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TUMOR NECROSIS FACTOR-α, INTERLEUKIN-1β AND INTERLEUKIN-6 IN THE CEREBROSPINAL FLUID OF NEWBORN WITH MENINGITIS

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ABSTRACT - *Objective*: To analyze the usefulness of determining the cerebrospinal fluid(CSF) levels of tumor necrosis factor-α(TNF-α),interleukin-1β(IL-1β) and interleukin-6(IL-6)for the early diagnosis and evaluation of the prognosis of neonatal meningitis. *Method:* We studied 54 newborn that underwent lumbar puncture.Thirty patients had meningitis and 24 were the control group.CSF and sera were obtained at the moment of suspicion of meningitis and stored at -70^{0}C.Cytokines were performed by enzyme-linked immunosorbent assay method. *Results:* CSF cytokines were detected in all the newborn with meningitis.TNF-α was detected in the CSF in 63.3% of the neonates, IL-1β in 73.3% and IL-6 in 96.6%.The CSF levels were significantly higher than serum in neonates with meningitis.There was no correlation between the CSF levels of cytokines and neurologic complications. *Conclusion:* The detection of TNF-α, IL-1β and IL-6 in the CSF is of great value in order to achieve a early diagnosis of neonatal meningitis.Among the three cytokines analyzed, IL-6 was the best indicator of meningeal inflammation.

KEY WORDS: meningitis, cerebrospinal fluid, newborn, tumor necrosis factor-α, interleukin-1β, interleukin-6.

Fator de necrose tumoral-α, interleucina-1β e interleucina-6 no líquido cefalorraqueano de recém-nascidos com meningite

RESUMO - *Objetivo:* Analisar a utilidade da dosagem dos níveis de fator de necrose tumoral-α(TNF-α), interleucin-1β(IL-1β) e interleucina-6(IL-6) no líquido cefalorraqueano (LCR) para o diagnóstico precoce e avaliação do prognóstico da meningite neonatal. *Método:* Foram estudados 54 recém-nascidos submetidos à punção lombar.Trinta pacientes apresentavam meningite e 24 constituíram o grupo controle. As amostras de LCR e sangue foram obtidas no momento da suspeita clínica de meningite e estocadas a - 70^{0}C.A dosagem de citocinas foi feita pelo método ELISA (*enzyme-linked immunosorbent assay*). *Resultados*: Foram detectadas citocinas no LCR em todos os neonatos com meningite. O TNF-α foi detectado em 63,3% dos casos, a IL-1β em 73,3% e a IL-6 em 96,6%. Os níveis liquóricos foram significativamente mais elevados do que os séricos nos neonatos com meningite. Não houve correlação entre os níveis de citocinas no LCR e complicações neurológicas. *Conclusão*: A detecção de TNF-α, IL-1β e IL-6 no LCR é de grande valor para o diagnóstico precoce de meningite neonatal. Entre as três citocinas analisadas, a IL-6 foi o melhor indicador de inflamação meníngea.

PALAVRAS-CHAVE: meningite, líquido cefalorraqueano, recém-nascido, fator de necrose tumoral-α, interleucin-1β, interleucina-6.

The incidence of neonatal meningitis varies from 0.22 to 2.66/1000 live births with mortality between 17% to 29% and sequelae in 15% to 68% of the survivors[1-3].The unfavorable course of the disease and frequency of sequelae are directly related to the difficulty in its diagnosis, due to the nonspecific signs and symptoms, especially at the beginning of the infectious process. Since the cell count and chemical analysis of cerebrospinal fluid (CSF) can present a normal result at the onset of the disease[4] and several days are necessary to obtain the result of the cultures, which may be negative in 30 to 50% of the cases, the importance of the development of fast and safe methods for the diagnosis of neonatal meningitis becomes evident.In older children and adults some studies indicate that cytokine levels in

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CSF can represent a valuable resource for the rapid recognition of the disease and for evaluation of the degree of neurological involvement[5,6]. Regarding immunologically immature newborn with meningitis, little is known about either the pattern of elaboration of the inflammatory mediators or its relationship with the course of the infectious process.

The objective of the present study was to analyze the usefulness of determining the CSF levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) for the early diagnosis and evaluation of the prognosis of neonatal meningitis.

METHOD

Fifty-four newborn that underwent lumbar puncture due to clinical suspicion of meningitis were studied.These had been admitted to the Neonatal Intensive Care Unit of the Children's Institute, Hospital das Clínicas, University of São Paulo, during January 1, 1997 to March 31, 2001. Thirty newborn presented meningitis and 24, without meningitis, constituted the control group.The diagnosis of meningitis was based on either the presence of bacteria in the CSF or CSF with an increase in the number of cells (>20 cells/mm^3), predominance of neutrophiles, increase in the concentration of protein (>100 mg/dL) and reduction in the concentration of glucose (<50% of the concomitant glycemia)[7].Neonates with toxoplasmosis, syphilis, cytomegalia, rubella, meningomyelocele, hydrocephalus, perinatal asphyxia, and those with a prior history of surgical procedure in the central nervous system (CNS) were excluded from the study.Ampicillin and third-generation cephalosporin was begun immediately after the lumbar puncture was performed, being modified, when necessary, in accordance with the result of the cultures and maintained during a minimum period of 21days.Skull ultrasound scan and encephalic computed tomography were performed for all of the newborn with meningitis.

Samples of CSF and blood for TNF-α, IL-1β and IL-6 detection were obtained at the time of clinical suspicion of meningitis. A quantity of 1.5 ml of blood and CSF was collected, immediately centrifuged under refrigeration and stored in a freezer at -70°C.All samples were tested by enzyme-linked immunosorbent assay (ELISA) in duplicate. ELISA tests were performed following the manufacturer instructions (R&D Systems, Minneapolis, USA, Quantikine human IL-1β, human IL-6 and human TNF-α). The lowest limit of sensitivity for the detection of the cytokines in the serum and CSF was 4.4 pg/ml (TNF-α), 1.0 pg/ml (IL-1β) and 0.7 pg/ml (IL-6). Free and informed consent of the parents was obtained for all of the infants. The study was approved by the Commission of Ethics for Analysis of Research Projects (Hospital das Clínicas,University of São Paulo School of Medicine).

Student's t test was utilized for the comparison of the two groups regarding age and birth weight and chi-squared test to compare the distribution between sexes. Mann-Whitney test was used to compare the groups regarding the levels of TNF-α, IL-1β and IL-6 in the CSF and blood, and for comparisons of the levels of cytokines in CSF and for the presence of complications.The values below detection limits were considered to be equal to zero.The serum and CSF values in the same group (meningitis or control) were compared by the Wilcoxon test. Spearman's coefficient of correlation was used to analyze the correlation between the CSF cell count, protein and glucose concentrations and levels of the cytokines; *p* values < 0.05 were considered statistically significant.

Table 1. Comparision between the values of age, birth weight, gestational age and gender in the group with meningitis and the control group.

	Meningitis (n=30)	Control (n=24)	p-value
Age (days)			
mean	13.2	12.5	0.796
minimum-maximum	1.0-28.0	0.0-28.0	
Birth weight (g)			
mean	2782.0	2578.1	0.297
minimum-maximum	1070-4810	700-3650	
Gestational age (weeks)			
Mean	36.9	36.6	0.400
minimum-maximum	29-40	27-40	
Term newborn	18 (60%)	17 (70.9%)	0.588
Preterm newborn	12 (40%)	7 (29.1%)	
Gender			
Female	19 (63.3%)	11 (45.8%)	0.198
Male	11 (36.7%)	13 (54.2%)	

Table 2. CSF findings, cytokines levels, bacteria, site of detection of the microorganism and complications in 30 newborns with bacterial meningitis.

Case	CSF findings*			TNF-α (pg/mL)		IL-1β (pg/mL)		IL-6 (pg/mL)		Bacteria (site of detection)	Complications****
	cells (/mm³)	protein (mg/dL)	glucose (mg/dL)	CSF	Serum	CSF	serum	CSF	serum		
1	640	105	56	47.7	<4.4	49.1	6.5	18.1	<1.0	Staphylococcus coagulase negative (blood)	No
2	4550	82	9	74.2	35.7	190.8	36.1	561.2	74	Streptococcus pyogenes (CSF-blood)	Thrombosis of saggital sinus, ventriculitis, convulsions
3	2000	93	35	21.2	<4.4	38.9	<1.0	35.7	<1.0	Klebsiella pneumoniae (urine)***	No
4	43	121	31	<4.4	<4.4	<1.0	<1.0	2.3	<1.0	Staphylococcus aureus (sinovial fluid)	Convulsions
5	47	54	36	16.4	50.1	38.9	43.3	14.9	11.7	—	No
6	185	839	29	18.8	<4.4	95.3	13.8	21.3	<0.7	Staphylococcus coagulase negative (blood)	Ventriculitis
7	87	2272	81	<4.4	<4.4	6.8	10.4	504.8	6.6	—	Convulsions
8	1680	237	23	28.4	<4.4	103.0	52.7	11.7	6.4	—	Ventriculitis, hydrocephalus
9	385	460	9	274.9	<4.4	307.0	52.3	900.2	8.2	Acinetobacter baumannii (CSF-blood)	Ventriculitis, hydrocephalus
10	176	148	34	38.7	<4.4	40.8	10.6	483.2	325.0	—	Thrombosis of saggital sinus, convulsions
11	3669	177	23	71.6	<4.4	34.7	32.8	964.6	7.5	—	No
12	143	85	45	<4.4	<4.4	1.2	35.7	190.0	7.2	—	No
13	110	53	83	<4.4	22.1	<1.0	<1.0	9.4	27.9	Staphylococcus aureus (blood)	No
14	570	282	31	<4.4	<4.4	5.8	<1.0	479.9	12.5	Escherichia coli (urine)***	Convulsions, intrventricular hemorrhage
15	32	180	67	42.9	21.2	195.0	341.0	16.5	45.3	—	No
16	1370	78	53	273.4	38.6	31.0	6.6	659.2	68.4	Staphylococcus aureus (blood)	Ventriculitis
17	3	49	54	<4.4	28.5	<1.0	<1.0	0.8	<0.7	Streptococcus viridans (CSF)	No
18	2720	303	34	52.0	<4.4	61.6	1.1	197.7	21.0	Staphylococcus aureus (blood)	No
19	137	313	5	14.6	<4.4	29.8	0.4	208.7	26.5	Staphylococcus aureus (sinovial fluid)	Ventriculitis, hydrocephalus
20	5	178	82	1920.7	134.5	506.0	31.5	1022.0	446.0	Alcaligenes xilosoxidans (CSF) Pseudomonas aeruginosa (blood)	Coma, death
21	240	282	9	1229.4	82.7	242.0	<1.0	1000.0	33.2	Gram-negative diplococcus** (CSF)	Coma, death
22	3	121	38	<4.4	<4.4	<1.0	<1.0	22.3	9.3	Klebsiella pneumoniae (CSF)	No
23	35	76	—	<4.4	<4.4	<1.0	<1.0	146.5	<0.7	Enterobacter cloacae (blood)	No
24	60	134	59	58.8	<4.4	3.3	9.8	43.0	19.0	—	Thrombosis of saggital sinus
25	126	50	—	42.1	<4.4	3.3	<1.0	3.8	24.0	Staphylococcus coagulase negative (blood)	No
26	22	151	24	65.7	46.7	11.5	5.5	123.6	46.5	Klebsiella pneumoniae (blood)	Periventricular leukomalacia
27	20	116	34	55.9	<4.4	<1.0	<1.0	<0.7	<0.7	Streptococcus viridans (CSF)	No
28	67	346	52	<4.4	<4.4	<1.0	<1.0	21.3	17.8	Staphylococcus aureus (CSF)	Subdural collection,hearing loss,convulsions
29	101	11	47	<4.4	<4.4	14.9	<1.0	162.1	12.2	Escherichia coli (urine)***	Periventricular leukomalacia
30	80	90	47	<4.4	<4.4	<1.0	<1.0	82.3	<0.7	—	No

*Mean values: leukocytes=643.6cells/mm³; protein=249.6mg/dL; glucose=40.3mg/dl. **Bacteria identified in Gram-stained smear. ***Urine obtained by supra-pubic puncture. **** Sixteen (53.4%) newborn had neurological complications and two (6.7%) died.

Table 3. Comparison between the CSF and serum levels of TNF-α, IL-1β and IL-6 in the group with meningitis and the control group.

Cytokine	Group	Median levels (pg/mL) CSF	Serum	p-value
TNF-α	Meningitis	23.6	0.0	0.003
	Control	0.0	0.0	0.069
IL-1β	Meningitis	13.2	6.0	0.042
	Control	0.0	0.0	0.878
IL-6	Meningitis	135.0	12.4	< 0.001
	Control	5.1	7.0	0.095

RESULTS

The two groups were considered to be similar regarding age, gender, birth weight and gestational age (Table 1).In most of the neonates with meningitis (63.3%) the signs and symptoms were nonspecific: fever, lethargy, irritability, poor feeding, vomiting, abdominal distention, jaundice, tachypnea, poor skin perfusion, apnea, and tachycardia. Neurological signs and symptoms occurred in 36.7% of the neonates, these presented convulsions, bulging anterior fontanel, coma, tremors, hypertonia and cranial nerve signs. The mean values for CSF findings, Gram-stained smears, cultures, neurological complications and deaths are presented in Table 2. TNF-α was detected in the CSF in 63.3% of the neonates, IL-1β in 73.3% and IL-6 in 96.6%.

In the newborn of the control group mean values for CSF findings were: leukocytes= 5.7 cells/mm^3, protein=80.9 mg/dL and glucose=50.8 mg/dl. Gram-stained smears and culture of the CSF were negative in all of the newborn.The definitive diagnoses were sepsis (50.0%), dehydration (16.7%), infection of the urinary tract (8.3%), fever without source (8.3%), convulsive syndrome (8.3%), hyperekplexia (4.2%) and polycythemia (4.2%).There was one death (4.2%).

The difference between CSF cytokines levels in the group with meningitis and the control group are shown in Figures 1 to 3.The comparison between the CSF and serum levels of cytokines in the group with meningitis and in the control group are shown in Table 3.The comparison between the levels of cytokines in the CSF and the presence of neurological complications, and values of cells, protein, and glucose in the newborn with meningitis are shown in Table 4.

DISCUSSION

We demonstrated the presence of cytokines in the CSF of all the newborn with meningitis, a fact which was not observed in the control group. Furthermore, the levels of the three cytokines analyzed were significantly higher in the newborn with meningitis.The detection of cytokines in the CSF was possible regardless of gestational age, indicating that even preterm newborn responded to stimulation of the infection with increased production of cytokines in the CNS. The wide variation of the CSF levels, also reported by other authors[8], probably reflects the timing of the collection of the sample and magnitude of the inflammatory response to the infection.

One of the questions to be discussed is whether the TNF-α, IL-1β and IL-6 detected in the CSF of neonates with meningitis were produced by cells of the CNS or if they originated from the systemic compart-

Table 4. Comparison between the levels of cytokines in the CSF and the presence of neurological complications, and values of cells, protein, and glucose in 30 neonates with meningitis.

	CSF TNF-α(pg/mL)*	CSF IL-1β(pg/mL)*	CSF IL-6(pg/mL)*
Neurological complication			
Present (n=16)	33.5	30.4	344.3
Absent (n=14)	18.8	2.3	20.2
P-value**	0.510	0.467	0.061
Spearman's coefficient (P-value)***			
Cells	0.312 (0.093)	0.495 (0.005)	0.414 (0.023)
Protein	0.215 (0.253)	0.403 (0.027)	0.361(0.050)
Glucose	–0.281 (0.148)	–0.302 (0.118)	–0.255 (0.189)

*Values are expressed as median. **No correlation was found between the levels of cytokines in the CSF and neurological complications or death. ***There were a positive correlation between the number of cells in the CSF and the levels of IL-1β and IL-6, as well as between the protein dosage and levels of these cytokines.

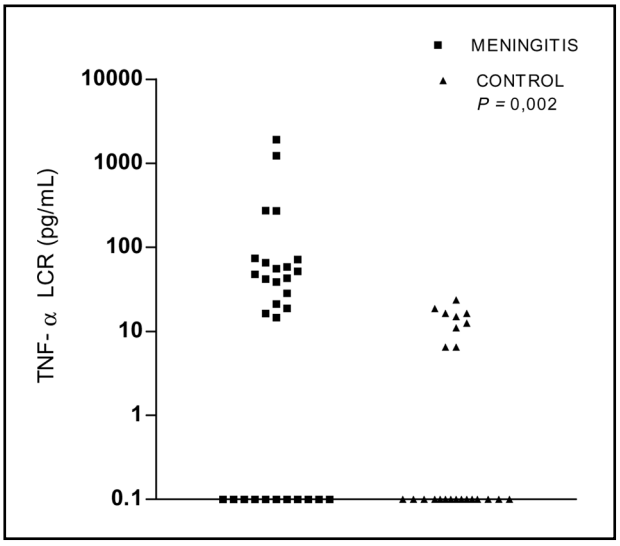

Fig 1. Levels of TNF-α in the CSF of 30 neonates with meningitis and 24 neonates of the control group. Neonates with meningitis=144.9 pg/ml±404.9(mean ± SD); 0.0-1920.7 pg/ml (minimum-maximum); 24.80 pg/ml (median). Control group= 5.3 pg/ml ±7.7(mean ± SD); 0.0-23.6 pg/ml (minimum-maximum);0.00 pg/ml (median); Mann-Whitney test: p = 0.002.

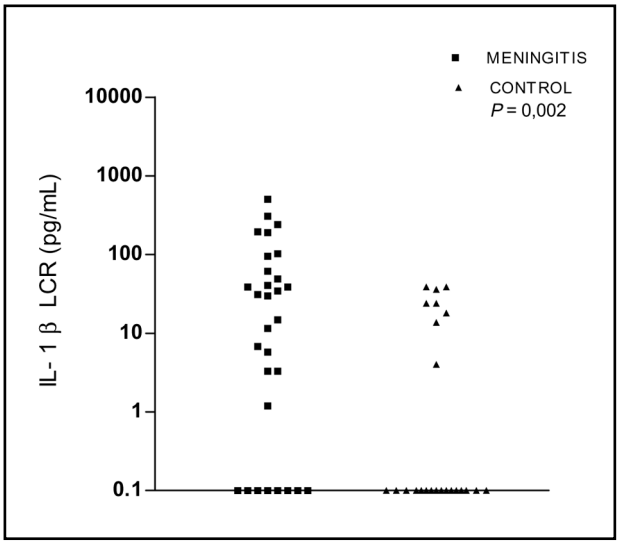

Fig 2. Levels of IL-1β in the CSF of 30 neonates with meningitis and 24 neonates of the control group. Neonates with meningitis =67.0 pg/ml ± 114.8(mean ± SD); 0.0-505.7 pg/ml (minimum-maximum); 22.4 pg/ml (median). Control group = 8.2 pg/ml ±13.8 (mean±SD); 0.0-38.9 pg/ml (minimum-maximum); 0.00 pg/ml (median); Mann-Whitney test: p = 0.002.

ment. Although the cytokines present in the blood circulation can reach the CSF through an impaired or even an intact blood-CSF barrier[9], it is known that the subarachnoid space and the circulation are separate compartments in respect to the production of these.High concentrations of TNF-α, IL-1β and IL-6 were detected in the CSF of rabbits after intracisternal inoculation of lipopolysaccharide (LPS) of *Haemophilus influenzae* or *Neisseria meningitidis*, yet no activity was detected in the serum[10]. In our patients the analysis of serum levels, obtained concomitantly with CSF levels, allowed us to establish the origin of the cytokines in the CSF. In neonates with meningitis the CSF levels of the three cytokines analyzed were significantly higher than the serum, a fact not observed in the control group. This behavior indicates the existence of local production of cytokines in the presence of meningitis, in response to the stimulus presented by the microorganism or their products. In older children, Ohga et al.[11] reported similar findings.

The production of TNF-α by the CNS seems to play a part of great importance in the pathogenesis of the initial lesion of the blood-CSF barrier. Although the mechanism of the action of TNF-α is still not fully clarified, experimental studies suggest that this cytokine is directly cytotoxic to the neurons. The cytotoxicity could also occur indirectly, through the release of secondary mediators, such as O_2 free radicals, that attack the double bonds of the polyunsatureted fatty acids of cellular membranes in the process of lipid peroxidation of the membrane. The brain of the newborn, in a phase of accelerated growth, contains high concentrations of polyunsaturated fatty acids, thus is particularly suscepti-

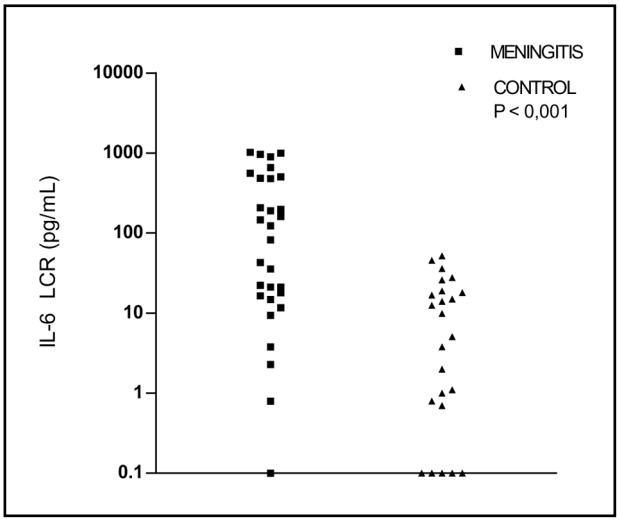

Fig 3. Levels of IL-6 in the CSF of 30 neonates with meningitis and 24 neonates of the control group. Neonates with meningitis =263.6 pg/ml ± 340.5 (mean ± SD); 0.0-1021.8 pg/ml (minimum-maximum); 103.0 pg/ml (median). Control group = 12.8 pg/ml ± 15.1 (mean ± SD); 0.0-51.7 pg/ml (minimum-maximum); 0.00 pg/ml (median); Mann-Whitney test: p = <0.001.

ble to the lesion induced by O_2 free radicals. In addition, these factors coincide also with the fact that the lowest levels of oxygen free radical scavengers and antioxidant enzymes are occurring in the neonatal period[12]. Experimental studies have demonstrated the sequential emergence of the bioactivity of TNF-α, IL-1β and IL-6 in CSF after injection of PLS or viable meningococci in the subarachnoid space[13]. Probably the increase of the production of TNF-α at the beginning of the inflammatory reaction[12,14] induces the secretion of IL-1β, both cytokines being responsible for the first steps of the inflammation cascade that leads to the destruction of tissue.

We observed that TNF-α was present in the CSF less frequently than IL-1β and IL-6. Similar findings have been reported by Mustafa et al.,[15] who demonstrated the presence of IL-1β in the CSF in 90% of 21 neonates with ventriculitis, but in the absence of TNF-α. This behavior can be attributed in part to the timing of the collection of CSF.It is known that the duration of the activity of TNF-α is short, such that it is impossible to detect this cytokine when the infectious process is in a more advanced phase. The presence of TNF-α in the CSF was registered 45 minutes after the intracisternal inoculation of lipo-oligosaccharide of *Haemophilus influenzae*, in rabbits, with a peak at two hours and persistence in the CSF for approximately five hours[10]. The increase of TNF-α in the CSF can be observed even before the increase of cell number, protein concentration and decrease of glucose levels[16].

IL-1β was detected in the CSF of 73.3% of newborn with meningitis. This finding is similar to that observed by other authors and suggests the role of this cytokine as mediator of meningeal inflammation[8,17]. López-Cortés et al.[17] considered this cytokine to be the best biochemical indicator of the disease in patients with neurosurgical pathologies, whose CSF IL-1β concentrations above 90 pg/ml were highly indicative of the disease. We observed that 23.4% of the neonates with meningitis and none of the control group presented such CSF levels. In the literature, only McCracken et al.[8] analyzed the levels of TNF-α and IL-1β in the CSF from a group of patients composed exclusively of neonates. In 42 newborn with meningitis due to enterobacteria, those authors observed detectable levels of IL-1β in the CSF in 95% of the cases at the time of the diagnosis in concentrations varying from 20 pg/ml to 2616 pg/ml.

The presence of IL-6 in 96.6% of the neonates with meningitis, and their CSF levels showing a highly significant difference in relation to the control group, indicate that CSF IL-6 detection is highly suggestive of meningeal inflammation. Similar results were obtained in studies of infants, older children and adults[6,18,19]. According to Dulkerian et al.[20], young infants respond to the bacterial invasion of the CNS with the release of cytokines at comparable levels to those observed in older children and adults. The results we have obtained demonstrate that neonates with meningitis are capable of producing TNF-α, IL-1β and IL-6 in a magnitude comparable to that observed in older age groups.

Bearing in mind that the increase in the CSF levels of cytokines can occur even before the onset of inflammatory alterations, its detection in the CSF can be of great value in the diagnosis of bacterial meningitis among patients with a normal or inconclusive CSF exam. Matsuzono et al.[19] consider that the CSF level of IL-6 is the only parameter capable of detecting the inflammatory process when the routine findings in the CSF are normal. Azuma et al.[21] highlighted that the measurement of IL-6 in the CSF might be particularly useful for the early diagnosis of meningitis in situations of immunological depression. Among our patients, four newborn (cases 17, 20, 22 and 27) with bacterial growth in the culture of CSF, presented a normal CSF exam or few alterations. In case 17, the CSF cells, protein and glucose were normal, but with growth of *Streptococcus viridans* in the culture and a detectable level of IL-6. In case 20, who died just a few hours after hospitalization, the CSF presented only protein 178 mg/dl, however an impressive increase was observed in TNF-α, IL-1β and IL-6. CSF culture, whose result was obtained after death of the patient, showed *Alcaligenes xilosoxidans*. Case 22 presented glucose 38 mg/dl, protein 121 mg/dl, *Klebsiella pneumoniae* in the culture of the CSF and detectable levels of IL-6. Case 27 presented protein 116 mg/dl, glucose 34 mg/dl, culture isolated *Streptococcus viridans* and detectable CSF levels of TNF-α and IL-1β.These results indicate that the detection of cytokines in the CSF is useful to confirm the diagnosis of meningitis, prior to obtaining a result from the cultures.

Although complications due to meningitis occurred in 53.4% of the neonates among our patients, the mortality was less than that reported by other authors[2]. These findings are in agreement with

the study of Harvey et al.[1] showing that mortality due to neonatal bacterial meningitis, in England, has decreased from 50%, in the 1970s, to less than 10%, in 1997.It is possible that the use of third-generation cephalosporin, in the treatment of the disease, could be related to the greater survival. In spite of the mortality in our study being relatively low, the frequency of serious complications was high, demonstrating the need to develop methods of early detection and evaluation of the clinical course of the disease. Low et al.[18] observed a correlation between the simultaneous presence of TNF-α, IL-1β and IL-6 in the CSF, with low values of glucose in five children with meningitis, and between the detection of TNF-α or IL-1β in the CSF and the duration of fever, convulsions, spasticity and death. McCracken et al.[8] verified a relationship between levels of IL-1β in CSF of over 200 pg/ml and the number of days in which endotoxin, antigen K_1 and bacteria persisted in 42 neonates with meningitis due to enterobacteria. We observed a positive correlation between the number of cells in the CSF and the levels of IL-1β and IL-6, as well as between the protein values and the levels of these cytokines, suggesting that these also are important in the evaluation of the magnitude of the inflammatory process in the central nervous system.

We conclude that the detection of TNF-α, IL-1β and IL-6 in the CSF is of great value in order to achieve a rapid and early diagnosis of neonatal meningitis, both for term and preterm newborn.Among the three cytokines analyzed, IL-6 can be considered the best indicator of meningeal inflammation.

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