a larger prospective clinical study. One might argue that most neonatologists treat possible neonatal sepsis immediately without waiting for laboratory results. However, there is a large group of patients that do not have obvious risk factors, who present postnatally with unspecific signs and symptoms, such as transient tachypnea, which then turns out to be due to mild respiratory distress syndrome. On the other hand, there are newborns with typical risk factors, such as maternal fever or premature rupture of membranes, but who do not present any signs and symptoms of bacterial infection. In both groups of patients, immediate antibiotic treatment—in our view—is not warranted. Nevertheless, the clinician is reassured when he has laboratory parameters at hand, which also do not indicate towards infection. We therefore believe that parameters such as LBP are worthwhile in the clinical routine and are helpful in reducing unnecessary antibiotic therapy. There is some hope that LBP, and to a lesser extent sCD14, might help obstetricians and neonatologists resolve the continuing dilemma of assigning the correct diagnosis of bacterial sepsis to an individual newborn and of treating or not treating the baby with antibiotics.

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