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Role of cytokines (interleukin-1β, 6, 8, tumour necrosis factor-α, and soluble receptor of interleukin-2) and C-reactive protein in the diagnosis of neonatal sepsis

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Aim: To investigate whether the serum levels of interleukin-1β, 6, 8, tumour necrosis factor-α and the soluble receptor of IL-2 are useful in the diagnosis of neonatal sepsis, and whether their diagnostic power is increased when in combination with classical markers such as C-reactive protein and white blood cell count. *Methods:* Blood samples were collected at admission from 40 neonates with suspected infection. Patients were included in different groups according to the bacteriological and laboratory results: Group I consisted of 20 newborns with positive blood cultures and other biological tests suggestive of infection. Group II included 20 neonates with negative blood cultures and biological tests not suggestive of infection. The control group included 20 healthy neonates with no clinical or biological data of infection. *Results:* Mean values of C-reactive protein were significantly higher in Group I. No differences were found between the groups for white blood cell count, with the exception of the presence of leucocytosis in Group II. Levels of interleukin-1β, 6, 8, tumour necrosis factor-α, soluble receptor of interleukin-2, and C-reactive protein were significantly higher in infected neonates than in the control groups. Detection sensitivity and specificity were 80 and 92% for C-reactive protein, 60 and 87% for interleukin-1β, 61 and 80% for interleukin-6, 62 and 96% for interleukin-8, 54 and 92% for tumour necrosis factor-α and 63 and 94% for soluble receptor of interleukin-2. The discriminant analysis showed that the best combination for sepsis diagnosis was C-reactive protein + interleukin-8 + soluble receptor of interleukin-2, with a sensitivity of 85% and a specificity of 97.1%.

Conclusion: Our study suggests that no individual test can on its own identify infected neonates, and that although the combination of C-reactive protein, interleukin-8 and the soluble receptor of interleukin-2 exhibits a high specificity, its sensitivity is limited.

Key words: *C-reactive protein, cytokines, interleukins, neonatal sepsis, tumour necrosis factor-α*

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Bacterial sepsis is still an important cause of morbimortality during the neonatal period 1, 2). Clinical manifestations are non-specific, and laboratory parameters such as white blood cell (WBC) count or C-reactive protein (CRP) are of limited value in identifying infected newborn babies (3–5). As a consequence, appropriate diagnosis and therapy could be delayed, worsening the prognosis of the patient.

In the past few decades, it has been observed that several mediators of inflammation tend to become elevated during sepsis. The study of interleukin (IL)-1β and tumour necrosis factor (TNF)-α, cytokines that are synthesized at the beginning of the inflammatory cascade, has rendered differing results (6–8). Levels of IL-6, a major inductor of hepatic protein synthesis, and of IL-8, one of the most potent chemotactic factors, have also been found to be elevated (6, 9, 10). IL-2 and its soluble receptor have been studied to a lesser extent in bacterial sepsis (11).

Despite the considerable number of publications related to cytokines in neonatal sepsis, there is no agreement about its diagnostic value in this condition. The objective of our study was to examine whether CRP, IL-1β, IL-6, IL-8, TNF-α and soluble receptor of

IL-2 (sIL-2R) are useful in identifying the infected neonate, and what combination, if any, exhibits the best diagnostic performance.

Patients and methods

We prospectively studied preterm and term newborn infants born between January 1996 and June 1997, admitted to our Neonatal Unit. These neonates exhibited non-specific clinical symptomatology that was attributed *a posteriori* to infection or non-infectious conditions, according to microbiologic and laboratory results. Samples were collected before the start of antibiotic therapy, simultaneously with blood for routine tests (WBC count, CRP, culture), as is the standard practice in our unit for babies with suspected sepsis. The second sample was collected between 72 h and 80 h after admission, at the same time that the routine control tests were ordered by the attending neonatologist. In healthy newborns only one first sample was collected during their stay in the nursery, at the time of routine metabolic screening or when an extraction for other purposes was decided on (glucose or calcium control). For ethical reasons, no second test was performed in these babies. The local ethics committee approved the study.

Patients

We collected a total of 382 samples from 218 neonates. Patients were included in Group I when sepsis was confirmed by a positive blood culture and a compatible clinical picture (respiratory distress, dark skin colour and poor perfusion, lethargy, poor feeding, apnoea, bradycardia, etc.) using a clinical score for sepsis (12). Sick babies without infection, whose blood cultures were negative and whose laboratory and clinical findings suggested other diagnoses, were included in Group II. Group III comprised healthy neonates without infectious risk factors admitted to the well-baby care nursery.

Fifty-one infants were excluded from the study: 34 because no pathogen could be isolated in the blood culture despite clinical and laboratory data highly suggestive of infection (clinical sepsis); 12 because their blood cultures were considered contaminated (isolation of more than one bacteria or delayed growth of a small number of colonies); and 5 patients for whom we were unable to establish a definitive infectious or non-infectious diagnosis. Of the 167 neonates included in the study, 50 corresponded to Group I, 67 to Group II and 50 to Group III. For operative reasons, we had to limit the number of samples to be analysed. Accordingly, 20 patients from each group were randomly selected by means of a random numbers table.

Laboratory determinations

WBC counts were performed in an automatic counter Coulter STKS (Coulter Electronics, Hialeah, Fla.), and differential counts were performed manually on Wright-stained blood slides. Standard criteria were used for recognition of the different cell subtypes (National Committee for Clinical Laboratory Standards on Cellular Morphology) (13). White blood cell values were scored according to the criteria of Manroe et al. (14).

Blood samples were collected in silicone vacuum-filled tubes (Vacutainer, SST model). After centrifugation at 3500 rpm for 3 min, the obtained sera samples were frozen and stored at −70°C until processing, with the exception of the samples for CRP, which were analysed immediately.

CRP was determined using an immunoturbidimetric method (Tina-quan CPR, Boehringer-Mannheim) and was quantified by means of an autoanalyser Hitachi 911. Reference values for healthy neonates, using quantitative techniques, are less than 1.6 mg/dl during the first 2 d of life, and less than 1 mg/dl for the remainder of the neonatal period (15).

IL-1β and TNF-α were determined by means of photometric immunoassay (ELISA, Boehringer Mannheim) and read by means of a microplate device (Whittaker. Reading filter: 450 nm. Reference filter: 690 nm). Intra- and interassay variation coefficients were 5.2% and 7.8% for IL-1β, and 6.7% and 9.9% for TNF, respectively. IL-6, IL-8 and sIL-2R were measured by chemoluminiscence enzymoimmunoassay in solid phase with an automated analyser (Immulite). The reagents and the analyser were supplied by "Diagnostic Products Corporation, Los Angeles" (CA: 90045-5597). All samples were assayed in duplicate. Intra- and interassay variation coefficients were 3.29% and 3.87% for IL-6, 3.38% and 3.61% for IL-8, and 2.8% and 5.9% for sIL-2R, respectively.

Statistical analysis

Data are expressed as means ± SEM, unless otherwise specified. Inference in the general population is shown as confidence intervals. Quantitative values were assessed through analysis of variance (ANOVA) when normally distributed, and by the Kruskal–Wallis test in other cases. *Post-hoc* analysis was performed in both cases: the Neumann–Keuls or Dunns test, respectively. Comparisons between two groups were made by means of the Mann–Whitney U-test or *t*-test, as appropriate. For qualitative analysis we used the chi-squared test (χ^2). Receiver operating characteristic (ROC) curves were used to assess the diagnostic value of the tests. We carried out a step-study of all the variables based on covariance analysis, followed by a discriminant analysis to estimate the combination of cytokines that best identify neonates with infection.

Table 1. Study population characteristics. Values of leucocytes, neutrophils and platelets in the first sample of blood, pre-antibiotic, in the three groups studied.

	Group I ($n = 20$)	Group II ($n = 20$)	Group III ($n = 20$)	p-value
Male/Female	8/12	11/9	9/11	0.07
Full-term/Preterm	11/9	13/7	10/10	0.062
Gestational age (wk) Median (range)	36 (29–42)	37 (31–40)	37 (30–41)	0.12
Birthweight (g), mean ± SD (range)	2595 ± 227.6 (800-4520)	2962 ± 152.5 (1340-4090)	2506 ± 181.1 (1470-3725)	0.22
Leucocytes	11020 ± 9608	23330 ± 10370*[a]	12770 ± 5618	
Total neutrophils	6841 ± 7370	12160 ± 5569*	6836 ± 4829	
Inmatures to total neutrophils ratio	0.04	0.08	0.05	0.109
Platelets	195 900 ± 94 180	220 400 ± 65 670	264 700 ± 69 320	0.08

(Data are means ± standard deviation).
* $p < 0.01$ vs control group
[a] $p < 0.001$ vs Group I.

Results

The general characteristics of the 60 patients studied are presented in Table 1. In Group I, 12 patients had early onset sepsis at a mean age of 12 h, and 8 patients late onset sepsis at a mean age of 557 h. The most frequently isolated pathogen in early onset sepsis was *Group B Streptococcus* [11 cases]. In the other patient with early onset sepsis, *Type III Haemophilus influenzae* was identified. The aetiology of late onset sepsis varied widely: *Staphylococcus epidermidis* [3], *Candida albicans* [2], *Escherichia coli* [1], *Klebsiella pneumoniae* [1] and *Salmonella typhimurium* [1]. All patients in this group survived, whereas 4 patients in Group II died (Table 2). A first sample was collected in all patients (20 in each group); in contrast, the second sample, collected 72 h after the initiation of therapy, was available in every patient in Group I, but only in 17 patients in Group II, because of three deaths before that time. In the control group (healthy babies), no second sample was collected, for ethical reasons.

WBC count, platelets and CRP values in the three groups studied are recorded in Table 1. Leucopenia was observed in 8 neonates with sepsis and 1 neonate in the control group, but in none of the patients in the second group. According to the Manroe criteria (14), only 2 infected patients and 1 with non-infectious pathology exhibited an elevated I/T ratio. The platelet count did not show any differences between groups.

CRP levels before therapy (first sample) were significantly higher in infected neonates than the levels in infants in the other two groups (Fig. 1), exhibiting a sensitivity of 80% and a specificity of 92%. The diagnostic power of CRP was higher for late onset sepsis (sensitivity 89.3%, specificity 94.8%) than for early onset sepsis (sensitivity 68%, and specificity 88.4%).

Cytokines

Mean levels, median, range and confidence intervals for each studied cytokine in the first collected sample in the

Table 2. Disease in Group II patients, postnatal age at the time of diagnosis and outcome.

No.	Postnatal age (h)	Gestational age (wk)	Diagnosis	Outcome
1	13	40	Perinatal asphyxia	Survived
2	23	38	Congenital heart defect	Survived
3	27	40	Neonatal seizures	Survived
4	7	32	Intracranial haemorrhage	Exitus 4th día
5	408	36	Congenital hypothyroidism	Survived
6	58	36	Non-immunologic jaundice	Survived
7	71	39	Immunologic jaundice	Survived
8	6	36	Pulmonary hypoplasia	Exitus 20 h. posnatal
9	168	42	Renal vein thrombosis	Survived
10	40	42	Congenital heart defect	Exitus 72 h postnatal
11	5	36	Intrauterine growth retardation	Survived
12	19	36	Paroxysmal tachycardia	Survived
13	1	39	Meconium aspiration syndrome	Exitus 12 h. postnatal
14	24	38	Polycythaemia	Survived
15	24	36	Drug withdrawal	Survived
16	4	41	Perinatal asphyxia	Survived
17	2	39	Meconium aspiration syndrome	Survived
18	3	42	Perinatal asphyxia	Survived
19	4	39	Congenital oesophageal atresia	Survived
20	6	41	Congenital heart defect	Survived

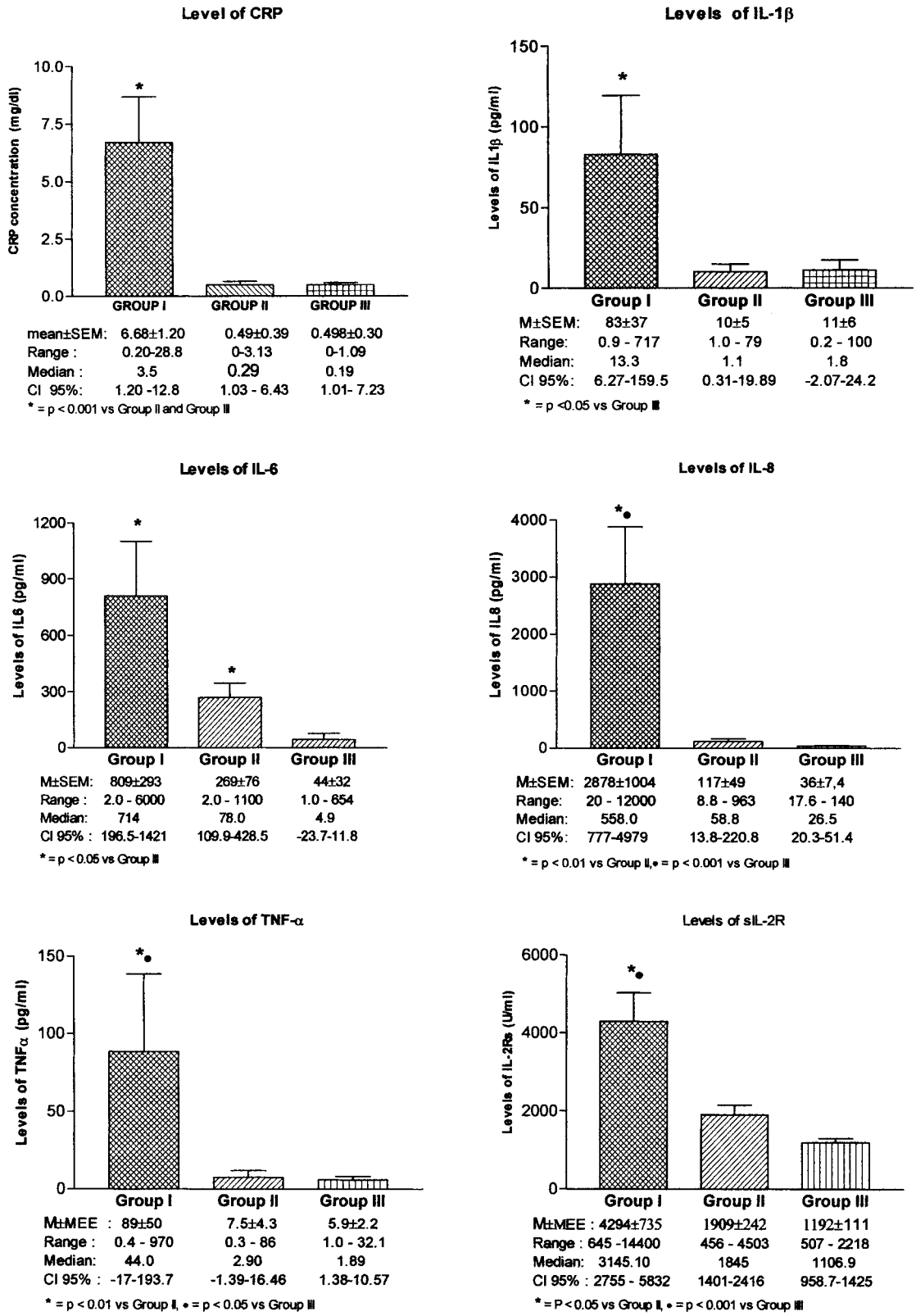

Fig. 1. Levels of C-reactive protein, interleukin-1β, 6, 8, tumour necrosis factor-α and soluble receptor of interleukin-2 in the three groups of patients studied.

Table 3. Levels of cytokines (mean ± standard error of the mean) in the second sample of blood in Groups I and II.

Cytokines	Group I ($n = 19$)	Group II ($n = 17$)	p-value
Interleukin-1β (pg/ml)	62.4 ± 48.1	24.4 ± 11.1	0.468
Interleukin-6 (pg/ml)	346.9 ± 146	261.9 ± 128	0.424
Interleukin-8 (pg/ml)	1166 ± 758	243.5 ± 194	0.254
Tumour necrosis factor-α (pg/ml)	26.65 ± 18.3	7.43 ± 1.83	0.453
Soluble receptor of interleukin-2 (U/ml)	2756 ± 409	2244 ± 376	0.176

three groups are presented in Fig. 1. IL-1β, IL-8, TNF-α and sIL-2R levels were significantly higher in infected neonates than in the other two groups. In contrast, IL-6 was higher in Groups I and II than in the control group. The mean levels of IL-1β, IL-8, TNF-α and sIL-2R in the second sample were higher in the infected group than in the non-infected patients, but these differences were not statistically significant (Table 3). IL6 remained elevated in the second sample in infected as well as in non-infected patients; this elevation was greater in the infected group but the difference was not significant.

Neonates with late onset sepsis exhibited higher levels of sIL-2R than infants with early sepsis: 5345 ± 626 (2465–7200) vs 3593 ± 1131 (645–14 400) U/ml ($p < 0.05$), but no differences were found in the other cytokines. As with CRP, the sIL-2R level exhibited a better diagnostic performance for late onset sepsis (sensitivity 84.3%, specificity 94.8%) than for early sepsis (sensitivity 59%, specificity 87.2%). Regarding the aetiology of the infection, we did not find any significant differences for any cytokine when comparing sepsis originating from Gram-negative or Gram-positive bacteria.

The area under the ROC curve was estimated for each cytokine (Table 4). CRP showed the highest area under the curve, with a sensitivity of 80% and a specificity of 92% for a cut-off value of 1.52 mg/dl. A stepwise selection of variables followed by a discriminant analysis showed that the best diagnostic power was achieved by a combination of CRP, IL-8 and sIL-2R, with a sensitivity of 85% and a specificity of 97.14%. This combination showed a sensitivity of 78% and a specificity of 93.2% for early sepsis, and 90.1% and 97.8% for late onset sepsis.

Discussion

The results of our work show that the different laboratory parameters studied have a low diagnostic sensitivity and specificity when used in isolation. The optimal combination of these parameters offers a good specificity but still an unacceptably low sensitivity for it to be recommended as a screening tool in the diagnosis of early sepsis, showing the limited value of CRP as well as cytokines for this purpose. Nevertheless, its diagnostic power is somehow better in cases of late onset sepsis.

As in studies elsewhere (3, 14, 16), the WBC count showed a low detection sensitivity in neonatal infection. Even the combination of total neutrophil count, I/T ratio and platelet count failed to reach an appropriate sensitivity and specificity in this pathology. CRP has been thoroughly studied as a diagnostic tool in neonatal sepsis and also as an indicator of response to therapy (17–19). However, its value early in the course of the disease or in cases of infections produced by some common pathogens, such as *Group B Streptococcus* infection, the most common agent in our study, is limited (20). The sensitivity and specificity of CRP were 80% and 92%, respectively, or even lower when considering only early onset sepsis, results that are similar to other previously published findings (21).

In our study, the TNF-α and IL-1β levels were significantly increased in neonates with sepsis, but while specificity was high, the sensitivity was unacceptably low for these cytokines to be recommended as a single tool for the early diagnosis of infection. Results of different published studies in relation to these cytokines are contradictory. Concerning IL-1β, results similar to ours have been reported by some researchers (22, 23), while not by others (24). Published data regarding TNF-α are also divergent. Some studies found the diagnostic utility of this cytokine (22, 23, 25), while others demonstrated similar or even lower levels in infected newborns compared to healthy neonates (7, 8). Discrepancies in results among different

Table 4. Sensitivity and specificity of the biochemical test using the calculated optimal cut-off values in the first sample of blood.

	CRP	IL-1	IL-6	IL-8	TNF	sIL-2R
Area*	0.75	0.71	0.69	0.73	0.732	0.737
Level of decision	1.52 mg/dl	24.5 ρg/ml	30 ρg/ml	63 ρg/ml	3.5 ρg/ml	2780 U/ml
Sensitivity	80	60	61	62	54	63
Specificity	92	87	80	96	92	94

* Area under the receiver operating characteristic (ROC) curves.

studies could be explained by the variations in laboratory methods in performing the analysis, the time of the sample collection, or the control population selected.

Several studies have demonstrated the diagnostic value of IL-6 in preterm and term neonates with infection (8, 10, 22–24, 26), even some days before the clinical signs are evident (27). In our work, IL-6 was not effective when used alone to diagnose neonatal sepsis because it was also elevated in neonates with non-infectious conditions, as has also been found in other studies (28, 29). This fact raises an important concern about the value of this cytokine in differentiating infected from non-infected ill newborns.

Infected neonates exhibited significantly higher levels of IL-8 than the other two groups in our study, but in contrast to other studies (6, 9, 30) the diagnostic power in the neonatal sepsis was limited (sensitivity 62%). What the few symptomatic patients without infection in which IL-8 was raised had in common was the presence of hypoxaemia and metabolic acidosis in their clinical picture. Hypoxaemia releases the inflammatory cascade and the level of several cytokines can rise (31), although the levels reached are usually not as high as those in infected neonates. On the other hand, antenatal steroids administered to the mother can diminish the inflammatory response in the foetus and, consequently, the synthesis of IL-8 (32). The only preterm neonate in our study whose mother was given antenatal steroids exhibited normal levels of IL-8.

We found a significant elevation of the sIL-2R levels in infected term and preterm newborns compared to healthy neonates or ill babies without infection. To our knowledge, there have been few studies to evaluate the sIL-2R level in neonatal sepsis. Elevated levels have been found in premature babies with early and late onset sepsis (11, 33). However, we are not aware of any previous study examining this receptor in term infants with infection or non-infectious pathology. In our work, this soluble receptor was moderately useful in the diagnosis of late onset sepsis.

In conclusion, as mentioned before, no parameter proved useful as a solitary tool to identify infected neonates. The best diagnostic value as an individual test was achieved by CRP. Owing to its unacceptably low sensitivity in identifying newborns with a highly threatening disease, the utilization of several tests in combination (CRP, WBC count and levels of cytokines) is warranted in order to ameliorate its diagnostic performance. The best combination also has a suboptimal diagnostic power for early onset sepsis, just moderately increasing the sensitivity, with a disproportionate increase in costs for a test that is necessary and frequently used in every neonatal unit. On the other hand, this combination offers a higher diagnostic power for late onset sepsis. We hope that future research will disclose a more sensitive and cheaper tool.

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