TLR2 and TLR4 expression on CD14^{+} cells from preterm infants with risk factors for neonatal sepsis development

Key words: Neonatal sepsis, toll-like receptors, sepsis diagnosis.

Palabras clave: Sepsis neonatal, receptores tipo toll, diagnóstico de sepsis.

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Abstract

Introduction: Neonatal sepsis (NS) is a systemic infection exhibiting 25% lethality among infected children, and it poses a higher risk in preterm than in full-term neonates. Routine microbiological culture, hematological quantification, IL-6, IL-8, C-reactive protein and procalcitonin determinations are the most commonly used tests to diagnose sepsis. However, these methods have substantial limitations due to the associated variation, low degree of sensitivity and long period of time required for execution. These factors decrease the efficiency of an early diagnosis. Toll-like receptors are involved in native immunity recognition and the stimulation of antimicrobial mechanisms. Therefore, they may be altered early during the systemic infection and may be useful in the diagnosis of sepsis. **Methods:** In this work, we analyze the expression of TLR2 and TLR4 on CD14^{+} cells as early markers for sepsis in preterm neonates. **Results:** Our results show that the percentage of CD14^{int} cells and their TLR2 expression were significantly altered in preterm infants with diagnosed

Resumen

Introducción: La sepsis neonatal (SN) es una infección sistémica con un 25% de mortalidad entre los niños infectados, y es más riesgosa en neonatos prematuros que en recién nacidos a término. Los exámenes más comunes para diagnosticar sepsis son cultivo microbiológico de rutina, cuantificación de hemoglobina, IL-6, IL-8, proteína C reactiva y marcadores de procalcitonina. Sin embargo, estos métodos tienen limitaciones sustanciales debido a la variación, el bajo grado de sensibilidad y el largo periodo requerido para su ejecución e impiden el eficaz diagnóstico temprano. Los receptores tipo Toll están involucrados con la respuesta de inmunidad natural y la estimulación de mecanismos antimicrobianos. Por lo tanto, pueden estar alterados en las etapas iniciales durante la infección sistémica y pueden ser útiles en el diagnóstico de sepsis. **Métodos:** En este trabajo analizamos la expresión de receptores TLR2 y TLR4 en células CD14^{+} como marcadores tempranos de sepsis en neonatos prematuros. **Resultados:** Nuestros resultados presentan que el porcentaje de células

infections and were not altered in the systemic inflammatory response syndrome (SIRS) patients. **Discussion:** These results suggest that TLR2 screening could be a helpful diagnostic test for bacterial infection in preterm infants.

CD14^{int} y sus expresiones de TLR2 estaban significativamente alterados en niños prematuros con diagnóstico de infección y no estaban alterados en los pacientes con síndrome de respuesta inflamatoria sistémica (SRIS). **Discusión:** Estos resultados sugieren que la expresión de TLR2 puede ser un examen diagnóstico de gran utilidad para infecciones bacterianas en niños prematuros.

Introduction

Neonatal sepsis is defined as a clinical syndrome of bacteremia with systemic signs and symptoms of infection in the first months of life. It is one of the main causes of neonatal death in developing countries[1,2] and mainly in very low birth weight (VLBW) infants.[3,4] *Klebsiella* sp., *Escherichia coli*, and *Staphylococcus aureus* are the most common causes of neonatal sepsis.[1,5] Neonatal sepsis is classified into two subtypes depending upon whether the onset of symptoms occurs before 72 hours of life (early-onset sepsis) or later (late-onset sepsis). Organisms prevalent in the maternal genital tract or in the delivery area cause early-onset infections. In addition, there are several associated factors for early-onset sepsis: low birth weight, prolonged rupture of the membrane, foul- smelling liquor, multiple *per vaginum* examinations, maternal fever, difficult or prolonged labor and the aspiration of meconium.[6]

Because 24-30% of VLBW infants experience septicemia,[7,8] it is extremely important to identify the infection within the first 72 hours of life. The inability to adequately exclude a diagnosis of sepsis results in the prolonged and unnecessary use of antibiotics, which increase hospitalization costs.[9] Routine microbiological and hematological analyses are currently used to confirm infections. However, these techniques usually have some limitations such as variability, difficulty in obtaining the sample and low sensitivity.[10,11]

For this reason, several biochemical markers have been described as predictors of early onset sepsis. Inflammatory molecules such as cytokines and chemokines mediate the host response to infection in many different ways. During bacterial infections, there is an activation of monocytes, macrophages, lymphocytes and endothelial cells, which secrete cytokines such as IL-1, IL-6, IL-8, TNF-α and IFN-γ.[12] Among these molecules, IL-6 is the most extensively studied and has been described as a diagnostic marker for neonatal sepsis.[13-18] Exposure to bacterial molecules results in a rapid and substantial increase in the blood concentration of IL-6.[19-21] However, because these molecules are also related to other inflammatory responses, such as delivery complications, stress, etc., other markers are needed to discriminate between infection-associated inflammation and other types of inflammation.

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs), which act as key components of the innate immunity response by recognizing pathogen- associated molecular patterns (PAMPs). Among the 10 members of the family, TLR2 and TLR4 have been associated with bacterial infections. TLR2 responds to bacterial lipoproteins from Gram-positive bacteria,[22-24] and TLR4 is described as the major recognition receptor for LPS,[25] which is a component of Gram-negative organisms. Because they participate as the first line of defense, these receptors should be upregulated in patients with bacterial infections. Several groups have analyzed this hypothesis by measuring the expression levels of TLR2 and TLR4 in adults and neonates. In a study with adult patients with Gram-positive, Gram-negative or polymicrobial sepsis, Armstrong *et al* concluded that TLR2 mRNA and protein were upregulated in monocytes from these patients. In contrast, there were no significant differences in either TLR4 mRNA or protein levels

in these patients when compared with control subjects.[26] In another study, Levy and collaborators isolated monocytes from blood samples of adults, term and preterm newborns and analyzed the expression of TLR4 before and after LPS stimulation. In basal conditions, there was a significant difference in the expression levels of TLR4 in each group of neonates compared with adults. However, in all the neonate groups, the expression of TLR4 was upregulated after *ex vivo* stimulation with LPS.[27] Another study compared TLR2 and TLR4 expression in phagocytic cells (monocytes and granulocytes) derived from blood samples of both neonates and adults. This study reported a mild deficiency of TLR2 expression in newborns and differential expression of this receptor in the course of sepsis. Finally, there were no differences in TLR4 expression among these groups.[28] Because there is contradictory data regarding the clinical value of TLR expression in the diagnosis of sepsis, we investigated the level of expression in neonates with bacterial infection.

Methods

Patient selection and clinical diagnosis. Infants with risk factors for neonatal sepsis development born at the Instituto Nacional de Perinatología, SSA, México (55 preterm patients) were included in this study. Systemic inflammatory response syndrome (SIRS) was diagnosed if newborns (0-7 days) exhibited two of the following parameters: 1) temperature (> 38.5 °C or < 36 °C); 2) tachycardia > 180 heartbeats/min (> 2 standard deviation according to age) or bradycardia < 100 heartbeats/min; 3) tachypnea (> 50 breaths/minute); and 4) alterations in leukocyte numbers (> 21 x 10^3/mm or < 5 x 10^3/mm). Sepsis was defined as the presence of pathogens as determined by microbiological culture.[29] The following pathogens were found in this study: *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus agalactiae*. Blood samples were taken at birth or when symptoms appeared. No deaths occurred, and all the patients were successfully treated with conventional antibiotics (ampicillin/amikacin). Informed consent was obtained from each set of parents, and the study was approved by the institutional ethics committee.

TLR2 and TLR4 determination. TLR2 and TLR4 were detected by flow cytometry according to the following protocol: 0.5 mL of blood was taken at birth or when symptoms appeared, and 25 μL of blood was incubated with the following antibodies: FITC-coupled anti-human TLR2 or anti-human TLR4 and PE-coupled anti-human CD14 (Beckman Coulter, Fullerton, CA, USA). Red blood cells were lysed, and the cells were fixed with FACS Lysing Solution (Beckman Coulter, Fullerton, CA, USA). All the samples were measured using an Epics Altra Flow Cytometer (Beckman Coulter, Fullerton, CA, USA), and the data were analyzed using FlowJo software. The relative mean fluorescence intensity (MFI) was determined by dividing the fluorescence values of samples stained for CD14 / TLR2 or CD14 / TLR4 by the values for CD14-stained samples.

Results

Patient classification. The patients were classified according to the clinical diagnosis into healthy/control, SIRS and culture-proven infection (CPI) groups. The data related to age, gender, gestational age, birth weight and Apgar score are presented in *table I.* The children were continuously evaluated by microbial culture, blood biometry, and serum CRP tests. Premature newborns (46/55) received the recommended antibiotic therapy management, which consisted of 3 days of ampicillin/amikacin administered at the standard doses. The treatment was extended to 7 days if the infant presented symptoms of SIRS and was extended to 10 days if the infection was confirmed (6/6). The infants (9/55) that did not receive the antibiotic therapy displayed favorable evolution. The infants (48/55) that received intravenous gamma globulin evolved

Table I. Demographic and clinical characteristics of the study groups.

	Control (n = 38)	SIRS (n = 11)	CPI (n = 6)	p
Sex (F/M)	19/19	5/6	3/3	0.9[b]
Birth weight (g)	1579 ± 510	1444 ± 357	1739 ± 946	0.56[c]
Gestational age (weeks)	33 ± 2	33 ± 2	33 ± 3	0.96[c]
Apgar score (1 min)	8[a] (4-9)	5[a] (1-8)	8[a] (8-9)	0.001[d]
Apgar score (5 min)	9[a] (7-9)	8[a] (6-9)	9[a] (9-9)	0.02[d]
Postnatal age	5.47 ± 10	3.09 ± 6	22 ± 14	0.001[c]
Preeclampsia	8	2	3	0.27[b]
Chorioamnionitis	4	1	0	0.9[b]
Antenatal steroids	17	3	2	0.7[b]
PRM	21	6	0	0.04[b]
Caesarean section	34	8	5	0.4[b]
Antibiotic treatment	32	8	6	0.6[b]
Gamma globulin	9	3	0	0.1[b]

SIRS = Systemic immune response syndrome. CPI = Culture-proven infection
[a] Median. [b] Chi-squared. [c] ANOVA. [d] Kruskal-Wallis H

as well as those that did not receive the treatment. Several children that were classified into the control/healthy group (5/38) and the SIRS group (8/11) presented asphyxia or obstetric trauma during delivery. However, because there were no differences in the percentage of CD14 cells or TLR expression when the children with asphyxia were compared with other children of the same age, these patients were classified as they were originally recorded. None of the analyzed children was diagnosed with early-onset sepsis.

Expression of CD14^{int} versus CD14^{hi} cells. To determine the role of TLR2 and TLR4 as early markers for neonatal sepsis, we analyzed the expression of these receptors on CD4^{+} cells. As described before, two subpopulations of granulocytes were found when they were stratified according to CD14 expression: CD14^{int} and CD14^{hi}.[28] The preterm CPI group exhibited a significant reduction in the percentage CD14^{int} cells compared with the control and SIRS groups (ANOVA, $p < 0.05$). However, there were no changes in the percentage of CD14^{hi} cells in any group *(figure 1)*.

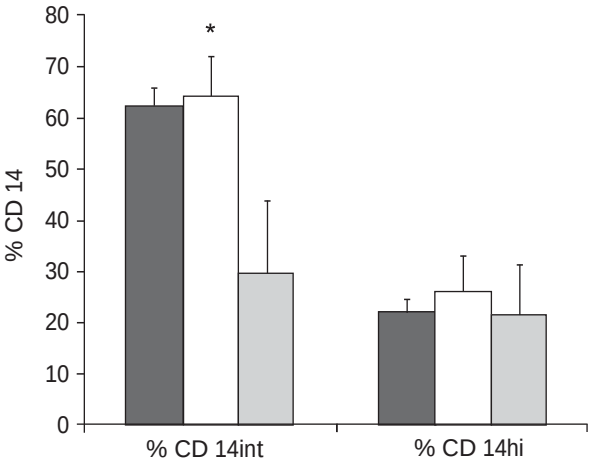

Figure 1. Percentage of CD14int and CD14hi cells in preterm and full-term patients. The percentages of CD14-positive cells, classified as CD14^{int} and CD14^{hi}, in preterm healthy (black bars), SIRS (white bars) or culture-proven infection (CPI) (gray bars). Asterisks represent a p value < 0.05 according to the ANOVA test.

TLR2 and TLR4 expression in term and preterm neonates. We were aware that steroids, particularly glucocorticoids, modulate immune function by exerting anti-inflammatory and immu-

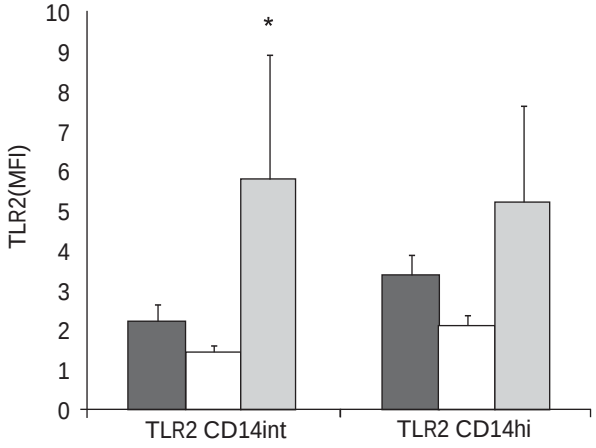

Figure 2. TLR2 expression in CD14^{int} and CD14^{hi} cells from preterm and full-term patients. TLR2 expression was determined by flow cytometry in CD14^{int} and CD14^{hi} cells in blood samples of preterm healthy (black bars), SIRS (white bars) or culture-proven infection (CPI) (gray bars). Asterisk represents a p value < 0.05 according to the ANOVA test.

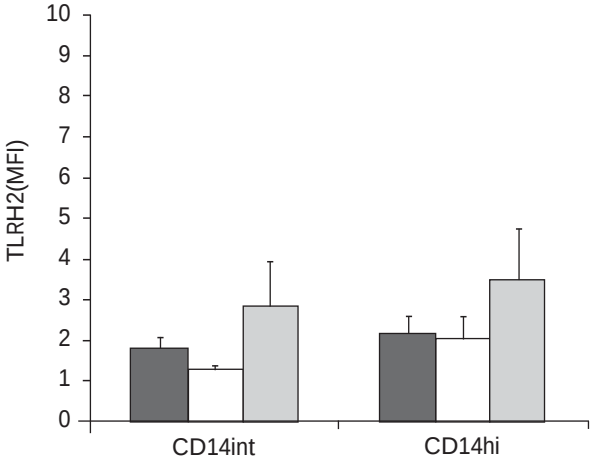

Figure 3. TLR4 expression in CD14^{int} and CD14^{hi} cells from preterm and full-term patients. TLR4 expression was determined by flow cytometry in CD14^{int} and CD14^{hi} cells in blood samples of preterm healthy (black bars), SIRS (white bars) or culture-proven infection (CPI) (gray bars).

nosuppressant effects.[30,31] Because the maturation treatment used for preterm patients comprises 1-3 doses of dexamethasone, we compared the percentage of CD14 cells and the TLR expression in healthy children that received hormonal treatment with healthy children that did not receive hormonal treatment. We did not find differences in any of the parameters measured (not shown).

To measure the normal TLR2 and TLR4 levels, we first analyzed the expression of these molecules on CD14^{int} and CD14^{hi} subpopulations of healthy patients and compared these values with the values obtained for the SIRS and CPI groups. Interestingly, the CPI patients exhibited a marked increase in the expression of TLR2 on the CD14^{int} subpopulation *(figure 2)*. However, when the TLR2 expression was analyzed in the CD14^{hi} cells, the pattern was similar to the pattern observed for the CD14^{int}cells *(figure 2).* The expression of TLR4, in contrast, showed no significant differences in either the CD14^{int} or the CD14^{hi} population *(figure 3).* The enhanced TLR2 expression observed on CD14^{int} cells of the CPI group supports the hypothesis that this receptor is only expressed in the presence of a bacterial infection. Therefore, it could be a useful marker to discriminate real infections from inflammatory responses caused by other factors such as delivery complications.

Discussion

The diagnosis of neonatal sepsis remains controversial because the imprecise laboratory methods lack sensitivity and specificity. This disease is often misdiagnosed, and infants are often treated with antibiotics even when sepsis is not confirmed.[9] One of the main problems with sample recovery is the relatively small volume of blood that can be obtained from these patients; in preterm newborns, this can avoid a reliable diagnosis. For this reason, it is important to identify a method that could discriminate infections from inflammatory responses caused by other causes such as delivery complications. Toll-like receptors are components of the innate immune response, are involved in the recognition of pathogen-associated molecular patterns (PAMPs), and activate most of the anti-

microbial mechanisms that are usually analyzed as diagnosis markers (CRP, procalcitonin, cytokines, etc.). Among the 10 TLRs described, only TLR2 and TLR4 are associated with bacterial infections.[22-28] For this reason, the over expression of these molecules on monocytes could be a reliable marker of bacterial infection in preterm newborns. In this work, we attempt to identify differences in TLR expression in patients with confirmed infections and patients that only exhibited an inflammatory response induced by other causes (asphyxia, obstetric trauma, hyaline membrane disease, etc.).

We analyzed infants that were born from mothers with risk factors for the development of neonatal sepsis. Most of the patients analyzed (37/44) did not develop early sepsis, probably because 3-day antibiotic treatment was indicated early when risk factors were described. Only 7 of the analyzed children continued the treatment (up to 10 days) when SIRS or infection was confirmed (n = 17). The elimination of antibiotics after 72 hours in the absence of SIRS may protect the child from the undesirable collateral effects of antibiotic therapy and may decrease the cost of treatment. In our study, we found the TLR expression values to be low. This may be due to the opportune administration of therapy, which was sufficient to control microorganism proliferation without the activation of an immune response. Alternatively, the effect may be due to the under development of the immune system in a premature infant.

We expected that patients with a confirmed infection might display an increased proportion of monocytes ($CD14^{hi}$) or an increase in the expression levels of TLR2 and TLR4 due to the effects of these proteins on proliferation and activation. However, we found no difference in the percentage of this subpopulation when comparing the control, SIRS and CPI groups. In contrast, we found a decrease in the percentage of $CD14^{int}$ cells during bacterial infection for children with CPI (50% reduction when compared with the control group). When we analyzed the expression of the TLRs, the $CD14^{int}$ subpopulation increased the expression of TLR2 (a 3-fold increase), whereas the $CD14^{hi}$ cells did not exhibit significant differences in the percentage or expression of both TLR2 and TLR4 when compared with the control patients. In addition, the patients that were diagnosed with a systemic inflammatory response but lacked a proven infection exhibited no differences in the TLR expression when compared with control subjects.

TLR2 mRNA and TLR4 mRNA were previously associated with sepsis in adult patients.[26] Previous authors have found that TLR2 was significantly upregulated in sepsis patients. In addition, an increase in TLR4 protein expression was previously observed in the $CD14^{+}$ cells of preterm and full-term neonates in response to lipopolysaccharide (LPS) in culture.[27] Skinner and collaborators analyzed the expression of these two receptors in monocytes derived from whole blood samples that were exposed to staphylococcal enterotoxin B, LPS or peptidoglycan. These authors described two subpopulations of monocytes classified by the expression of CD14 and CD16 ($CD14^{+}$ $CD16^{-}$ and $CD14^{dim}$ $CD16^{+}$) and found that exposure to bacterially derived products increased the percentage of the $CD14^{dim}$ $CD16^{+}$ subpopulation and increased the expression of TLR2 in response to LPS, which corresponded with an increase in TNF-α.[32] In contrast, our results described a reduction rather than an increase in this subpopulation. This could be due to the inclusion of other subpopulations in the $CD14^{int}$ classification and further analysis; including the use of other markers or the use of adult blood samples.

These factors and the enhanced TLR2 expression observed on the $CD14^{int}$ cells in the CPI group suggest that TLR2 overexpression is restricted to bacterially derived inflammatory responses and suggests that it could be a useful marker to discriminate real infections from inflammatory responses caused by other factors such as delivery complications.

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