

Breastfeeding reduces immune activation in primary respiratory syncytial virus infection

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ABSTRACT. In epidemiological studies of respiratory syncytial virus (RSV) disease, breast milk has proven to be beneficial. However, a host mechanism that is associated with both disease severity and that is capable of being modulated by breast milk, has not yet been identified. Both the predominance of interleukin-10 (IL-10) over interferon- γ (IFN- γ), and high soluble interleukin-2 receptor antagonist (sCD25) concentrations have been associated with RSV severity. We explored if they were modulated by breastfeeding. Previously healthy Chilean infants from Santiago with RSV infection (n = 349) were consecutively enrolled in the study if they were term births, without underlying pathology. Breastfeeding was described as absent or present, and if partial or exclusive. Immune response was expressed through plasma concentrations of IFN- γ , IL-10 and sCD25, obtained both in the acute and the recovery phase. The acute phase sCD25 concentrations were lower in the breastfed (13.8 ng/mL, n = 133), compared with the non-breastfed infants (15.9 ng/mL, n = 27, p = 0.015). The difference increased in infants below 3 months of age (p = 0.006) and with exclusive (p = 0.004), compared to partial breastfeeding (p = 0.025). When analyzed together with age, sex, severity and environment, breastfeeding was the only independent predictor of high sCD25 concentrations (above mean + 1SD, OR 4.6, 95% CI 1.8-11.9, p = 0.0015). The recovery phase IFN- γ /IL-10 ratio was higher in the breastfed infants, but when analyzed with potential confounding factors, only female sex was associated with an increased ratio (OR 2.32, 95% CI 1.02-5.29, p = 0.045). High sCD25 concentrations during the acute phase of infection, previously associated with severe RSV disease, were significantly and independently reduced in association with breastfeeding, whereas the Th1/Th3 balance was only modified in the recovery phase.

Keywords: breastfeeding, immune response, primary RSV infection

Epidemiological studies show that breastfeeding can reduce the frequency, severity and mortality of respiratory disease in infants [1, 2]. In respiratory syncytial virus (RSV) infection – the leading cause of bronchiolitis and hospitalization of infants in the world – it protects against severe disease, and this is seen best in developing countries [3]. The pleiotropic, biological activity of breast milk has been extensively studied [4]. Among its immunological properties, breast milk exhibits direct antiviral activity [5], contains neutralizing antibodies and other anti-infectious components, and stimulates the offspring immune system by transferring cytokines and growth factors [6, 7]. The relative contribution of these mechanisms in the protection against RSV infection is unknown. Most importantly, a host mechanism that is both involved in the severity of RSV disease and capable of being modified by breastfeeding has not yet been identified.

In the present study, we explore the relationship of breastfeeding with cytokine responses in RSV-infected infants. Both the type of immune response (Th1 in relation to Th3) [8, 9], the plasma interleukin-10 (IL-10) concentration, and the degree of immune activation measured by soluble

interleukin 2 receptor (sCD25) concentration [10] have been implicated in the pathogenesis of hypoxic, severe, RSV bronchiolitis. In our study, we examined if the host immune response, as expressed through plasma IFN- γ , IL-10 and sCD25 concentrations, both in the acute and the recovery phase of primary RSV infection, differed between breastfed and non-breastfed infants.

MATERIALS AND METHODS

Patients

The study was conducted in Santiago, Chile, in a general Pediatric Hospital that is part of the National Health Service, and which is attended by the lowest socioeconomic group in the surrounding population, free of charge. Three hundred forty nine patients from the outpatient department and the infants ward, were consecutively enrolled into the study over three RSV seasons. Inclusion criteria were: term birth, no underlying illness, seen within the first eight days of the first respiratory infection, informed consent obtained from the guardian, and a rapid, positive RSV test

(BD Directigen RSV[®]) result using a nasopharyngeal aspirate (NPA) sample.

Two patient cohorts were formed, one with severe disease defined as being hospitalized because of oxygen saturation below 95% (criterion for admission at our institution) and the other with mild disease defined as an outpatient course of disease with oxygen saturation equal to or above 95%. Most of the enrolled infants (300/349, 86%) were breastfed. In 222/300 (74%) breastfeeding was exclusive, in 40/300 (13%) partial and in 38/300 (13%) the type of breastfeeding had not been specified. The 49 patients who were not breastfed had usually received some breast milk (33 of 46 patients, missing data from 3), but the breastfeeding had stopped (in 30/33 patients, missing data from 3) more than a month earlier.

The breastfed infants (partial or exclusive) did not differ from the non-breastfed infants as regards sex or mother's educational level (*table 1*). Not being breastfed was linked with crowding ($p = 0.004$), having more family members who smoked ($p = 0.04$), and more sibling contacts with a cold ($p = 0.01$, *table 1*). As expected, increasing age of the infants clearly diminished the frequency of breastfeeding ($p < 0.0001$). Therefore, the main findings were analyzed comparing groups of infants of the same age (*table 2*) and results were controlled for age (*table 3*).

Data collected

Clinical examination, history including the duration of symptoms in days, description of the environment and

Table 1
Basic characteristics of previously healthy infants, their environment, course and diagnostic results of primary RSV infection as a function of breastfeeding

Variable	Breastfeeding		p value
	Yes	No	
n	300	49	
Females (%)	133 (44)	22 (45)	0.96
Mean age, months	2.3	4.4	< 0.0001
Born in the first half of RSV season	252 (84)	39 (80)	0.21
Mother with basic education	83/289 (29)	12/44 (27)	0.84
Smoking family members	1.4	1.9	0.04
Number of cohabitants	6.7	8.3	0.004
Number of siblings with a respiratory infection in the past week	1.0	1.5	0.01
Pulse oxymetry reading, %			
- On admission	94.2	93.9	0.60
- 1 week follow-up visit	96.3 (N225)	95.2 (N41)	0.18
Nasopharyngeal aspirate positive ^a by:			
- Immunofluorescence	151/286 (53)	24/47 (51)	0.83
- Culture	137/280 (49)	32/47 (68)	0.03

^a All patients had a rapid ELISA- (BD Directigen RSV[®]) positive RSV test using the nasopharyngeal aspirate.

Table 2
Immune response in the acute and the recovery phase of primary RSV disease as a function of breastfeeding

Plasma concentrations	Breastfeeding		p value
	Yes	No	
Acute phase			
Day of symptoms when sample taken	4.5	4.5	0.85
IFN- γ ^c	29.8 ^a (98) ^b	33.4 (21)	0.62
IL-10	14.2 (134)	14.5 (27)	0.88
IFN- γ /IL-10	1.8 (98)	2.2 (21)	0.50
SCD25			
- in all infants	13.8 (133)	15.9 (27)	0.015
- in infants below 4 months of age	13.9 (108)	16.8 (18)	0.008
- in infants below 3 months of age	14.0 (95)	18.1 (10)	0.006
Recovery phase			
Day of symptoms when sample taken	30.0	29.5	0.83
IFN- γ	13.7 (127)	9.4 (25)	0.10
IL-10	11.7 (127)	15.2 (25)	0.07
IFN- γ /IL-10	1.2 (127)	0.6 (25)	0.04
SCD25	12.4 (126)	11.9 (25)	0.78

^a Geometric mean values.

^b Number of patient samples.

^c IFN- and IL-10 were measured in pg/mL and sCD25 in ng/mL.

Table 3
Odds ratios of patient characteristics, environment and various aspects of breastfeeding, and having a high sCD25 plasma concentration

Variable	SCD25 above mean + 1SD		
	OR	95% CI	p
Age	0.82	0.64-1.07	0.14
Sex - female	1.26	0.54-2.97	0.59
Siblings with a cold	0.86	0.65-1.15	0.32
Smokers	1.02	0.77-1.36	0.88
Outpatient	0.71	0.30-1.68	0.43
Days of symptoms	1.08	0.88-1.33	0.44
Breastfeeding ^a	0.22	0.08-0.56	0.0015 ^b
- days 1-4 of symptoms	0.74	0.14-3.98	0.72
- days 5-7 of symptoms	0.05	0.01-0.22	< 0.0001
Partial breastfeeding (n)	0.09	0.01-0.73	0.025
Exclusive breastfeeding (n)	0.24	0.09-0.64	0.004

^a Partial or exclusive.

^b Remained an independent predictor in multiple regression analysis.

details of breastfeeding were obtained upon enrolment. A NPA sample was taken into Hank's solution by gentle suction of the nasopharyngeal space, at least two hours after the infant's last feed. A heparinized blood sample was obtained from 160 infants (during the first two study years). A second blood sample was taken from 154/160 infants (96%), 3-4 weeks after the enrolment sample, at a follow-up visit. All samples were kept cold until delivery, within maximum 2 hours, to the laboratory. There plasma, obtained by refrigerated centrifugation, was kept frozen at -70°C until analyzed at the same time, to determine IL-10, IFN- γ , and sCD25 concentrations using commercial ELISA, as previously described [10].

The NPA sample was centrifuged, and the sediment used for indirect immunofluorescence (IIF) of RSV; 0.2 ml of the solution was cultured for 10 days in a monolayer of Hep-2 cells, with addition of Dulbecco medium with antibiotics and a fungicide. The cultures were examined daily for the characteristic cytopathogenic effect (CPE). In the CPE-positive cultures, the presence of RSV was confirmed by IIF and monoclonal antibodies against protein F. The CPE-negative cultures were examined for RSV at 10 days by immunofluorescence. If negative, they were considered negative, or else subcultured.

Statistics

Categorical variables from breastfed and non-breastfed infants were compared using χ^2 and continuous variables by Student's *t*-test. Immune response marker results were logarithmically transformed before analysis. The term "breastfed" refers to all infants receiving breast milk at the time of the study, either partially or exclusively, if not specified otherwise. The association of breastfeeding with severe disease was examined, both by a univariate test and together with previously recognized confounding variables (age, date of birth, sex, crowding, smoking) by logistic regression analysis. The variables that distinguished breastfed from non-breastfed infants in univariate analysis (p value less than 0.10) were further tested by logistic regression, together with putative confounding factors (age, sex, severe disease), to examine their role in relation to high cytokine concentrations. For this purpose, the cytokine result was divided into the categories of "high"

and "not high" taking as the cut-off level, the mean plus one standard deviation of the concentration in all, 160 patients.

Ethics

The study was approved by the Ethics Committee, Hospital Luis Calvo Mackenna, Santiago, Chile, and patients were enrolled provided written informed consent was obtained from the guardian of the child.

RESULTS

Thirty-three/49 children (67%) not breastfed, 24/40 children (60%) partially breastfed and 124/260 children (48%) exclusively breastfed were hospitalized because of a pulse oxymetry reading below 95% (contingency table, $p = 0.023$). Being breastfed reduced the risk-of hospitalization (table 1, OR 0.47, 95% CI: 0.25-0.89, $p = 0.021$), but only when this factor was tested alone. When the role of breastfeeding was analyzed together with the other variables, which in our study significantly increased the risk of hospitalization (table 1), the number of family members who smoked (OR 1.21, 95% CI 1.04-1.39, $p = 0.012$) and the number of sibling contacts with a cold (OR 1.32, 95% CI 1.06-1.64, $p = 0.015$) prevailed as independent predictors of the need for hospitalization, whereas breastfeeding lost its significance (OR 0.55, 95% CI 0.28-1.09, $p = 0.087$).

The type of immune response as measured by IFN- γ and IL-10 concentrations and their ratio, did not differ in the acute phase of disease between the breastfed and the non-breastfed infants (table 2). In the recovery phase, the IFN- γ /IL-10 ratio was greater in the breastfed infants according to a univariate test ($p = 0.043$). However, when the effect of breastfeeding on a high IFN- γ /IL-10 ratio (above mean+1SD, table 3) in the recovery phase, was tested together with sex, age, and the environment, only sex remained an independent predictor. Female sex increased the possibility of having a high value by 2.32 (95% CI 1.02-5.29, $p = 0.045$) in the recovery phase.

The magnitude of the immune response activation, measured by the sCD25 concentration, was smaller in the breastfed infants (geometric mean 13.8 ng/mL) compared

with the non-breastfed infants (geometric mean 15.9 ng/mL, $p = 0.015$, *table 2*). Each ng/mL increase in sCD25 increased the risk of requiring hospitalization by 1.10 (95% CI 1.02-1.19, $p = 0.016$). The difference between the breastfed and non-breastfed infants increased with decreasing age ($p = 0.015$ to $p = 0.0016$, *table 2*). The risk of presenting high sCD25 concentrations (above mean+1SD), was 4.6 times higher in the non-breastfed infants (95% CI 1.8-11.9, $p = 0.0015$) compared with breastfed infants, and increased to 11.5 times (95% CI 2.8-47.1, $p = 0.0007$) in infants below 3 months of age. Exclusive breastfeeding reduced the risk of having a high sCD25 concentration ($p = 0.004$) more substantially than partial breastfeeding ($p = 0.025$, *table 3*).

The effect of breastfeeding on the sCD25 levels was influenced by the number of days that the infant had presented symptoms (*table 3*) when the blood sample was taken. This was very clear ($p < 0.0001$) on days 5-7 of symptoms, but was insignificant on days 1-4 of symptoms ($p = 0.722$). Not breastfeeding was the only independent predictor of high sCD25 concentrations (*table 3*).

Breastfeeding was also associated with the NPA results (*table 1*). Viral cultures were more often negative in the breastfed ($p = 0.03$) compared with the non-breastfed infants.

DISCUSSION

Breastfeeding is one of the factors that influence severity of RSV disease, in addition to RSV genotype [11], viral load [12], host response [8-10] and environment [3]. Previous studies have shown a clear, protective role of breastfeeding in poor hygiene conditions but less so in more favorable ones [3]. Our results show partial protection against severe RSV disease requiring hospitalization afforded by breastfeeding in urban, Latin American infants living in improving hygienic conditions. Finding local differences underlines the importance of local studies.

The influence of breast milk on the RSV cellular immune response has been little explored previously. The clearest effect of breastfeeding on the immune response seen in our study was the low sCD25 plasma level (*tables 2 and 3*) compared with the non-breastfed infants.

Breast milk contains a series of components with immunomodulating properties, which represent a benefit for the infant. Its antimicrobial, anti-inflammatory, and immunomodulatory agents are multifunctional and act synergistically [13]. Taking into account that immune activation and CD25 solubilization have been shown to depend on stimulation by antigen or polyclonal activators [14], one of the mechanisms by which breast milk could diminish sCD25 plasma levels is by reduction of the antigen load by neutralizing antibodies in breast milk. Alternatively, the anti-inflammatory and immunomodulatory properties of breast milk, mainly exerted by transforming growth factor-beta (TGF- β), could inhibit some other immune activation pathways [15]. For example, TGF- β is known to inhibit production of IL-2 and T lymphocyte proliferation [15]. A previous study showed that breastfeeding in RSV infection stimulates production of antiviral interferon- α , but suppresses lymphocyte transformation *in vitro* [16].

In rotaviral infection, breast milk lactadherin specifically binds to the virus, reduces the infecting dose and thus ameliorates the disease [5]. The same study also showed

how breast milk lactadherin concentrations vary in different mothers, and that the protective effect was concentration-dependent. The latter finding can, at least in part, validate the clinical observation of varying efficacy of breast milk.

The greatest beneficial effect of breastfeeding on the sCD25 response was seen in the youngest babies, suggesting, in part, that breast milk during the first months of life contains a higher concentration of the elements that achieve the immunomodulating benefits. The greatest benefits *vis-à-vis*, preventing elevations of plasma sCD25 and producing negative cultures, were obtained from exclusive breastfeeding and when given on days 5-7 of symptoms, rather than days 1-4. Possibly, the full effect of breastfeeding on modulating the immune response takes some days to become evident. Also, milk produced after some days of a concomitant RSV infection by the mother, may contain a higher virus-neutralizing capacity. All these observations have potential practical importance.

Reaching a favorable, Th1-predominant response (higher IFN- γ /IL-10 ratio) in the recovery phase was partially bolstered by breastfeeding. This observation may have importance for determining if breastfeeding prevents the development of allergic sensitization and obstructive airway disease, both conditions characterized by decreased production of IFN- γ and increased IL-10 production [17, 18].

Other mechanisms possibly related to both RSV severity and breastfeeding, are RSV genotype [11] and - as also suggested in our study, by its surrogate marker of negative cultures - the infecting dose [12]. If breast milk RSV antibodies are as genotype-specific as serum RSV antibodies [19], protection may be limited for some genotypes. The importance of the infecting dose, or pathogen genome count to the severity of disease, is a principle also demonstrated in bacterial meningitis [20] and also contributes to the severity of measles in Africa [21].

In summary, our results help to clarify how the cellular immune response, a host mechanism associated with RSV severity, is influenced by breastfeeding.

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