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Analysis of the cell population in 8-month-old mature human milk: Modulation of the presence of Vγ2Vδ2 T cells: A case study

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Abstract

Background: Most of the studies aimed at analyzing the presence of immune cell populations in breast milk focusing mainly on colostrum and transitional milk, and a few on mature milk, few studies analyze the presence of immune cells during supplemental feeding of infants after 6 months. The purpose of this work was to analyze the presence of some populations of immune cells, including γδ T cells, in 8-month-old mature breast milk.

Patient: Apparently healthy 19-year-old woman, who does not report pregnancy complications and states that she has no history of any chronic or degenerative disease. Within the first 12 days of sampling, the health status of the mother and the baby is apparently healthy. On day 13 the infant presented respiratory tract infection and between days 22-40 the mother reports a state of asthenia.

Materials and methods: The subpopulations of CD45, CD3, CD4, CD8 and T γδ cells were analyzed in 8-month-old mature breast milk, 10 mL of milk was collected for 60 days after ethical approval and informed consent. Flow cytometry was used for the analysis of the subpopulations.

Discussion and conclusions: the data revealed the importance of dyad factors on the regulation of unconventional T cells present in breast milk, such as infections and depression, being able to observe how the subpopulation of γδ T cells is modified in the face of an active infection of the infant and in a state of asthenia of the mother.

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Introduction

When a child is born, it has a complete immune system, but immature, it lacks immune memory, however, it is capable of responding to different antigenic stimuli, since, under normal conditions, the fetus is not exposed to antigens, so its immunological memory acquires it when it comes into contact with the different antigens [1]. In this sense, breast milk, in addition to providing bioactive molecules and microbiota, provides immune cells that are responsible for programming the newborn's immune response, optimizing the maturation of the neonatal and infant immune systems, inducing immune tolerance, and tissue repair [2]. Lymphoid cells $CD45^+$, $CD16^+$, NK cells, basophils, myeloid precursors, B cell precursors, B cells, neutrophils, eosinophils, immature granulocytes, $CD4^+$ and $CD8^+$ T cells and stem cells have been identified in breast milk [3]. In addition, temporary changes have been reported in the composition of breast milk, observing large differences in colostrum, transitional milk and mature milk [4]. Since the 1990s, the presence of γδ T cells has been identified in human milk [5]. In humans, there are two subpopulations identified by their Vδ chain. Vδ1 T cells are predominant in the thymus and peripheral tissues and recognize stress-related antigens, mostly uncharacterized. Vδ2 T cells make up most of the blood γδ T cells. They are generally associated with the Vγ9 chain in adults and mainly recognize phosphorylated non-peptide molecules [6,7]. Both subpopulations of human γδ T cells possess a cytotoxic potential mediated by the release of soluble mediators such as perforin and granzyme, they can also produce granulysin and induce apoptosis by the CD95L pathway, and they can also exert antibody-dependent cellular cytotoxicity (ADCC) [8]. However, its properties and function in the breast, during lactation, and for the breastfed infant are still far from fully understood, and the maternal transmission of T cells to the newborn is a subject of intense debate. To date, most of the studies aimed at analyzing the presence of immune cell populations in breast milk focusing mainly on colostrum and transitional milk, and a few on mature milk [9], few are the studies that analyze the presence of immune cells during the supplementary feeding of infants after 6 months. The purpose of this work was to analyze the presence of some immune cell populations including γδ T cells in 8-month-old mature human milk.

Case report

Apparently healthy 19-year-old woman, 62 kilos, 1.67 m tall, who reported no complications in pregnancy and manifests not having a history of any chronic or degenerative disease. Within the first 12 days of sampling, the health status of the mother and the infant is apparently healthy. On day 13, the infant presented respiratory tract infection, fever 38.1°C, the pediatrician reported a viral infection, only prescribed paracetamol. The mother did not present contagion, the mother expresses 20 to 25 milliliters of milk in half an hour. Between days 22-40 the mother reports a state of asthenia, manifests having personal problems. Milk production decreases, she extracts 11 milliliters in half an hour.

Materials and methods

Obtaining the samples

19 samples of mature breast milk (10 mL) were collected in an interval of 60 days prior to obtaining informed consent, starting the sampling at 8 months of lactation and ending at 9.5 months of lactation. The samples were collected by manual extraction after hygiene and asepsis from the mother's breast. All samples were drawn between 6 and 7 AM. The samples were placed in sterile tubes and stored at -20°C until use.

Obtaining cells from mature breast milk

The milk samples were thawed to avoid cell breakdown by the crystals formed during storage. Subsequently, the samples were centrifuged at 327 X g for 15 min at 4°C, the fat and the supernatant were discarded. The cell pellet was washed with phosphate saline buffer (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4, pH 7.4) by repetitive pipetting and the cells were centrifuged at 327 X g for 15 min at 4°C, at least 3 washes were performed, or until the supernatant was clear.

Assessment of cell viability

Cell suspensions obtained from milk human mature were centrifuged at 1200 X g at room temperature for 10 min. The supernatants were thoroughly removed by aspiration and the tubes tapped vigorously to disperse the pellets. A sterile-filtered solution of 50 µg/mL propidium iodide (PI) in 100 mM Tris, pH 7.0 containing 5 mM $MgCl_2$ was added slowly. The tubes were tapped vigorously during the addition to help suspend the pellets. Vortexing was avoided. One milliliter of the PI solution was added for every 10^6 cells. After staining, the cell population was analyzed by flow cytometry.

Analysis of cell populations by flow cytometry

The phenotype of the cells was evaluated by flow cytometry. Compensation beads and blood cells were used for antibody titration by individual stains to determine compensation settings, A dispersion threshold level (SSC) was established at 2800 and (FSC) at 1950 units to eliminate debris. Briefly, 1 X 10^6 cells were independently placed in 1.5 mL tubes and blocked with a 2% solution of SFB in PBS, and incubated at room temperature for 30 min. Cells were centrifuged at 327 × g for 10 min. Cells were washed twice with 500 µL of PBS-10% SFB and centrifuged at 327 X g for 10 min. For immunophenotyping using fluorochrome-conjugated monoclonal antibodies PE anti-human CD45 antibody (BioLegend CNS Inc. CAT 368510), FITC anti-human CD3 antibody (BioLegend CNS Inc. CAT 300306), PE anti-human CD4 antibody (BioLegend CNS Inc. CAT 357404), FITC anti-human CD8 antibody (BioLegend CNS Inc. CAT 344704) and FITC TCR anti-human TCRγδ antibody (BioLegend CNS Inc. CAT 331208) were added to a final concentration of 1 µg/100 µL in staining buffer (Invitrogen ™ eBioscience Flow Cytometry Staining Buffer). The cells were incubated for 40 min at 4°C, at the end of the incubation the samples were brought to a volume of 1 mL with 1X PBS, washed twice, the cells were centrifuged at 327 X g for 10 min, finally they were resuspended in 1 mL of 1 X PBS. cells were read according to their fluorochrome on a flow cytometer (Attune Acoustic Focusing Cytometer, Applied Biosystems). A minimum of 10,000 events was collected (referring to the number of cells in the sample passing the cytometer's laser beam for counting and analysis). Recorded data was post-hoc compensated and analyzed with FlowJo software. Statistical tests were performed on Prism for Mac (version 9.1.2, GraphPad, La Jolla CA, USA).

Statistical analysis

The values obtained from all the experiments are expressed as means and standard deviation (mean ± SD). Cell populations were reported according to the median and their interquartile

range (IQR). The differences between the proportions of cells (CD8 + and T gd) concerning the days were analyzed using the paired Student's t-test (GraphPad Prism, Version 9.1.2, La Jolla CA, USA). Statistical significance values were established at p <0.05.

Ethical approval

This study was approved by the ethics committee of the Faculty of Chemical Sciences of the Veracruzana University, and the participant provided her written informed consent.

Results

The presence of $CD45^+$ cells was monitored, observing a median of 54.2% (IQR [39.4-69.0]), from these cells, different subpopulations of T cells were identified and a median of 30.3% (IQR [24.7-35.9]) of $CD3^+$ T cells was found, 19.2% (IQR [15.3-23.1]) of $CD4^+$ T cells, 10.5% (IQR [5.1-15.9]) of $CD8^+$ T cells and 2.9% (IQR [0.9-4.9]) of γδ T cells (Figure 1A). No differences were observed in the percentage of the subpopulations analyzed during the days of my sampling except for what was observed in the subpopulation of γδ T cells, where an increase in the percentage of cells of 9.9 times was observed on day 16 (2.9% Vs 28.8%), this increase coincides in the days where the infant suffered a process of viral infection, subsequently, a decrease in the percentage of cells of 0.6 times was observed from day 22 to day 42 concerning the median of T cells γδ observed (2.9% vs 1.87%) (Figure 1B), this decrease was observed in the days in which the mother reported a state of asthenia.

The number of γδ T cells was analyzed during the observed modulation, finding that on day 15 the concentration of these cells was 31,881 cells/mL, while the concentration on day 40 was 1169 cells/mL, after this decrease in the number of cells a recovery of the percentage of these cells was observed at day 50 (Figure 2A).

$CD8^+$ populations (T αβ and T γδ) were analyzed, firstly, a comparison was made in the concentration of $CD8^+$ γδ T cells on day 1 *Vs* day 15 [9,922 cells/mL (7.9%) *Vs* 31,881 cells/mL (28.85 %); P = 0.02], from day 1 *Vs* day 40 [9,221 cells/mL (7.9%) *Vs* 1,169 cells / mL (1.85); P = 0.03], from day 40 *Vs* day 50 [1,169 cells/mL (1.8%) *Vs* 5.575 cells/mL (5.7%); P = 0.01], from day 15 *Vs* day 40 [31,811 cells/mL (28.8) *Vs* 1,169 cells/mL (1.8%); P = 0.006] statistically significant differences were observed (Figure 2B). However, when the $CD8^+$ αβ T cell concentration of day 15 (35,000 cells/mL) was compared to the $CD8^+$ αβ T cell concentration of day 40 (29,583 cells/mL); (P = 0.08) or *Vs* day 50 (34,412 cells/mL); (P= 0.66) no statistically significant differences were observed. The results showed only a significant change in the concentration of $CD8^+$ γδ T cells (Figure 2B).

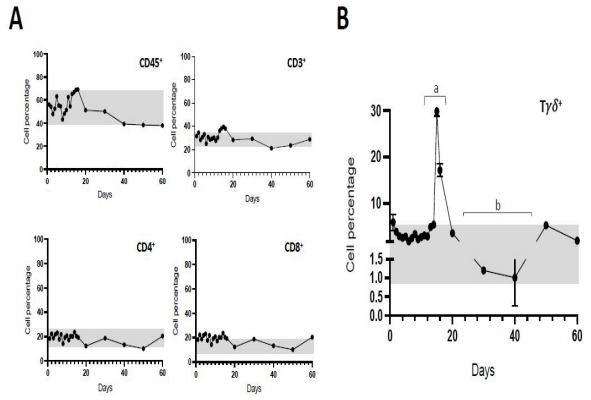

Figure 1: Ratio of leukocytes in 8-month-old mature breast milk. A) Charts showing the percentage of CD45, CD3, CD4, and CD8 cells in breast milk. B) Behavior of the Vγ2Vδ2 T cell subpopulation. The bracket (a) indicates the period in which the infant had a viral infection, the bracket (b) indicates the period in which the mother manifested feeling asthenia. The gray band in the graphs indicates the median ± IQR cell percentage for each subpopulation.

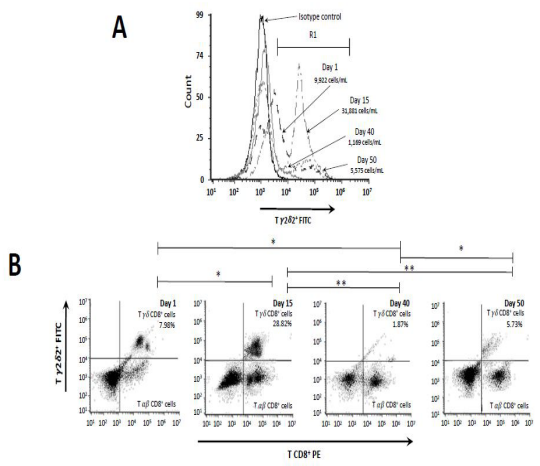

Figure 2: Analysis of modulation in the percentage of γδ T lymphocytes by flow cytometry. A) The histogram shows the number of Vγ2Vδ2+ T cells/mL present on day 1, 15 (infection in the infant), 40 (state of asthenia in the mother), and 50. B) The percentage of Vδ2+ lymphocytes was analyzed by flow cytometry as a fraction of CD8+ cells in mature breast milk, TCR γδ cells were defined by TCR γδ+ region, gated on $CD45^+/CD8^+$. The scatter diagrams show the percentage of T cells on days 1, 15, 40, and 50. Differences between the percentage of T cells were analyzed by the unpaired t-test. P=*,**; < 0.05, 0.001.

Discussion

This is one of the few studies in 8-month-old mature milk that aimed to characterize $CD45^+$ leukocytes and describe the modulation of the presence of unconventional T lymphocytes. A high interindividual variability has been reported in the number of cells in breast milk and the total number of cells in breast milk varies greatly in the different stages of lactation, the percentage of cells found in this study is consistent with that reported in other studies with mature milk [10], however, an increase in the number of γδ T cells was observed during the course of viral infection in the infant. Viral infections are common in people of all ages, but they often seem to be concentrated in infants and children on a regular and unavoidable basis due to their immaturity of immune systems, yet most children with viral infections get better without treatment [11], this improvement could be due to the participation of immune cells that provide breast milk.

Breast milk transmission of T cells to the newborn is a subject of intense debate, the results obtained in this work denote bidirectional immune crosstalk between breastfeeding and the newborn, suggesting that the number of γδ T cells rise in response to dyad infections, these cells pass through breast milk favoring the infant's immunity and resolving the infection and later returning to basal concentrations upon recovery of health of the infant [12].

Vγ2Vδ2 T cells is a subpopulation of γδ T cells also known as Vγ2Vδ2 cells, as they can recognize a broad range of antigens without the presence of major histocompatibility complex

(MHC) molecules. They can attack target cells directly through their cytotoxic activity or indirectly through the activation of other immune cells [13]. γδ T cell subsets have been described as potent effector populations against pathogens including viruses [14].

On the other hand, a decrease in the number of γδ T cells in breast milk was observed during the period in which the mother mentioned having problems with discouragement. Depression is known to increase the risk of cessation of breastfeeding in several ways [15,16], however, there is little evidence on the influence of emotional variables such as anxiety and depression on breastfeeding and its association with the number of cells present in breast milk [17]. The relationship of depression with immunological functions shows a predominance of heterogeneous findings for many of the immunological parameters since there are variables such as sex, age, ambulatory status, and the severity of depressive symptoms that are involved in this process. Alterations in humoral and cellular immunity have been reported, some of which have been interpreted as hyperactivity and others as depression of the immune system [18], however, more studies are needed aiming at the effect of depression on the modulation of the presence of cells in the breast milk. Although these data are limited to a single sample, they reveal the importance of dyad factors on the regulation of unconventional T cells present in breast milk, such as infections and depression, being able to observe how this cell subpopulation is present in milk it is modified in the face of an active infection of the infant and a state of asthenia of the mother.

Conclusion

The molecular communication between the infant and the mother has not been fully described, the cellular content of the milk depends on several factors, such as the fullness of the mammary gland, the stage of lactation, the health status of the mother/baby dyad, the permeability of the basement membrane and the development of the mammary epithelium [19]. This suggests that there is great heterogeneity in the composition of milk from one woman to another and that it changes as it adapts to the needs of her baby. However, these results may give an indication and try to explain how newborns with an immature immune system cope with exposure to pathogens, highlighting the importance of T γδ cells in this process.

Declarations

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References

1. Ygberg S, Nilsson A. The developing immune system - from foetus to toddler. Acta Paediatr. 2012; 101: 120-7.

2. Camacho-Morales A, Caba M, García-Juárez M, Caba-Flores MD, Viveros-Contreras R and Martínez-Valenzuela C. Breastfeeding Contributes to Physiological Immune Programming in the Newborn. Front. Pediatr. 2021; 9: 744104.

3. Trend S, de Jong E, Lloyd ML, Kok CH, Richmond P, et al. Leukocyte Populations in Human Preterm and Term Breast Milk Identified by Multicolour Flow Cytometry. PLoS ONE. 2015; 10: e0135580.

4. Kaingade P, Somasundaram I, Sharma A, Patel D, Marappagounder D. Cellular Components, Including Stem-Like Cells, of Preterm Mother's Mature Milk as Compared with Those in Her Colostrum: A Pilot Study. Breastfeed Med. 2017; 12: 446-449.

5. Bertotto A, Castellucci G, Fabietti G, Scalise F, Vaccaro R. Lymphocytes bearing the T cell receptor gamma delta in human breast milk. Arch Dis Child. 1990; 65: 1274-5.

6. Vantourout P, Hayday A. Six-of-the-best: unique contributions of γδ T cells to immunology. Nat Rev Immunol. 2013; 13: 88–100.

7. Constant P, Davodeau F, Peyrat MA, Poquet Y, Puzo G, et al. Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. Science. 1994; 264: 267–70.

8. Mincheva-Nilsson L, Nagaeva O, Sundqvist KG, Hammarström ML, Hammarström S, et al. gammadelta T cells of human early pregnancy decidua: evidence for cytotoxic potency. Int Immunol. 2000; 12: 585-96.

9. Cacho NT, Lawrence RM. Innate Immunity and Breast Milk. Front Immunol. 2017; 8: 584.

10. Li S, Zhang L, Zhou Q, Jiang S, Yang Y, Cao Y. Characterization of Stem Cells and Immune Cells in Preterm and Term Mother's Milk. J Hum Lact. 2019; 35: 528-534.

11. Mulholland K. Global burden of acute respiratory infections in children: implications for interventions. Pediatr Pulmonol. 2003; 36: 469-74.

12. Hassiotou F, Hepworth AR, Metzger P, Tat Lai C, Trengove N, et al. Maternal and infant infections stimulate a rapid leukocyte response in breastmilk. Clin Transl Immunology. 2013; 2: e3.

13. Cairo C, Longinaker N, Cappelli G, Leke RG, Ondo MM, et al. Cord blood Vγ2Vδ2 T cells provide a molecular marker for the influence of pregnancy-associated malaria on neonatal immunity. J Infect Dis. 2014; 209: 1653-62.

14. Lawand M, Déchanet-Merville J, Dieu-Nosjean MC. Key Features of Gamma-Delta T-Cell Subsets in Human Diseases and Their Immunotherapeutic Implications. Front Immunol. 2017; 8: 761.

15. Stuebe AM, Grewen K, Pedersen CA, Propper C, Meltzer-Brody S. Failed lactation and perinatal depression: Common problems with shared neuroendocrine mechanisms?. J Womens Health (Larchmt). 2012; 21: 264-72.

16. Kendall-Tackett K. The new paradigm for depression in new mothers: current findings on maternal depression, breastfeeding and resiliency across the lifespan. Breastfeed Rev. 2015; 23: 7-10.

17. Singh M, Stacey T, Abayomi J, Simkhada P. Maternal mental health and infant and young child undernutrition: protocol for a systematic review. BMJ Open. 2021; 11: e044989.

18. Navas, Carmen, & Zapata, Deliana. Aspectos inmunológicos en la depresión. Revista Venezolana de Oncología. 2009; 21: 244-252.

19. Hassiotou F, Geddes DT, Hartmann PE. Cells in human milk: State of the science. J Hum Lact. 2013; 29: 171-82.