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Prospective evaluation of cytokine in saliva of preterm and fullterm neonates

Maria Lucia Talarico Sesso[a], Mariana Castro Loureiro Borges[a], Virginia Paes Leme Ferriani[a], Vinicius Rangel Geraldo-Martins[b], Denise Bertulucci Rocha Rodrigues[b], Ruchele Dias Nogueira[b,*]

[a] Department of Pediatrics, Medical School of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil
[b] Uberaba University, Uberaba, Minas Gerais, Brazil

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ABSTRACT

Little is known about the ontogeny of the cytokines in saliva of newborn. Previous studies showed that levels of immunoglobulin A (IgA) in saliva could be influenced by prematurity. So, the aim of this study was to analyze the levels of interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12 (IL-12), and interferon gamma (IFN-γ) in sample saliva of fullterm (FT) and preterm (PT) neonates at birth (T0) and after 3 months of age (T3). Saliva from 50 infants (25 FT and 25 PT) were collected at T0 and T3 and analyzed by Luminex Corporation (Austin, Texas, United States) multiplex assay. Clinical characteristics and social–economic data were assessed through questionnaires. All cytokines could be detected at birth in levels higher than found in T3. The mean levels and frequency of detection of cytokines were significantly higher in PT than FT at T0 ($P < 0.05$). There were a positive association between IL-10 and infection ($P < 0.05$) and IL-6 and stress ($P < 0.005$). Salivary cytokines were detected within the first hours after birth and their levels decreased after 3 months. The cytokine levels were different between PT and FT children and appear to be influenced by stress situation and/or antigenic microbial challenge. The results confirm the necessity for further studies about the mucosal immune system by using of saliva as a source of diagnostic by identification of biomarkers of the status of the immune.

Introduction

Since mucosal surfaces represent the interface between the host and the environment and are the most common portal of pathogen entry, early development of functional mucosal immune defense is essential for survival. Several factors may influence the development of an effective mucosal immune response, including breastfeeding, gestational age, contact to antigens, genetic factors, and stress (Maruyama et al., 2009).

Cytokines are peptides secreted by cells of immune response to regulate the activation and effector phases of inflammatory response (Clapp, 2006). Proinflammatory cytokines, such as: IL-6, IL-8, IL-12, IFN-γ, stimulates the inflammation (Meirovitz et al., 2010). Also, there are the anti-inflammatory cytokines: IL-10 and IL-13, which attenuate the inflammation by restricting the production of inflammatory cytokines and increase expression of soluble receptors (Sarrouh et al., 2007). These peptides can be released in response to microorganisms (Rang et al., 2001) and are important in controlling the response to antigenic challenges. Several cytokines play an important role in the protection of oral mucosa together with the adaptive immune response, i.e, IL-12 and IFN-γ can induce activation of secretory IgA response against different infectious agents (Bradney et al., 2002).

Saliva represents an important biofluid that have several functions in the first line of defense because it presents satisfactory levels of immunoglobulin A, cytokines, and antimicrobial peptides (Nurkka et al., 2001). Salivary levels of selected cytokines were associated with both oral diseases, as well as systemic diseases in adult's saliva in Crohn's disease (Szczeklik et al., 2012) and lichen planus (Zhang et al., 2008). In patients with periodontal disease IL-6 concentrations are elevated in samples of saliva (Costa et al., 2010; Johnson and Serio 2005; Teles et al., 2009). In children, increased levels of IL-6 were found in subjects submitted to some kind of

* Corresponding author at: Faculdade de Medicina de Ribeirao Preto, Departamento de Puericultura e Pediatria, Av. dos Bandeirantes, 3900, CEP 14049-900 Ribeirao Preto, SP, Brazil. Tel.: +55 16 3602 2902; fax: +55 16 3602 2297.
E-mail addresses: malutase@yahoo.com.br (M.L.T. Sesso), mariana.ped@hotmail.com (M.C.L. Borges), vplferri@fmrp.usp.br (V.P.L. Ferriani), vinigmartins@hotmail.com (V.R. Geraldo-Martins), denise.rodrigues@uniube.br (D.B.R. Rodrigues), ruchele_nogueira@yahoo.com.br (R.D. Nogueira).

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stress, such as sleep deprivation (Redwine et al., 2000) and mental problems (Keller et al., 2010).

Newborns are known to have a higher frequency of microbial infections than older children and adults, due to the immaturity of the immune system (Sadeghi et al., 2007), especially babies born prematurely who have five times higher susceptibility to bacterial infections (Clapp et al., 1989). Our previous study showed that salivary IgA could be detected in newborns and levels were higher in fullterm than in preterm children (Nogueira et al., 2012) in the first hours of life, suggesting that the prematurity could influence the ontogeny of the immune response (Nogueira et al., 2012).

Studies focused on the analysis of cytokines in samples of serum, plasma, amniotic liquid, and cervicovaginal fluid (Dammann and Leviton, 1997; Mehr and Doyle, 2000; Romagnoli et al., 2001; Holst et al., 2005; Jacobsson et al., 2005; Lyon et al., 2010; Wei et al., 2010), showing a positive correlation between the increase of proinflammatory cytokines, particularly IL-6 with prenatal intrauterine infection (Dammann and Leviton, 1997; Holst et al., 2005), preterm birth (Holst et al., 2005; Jacobsson et al., 2005; Lyon et al., 2010; Wei et al., 2010), and neonatal brain damage (Dammann and Leviton, 1997). Also, increased levels of IL-10 (Romagnoli et al., 2001; Cancelier et al., 2009) and IL-6 (Mehr and Doyle 2000; Cancelier et al., 2009) have been considered as prognostic indicators of neonatal sepsis in preterm infants.

So, there are few studies that elucidate the ontogeny of mucosal immune system in recent life, especially about cytokines. In this study, we characterized the levels of proinflammatory (IL-6, IL-12, and IFN-γ) and anti-inflammatory (IL-10) cytokines in saliva samples of fullterm (FT) and preterm (PT) children at birth and after 3 months of life.

Material and Methods

Patients and study design

A total of 123 (70 FT and 53 PT) children born in the Clinical Hospital of the Ribeirao Preto Medical School, in Brazil were enrolled in this study. Fifty children (25 FT and 25 PT) that had enough amounts of saliva were performed in Luminex® procedure. Those samples were collected in two visits: T0 and T3. Only healthy newborns with less than 10 h of life were included in this study. Children with congenital malformations, perinatal hypoxia, intracranial hemorrhage, with length or weight incompatible with gestational ages, or under antibiotic therapy were excluded.

The Ethical Committee of the Medical School of Ribeirao Preto (process: 2963/2007) approved this study and mothers gave written consent for participation. Gestational age was estimated from the reported date of last menstruation period and also by somatic evaluation.

Data collection

Information on gestational background, health of mother, and of newborn was obtained by interview with mother and clinical data from hospital. Analysis of interviews with mothers and of clinical evaluation allowed collecting several data about maternal and newborn health during the gestation and the delivery. We obtained information about presence of infection (i.e., chorioamnionitis and urinary infection), systemic and oral disease, and use of antibiotics or corticoids from mother during the pregnancy. At birth were evaluated the time of rupture of amniotic membrane, type of delivery, development hypoglycemia, apgar score, weight and height of newborn. After 3 months, other informations were obtained from mother about the baby's health such as, infection, respiratory disease, breastfeeding, and use of pacifier. Those data collected allowed knowing if the newborn was submitted to some type of stress (such as hypoglycemia, time of rupture of membrane higher than 18 h) and/or of contact with bacterial infection during the intrauterine life or after birth. So, those data were associated with levels of interleukin found in the samples of saliva.

Collection of samples

Samples of whole unstimulated saliva were collected using sterile polypropylene transfer pipettes. Collections were performed in all children at approximately 4–10 h after birth in order to standardize the collection and the oral microbial exposure, and at least 3 h after breastfeeding to avoid contamination with non-salivary components. Solution of 250 mM EDTA, pH 5.2 (Sigma, St Louis, MO, USA) was added to each sample prior to transport on ice to the laboratory where they were stored at −80 °C until analysis.

Quantification of cytokines using Luminex®

Cytokine levels were determined using a multiplexed bead immunoassay as described by Teles et al (2009). Saliva were diluted 1:16 in PBS-BN (PBS [Sigma], 1% bovine serum albumin [Sigma], 0.05% sodium azide [pH 7.4]), then filtered on 0.22 μM centrifuge tube filters (Spin-X, CoStar) at 10,000 RPM for 10 min. Four cytokines: IL-6, IL-10, IL-12, and IFN-γ were measured using the human high-sensitivity cytokine of four-plex antibody bead kit (Millipore Corp., Bedford, MA, USA). Briefly, the beads coated with monoclonal antibodies against the 4 different cytokines were added to the wells. Samples and standards were pipetted into the wells and incubated for 2 h with the beads. The wells were washed and biotinylated secondary antibodies were added. After incubation the wells were washed followed by an incubation of 30 min with streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin-RPE). After washing to remove the unbound streptavidin-RPE, the beads (minimum of 100 per analysis) were analyzed in the Luminex 100™ instrument (MiraiBio, Alameda, CA). Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification (ULQ) of the standard curves were assigned the highest value of the curve.

Statistical analysis

Differences in levels of salivary cytokines and clinical data related to mother and infant health between groups (PT and FT) and visits (T0 and T3) were examined using the ANOVA test. chi-square test was used to analyze differences in the frequency of detection of each cytokine between groups and data obtained from questionnaires. Pearson correlation was used to assess correlation between levels of cytokines and data on mother and infant health. A P-value of <0.05 was considered statistically significant.

Results

Population data

Fullterm and PT children were born at mean gestational ages of 39.1 (SD: 1.22, range: 37–41) and 34.3 (SD: 2.19, range: 30–36.5) weeks, respectively. All children were delivered by cesarean section.

Levels of interleukins: IL-6, IL-10, IL-12, and IFN-γ

The distribution of cytokine levels in saliva samples in PT and FT infants at birth (T0) and at 3 months (T3) are shown in Fig. 1. Comparison of cytokine levels and frequency of positive detection