

ESCHERICHIA COLI SPECIFIC SECRETORY IgA AND CYTOKINES IN HUMAN MILK FROM MOTHERS OF DIFFERENT ETHNIC GROUPS RESIDENT IN NORTHERN ITALY

L. CIARDELLI, F. GAROFOLI, M.A. AVANZINI, A. DE SILVESTRI¹,
A. GASPARONI², G. SABATINO³ and M. STRONATI⁴

Research Laboratories (Neonatal Immunology and Paediatric Oncohematology), IRCCS Policlinico San Matteo, Pavia; ¹Biometric Unit, IRCCS Policlinico San Matteo, Pavia; ²Neonatal Intensive Care Unit, Spedali Civili, Brescia; ³Neonatal Intensive Care Unit, University G. d'Annunzio, Chieti; ⁴Neonatal Intensive Care Unit, IRCCS Policlinico San Matteo, Pavia, Italy

Received August 1, 2006 – Accepted February 1, 2007

Breast milk supplies many bioactive components. Neonates protection from pathogenic bacteria is mainly attributable to secretory IgA antibodies present in human milk in an amount depending on previous antigenic exposure. To bring new details into the field of immunological memory in secretory immunity, we evaluated the production of s-IgA specific for *E. coli* (*E. coli* s-IgA), and of pro-inflammatory (IL-6 and IL-8) or anti-inflammatory (IL-10) cytokines in the milk of mothers of different ethnic groups exposed in the past to poor conditions, but nowadays living in Italy in adequate conditions. Mothers from Italy, Africa, Asia and Eastern European Countries were included in the study. Anti-*E. coli* s-IgA, IL-6, IL-8 and IL-10 were determined by ELISA. Breast milk of all the foreign mothers presented higher levels of *E. coli* s-IgA than Italians, and for Asian and African mothers were significant ($p=0.031$ and $p=0.015$, respectively). Milk from women of Eastern European Countries revealed the highest IL-8 levels ($p=0.026$), while milk from Asian women presented the greatest concentration of IL-6 ($p=0.04$); however, the Africans reported the lowest concentrations of IL-10 ($p=0.045$). Since all the mothers had been living in Italy for some time, we believe that the presence of high levels of *E. coli* s-IgA, supported by high levels of pro-inflammatory cytokine, is part of a persisting immunological secretory memory.

Breast milk supplies newborns not only with nutrients, but also with very important bioactive components that modulate the immune response and ameliorate the development of the gastrointestinal tract. Breastfed infants develop less infections than bottle-fed infants and this is related to the different agents with antimicrobial, anti-inflammatory and immunomodulating action, unique to human milk (1-3). The protection of the newborn's intestinal and respiratory tract from pathogenic bacteria is also

attributable to antibodies present in milk (4-5).

In the so called entero-mammary link, once antigens are presented to the mother's gut, the responding B cells switch to the production of IgA that, leaving the Peyer's patches, migrate to the mammary glands and passing through, appear in milk, stabilized by the secretory component (4).

In human milk, secretory IgA (s-IgA) is the predominant isotype and acts as first line defence in mucosal areas of the infant (5-6) against the

Key words: human milk, ethnic group, E. coli s-IgA, IL-6, IL-8, IL-10

Mailing address: Dr. Laura Ciardelli,
Patologia Neonatale,
IRCCS Policlinico San Matteo,
Piazzale Golgi 1,
27100 Pavia, Italy
Tel: ++39 382 502.865 Fax: ++39 382 502802
e-mail: l.ciardelli@smatteo.pv.it

Table I. *E. coli* s-IgA and cytokine concentrations in human milk of mothers from different countries and resident in Northern Italy

Country of origin		S-IgA (AU/ml)	IL-10 (pg/ml)	IL-8 (pg/ml)	IL-6 (pg/ml)
Italy	Mean	7.44±8.62	71.5±114.9	5439±5831	25.5±38.4
	Median	4.5	6.81	2532	8.3
	IQ range	1.9-8.3	0.29-106	931-8077	4.5-25.1
Africa	Mean	23.5±30.89	7.8 #±18.7	5115±5940	110±448.1
	Median	11.7	0.42	2223	4.35
	IQ range	5.2-27.8	0.17-4.3	1534-6298	1.8-25.7
Asia	Mean	38.4 [§] ±36.20	85.6±196.6	7881±7334	2077*±4680
	Median	13.2	3.86	4676	7
	IQ range	1.4-42	0.25-85.7	1483-15978	3.6-41.8
East Europe	Mean	20.4±24.57	44.5±99.7	9493 °±7699	165±444
	Median	8.1	3.31	10477	9.6
	IQ range	3.1-36	0.37-28.2	2081-16081	1.8-48.1

Results are expressed as mean, median and interquartile range (IQ)

[§] $p = 0.007$; # $p = 0.045$; ° $p = 0.026$; * $p = 0.004$

environmental antigens. A long-term maternal immunological memory in respect to mothers' antigen contacts has been reported (4, 7). In this context, the place of residence, both developed and developing countries, influences gut humoral immunity (8). Elsewhere, it has been shown that mothers from Africa are capable of producing higher levels of secretory antibodies than mothers from Western European Countries (9).

In contrast, other studies demonstrate that privileged mothers from different ethnic groups, produce more s-IgA than underprivileged mothers in comparable period of lactation (10). In human milk, s-IgA work in synergy with other soluble components, like cytokines. It is known that IL-6 and IL-10 enhance IgA production, and IL-10 and IL-8 are very important for the intestinal development and for the response to pathogenic agents (11-14).

In order to evaluate immunological memory in secretory immunity, we studied the production of s-IgA specific for *E. coli* (*E. coli* s-IgA), in the milk of mothers of different ethnic groups now living in Northern Italy. In addition, we explored whether mothers' milk presents a different array in pro-inflammatory or anti-inflammatory cytokines.

MATERIALS AND METHODS

Subjects

One hundred and twelve mothers were included in

the study: 40 women were from Italy, 24 from Eastern European Countries, 24 from Asia and 24 from Africa. All the mothers delivered full-term infants (38.5 ± 1.91 weeks); the offspring's weight was normal for gestational age (3234 ± 529 g). Mother's age was 28.4 ± 5.6 years. Before living in Italy the foreign mothers were exposed to poor conditions.

58.3% of the foreign mothers have been living in Italy for more than two years, 37.5% of them for more than 1 year and only 4.1 % for more than 6 months. Moreover, 64% have not been back to their country of origin in the past two years. All mothers received a case report form, set up by an epidemiologist, asking about family unit, number of rooms and bathrooms of habitation, school-attendance rate and kind of job in Italy. With reference to data obtained from the case report forms, all the foreign women, except one from Eastern Europe, had been in adequate living conditions since arriving in Italy. We therefore decided to exclude this data.

Samples

Milk samples were collected after 74.7 ± 6.9 hours after delivery at the Neonatal Unit of "Spedali Civili" Hospital of Brescia and at IRCCS Policlinico San Matteo Hospital of Pavia, Italy. Ethical approval was received by the relevant Ethical Committee of each Hospital. Informed written consent was obtained from all mothers attending the study.

Milk samples were taken by manual expression after cleaning the breast with sterile water and collected into 50 ml sterile polypropylene tubes. After centrifugation to eliminate lipid phase, aqueous samples were stored at -20°C until analysis.

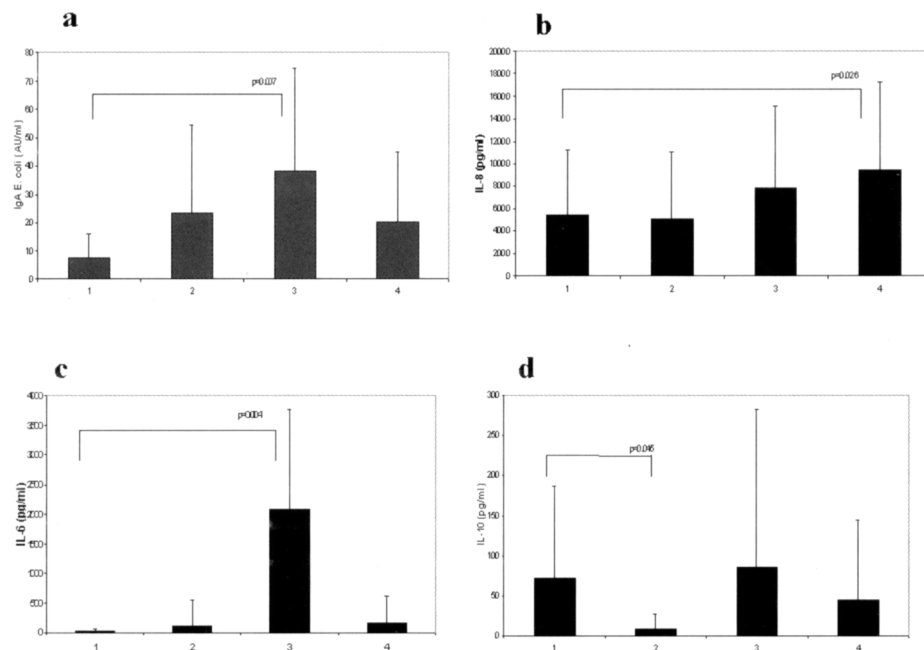


Fig. 1. *E. coli* s-IgA (a), IL-8 (b), IL-6 (c) and IL-10 (d) concentrations in milk of mothers from different ethnic groups resident in Northern Italy. 1=Italy; 2=Africa; 3=Asia; 4=Eastern European Countries. Anti-*E. coli* s-IgA and cytokines were determined by ELISA technique. Asian milk reported the statistically highest s-IgA (a), and IL-6 (c) concentrations ($p=0,007$ and $p=0,004$ respectively), Eastern European Countries milk reported the statistically highest IL-8 (b) concentration ($p=0,026$), while African milk accounted the lowest values of IL-10 ($p=0,045$).

Methods

Anti-*E. coli* s-IgA were determined in milk by ELISA, as described previously (15-16). Briefly, 100 μ l of undiluted breast milk were added to microtiter plates (Costar Corning Inc., Corning, NY, USA) coated with the appropriate antigen concentrations. After incubation, the conjugated anti-human IgA (Dakopatts AG, Copenhagen, Denmark) was applied. A working standard curve was constructed using a milk pool and antibody content of unknown samples was expressed as Arbitrary Units (AU). Plates were read on an ELISA reader at 492 nm (Titertek Plus MS 212M, Tecan Italia srl, Cologno Monzese, Italy).

IL-6, IL-8 and IL-10 were quantified in all milk samples using ELISA techniques. Plates (Costar) were coated with purified antibodies at the appropriate concentrations (Pierce Endogen, Rockford, IL, USA).

Standard curves were prepared with recombinant human cytokine (Pierce Endogen). Biotin-labelled antibodies (Pierce Endogen) were added and HRP-conjugated, streptavidine (Pierce Endogen) was used to develop the reactions. Plates were read at 450 nm (Titertek Plus MS 212M).

Statistical Analysis

Analyses were performed using STATA v. 8.0 (STATA Corp, College Station, TX)

Univariate analysis was performed to assess the difference in s-IgA against *E. coli* and in cytokine production in milk in mothers of different ethnic groups compared to Italian mothers.

Four multiple regression models were performed: using *E. coli* s-IgA, IL-8, IL-6 and IL-10 levels as dependent variables. While countries of origin, residence period and return home were used as explicatory variables.

Finally, a multivariate regression analysis was performed using *E. coli* s-IgA as dependent variable, while, the period of residence in Italy, return home and cytokine concentration were used as explicatory variables.

A p-value $<0,05$ was considered statistically significant.

The regression coefficient β represents the independent variable change that corresponds to a single-unit increment of the quantitative dependent variables or, for categorical variables, represents the difference in respect to the reference group.

RESULTS

Breast milk of all the foreign mothers presented higher levels of *E. coli* s-IgA than Italians. For Asian women the difference was significant ($p=0,007$,

Fig. 1a, Table I). When the residence period in Italy and the return frequency to the country of origin is added to the model, the Asian mothers still had a significantly higher concentration of *E. coli* s-IgA in their milk than Italian women ($\beta=18.5$, $p<0.001$), while no significant differences are observed for African ($\beta=7.2$) or Eastern European ($\beta=3.6$) women.

Nevertheless, a significant higher concentration of s-IgA was found in Asian and African mothers' milk than that of Italians ($\beta=26.6$, $p=0.031$; $\beta=33.1$, $p=0.015$, respectively), when data were corrected for the residence period in Italy and the return frequency to the country of origin and cytokine concentrations.

A positive correlation between s-IgA from all the mothers and IL-8 levels is also observed ($\beta=7.7$, $p=0.01$).

Milk from women of Eastern European Countries reveals the highest IL-8 levels ($p=0.026$, Fig. 1b, Table I). When the residency period in Italy and the return frequency to the country of origin is added to the model, the Eastern European mothers still had a significantly higher concentration of IL-8 in their milk than Italian women ($\beta=726$, $p=0.001$), while no significant differences are observed for African ($\beta=-32$) or Asian ($\beta=214$) women.

Of the different countries, milk from Asian women presents the greatest IL-6 concentration ($p=0.004$, Fig. 1c, Table I). When the residency period in Italy and the return frequency to the country of origin is added to the model, the Asian mothers still had a significantly higher concentration of IL-6 in their milk than Italian women ($\beta=41.8$, $p=0.006$), while no significant differences are observed for African ($\beta=5.8$) or Eastern European ($\beta=24.6$) women.

Most of the samples of all the ethnic groups showed detectable levels of IL-10 (ranging from 75% of the Eastern European mothers to 100% of the Asians), but the Africans reported the lowest concentrations ($p=0.045$, Fig. 1d, Table I). When the residency period in Italy and the return frequency to the country of origin is added to the model, the African mothers still had a significantly lower concentration of IL-10 in their milk than Italian women ($\beta=-6.7$, $p=0.016$), while no significant differences are observed for Asian ($\beta=-3.2$) or Eastern European ($\beta=-3.6$) women.

DISCUSSION

It is well known that breast feeding is very important for the presence of antimicrobial, anti-inflammatory and immunomodulating agents protecting the infant (1-3). It has been demonstrated that breast feeding has a dramatic protective effect against septicaemia in neonates, even preterms, by influencing the intestinal milieu to become hostile to some bacteria, but favourable for others (2, 17-18). All the cellular and soluble compounds of human milk work in synergy to exert these protective effects.

Different antigenic exposure due to different environments has been reported to influence the immunologic composition of milk (4, 19-20).

We observed higher levels of *E. coli* s-IgA in women coming from developing countries than in Italians, often related to the hygienic and environmental conditions. Since all mothers are now living in Italy in adequate environmental conditions, we believe that high levels of *E. coli* s-IgA could be the expression of a persisting secretory immunologic memory (7, 16). In fact, previous studies reported higher milk concentrations of s-IgA in African and Asian mothers living in their country, than in European mothers (8-9, 21).

We found very high amounts of IL-8 in all the mothers' milk, particularly in women from Eastern European Countries. Immunological properties of IL-8 are widely reported: it is a very important stimulation factor for neutrophil migration, activation and regulation of the expression of adhesion molecules (22). We believe that the presence of such cytokines may promote the high percentage of neutrophils in human colostrum, important in counterbalancing the deficiency in newborns (3, 23). Moreover, IL-8 has a trophic function in the developing human gut: this cytokine seems able to stimulate intestinal cell migration, proliferation and differentiation (11) and also protects intestinal cells against chemical injuries (12). IL-10 was detectable in most of the milk samples, as reported in literature (24-25). It is a cytokine with anti-inflammatory, tolerogenic and inhibitory properties. It has been reported that IL-10 plays an important role in the amplification of humoral responses (26). Moreover, IL-10 exerts a protective action for the intestinal

cells. In fact, it has been hypothesized that a low concentration of IL-10 in preterm milk contributes to the development of necrotizing enterocolitis (14). In our study, the milk of African mothers reported significantly lower levels of IL-10 than all the others. This may be explained as a mechanism of protection because this cytokine is a potent down-regulator of pro-inflammatory cytokine production, but stimulatory mediators are required to defeat environmental infections.

In agreement with other studies, IL-6 was also detected in the majority of the colostrum (27), in particular in milk from Asian women.

It is known that this cytokine can enhance s-IgA production in breast milk (1, 6) and it is positively correlated with IgA concentration (24, 27). IL-6 is mainly a pro-inflammatory cytokine that promote an activation of the immune system (28), and acts together with IL-10 to protect offspring mucosa.

Our findings support an immunological memory of secretory immunity, particularly useful for the babies born in countries where infections are frequent and severe and still present when the mother changes her place of living.

ACKNOWLEDGEMENTS

The authors thank Mrs. Claudia Cova of the Research Laboratories, Neonatal Immunology, IRCCS Policlinico San Matteo, Pavia, Italy, for her technical assistance and expertise.

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