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Breastfeeding is associated with the production of type I interferon in infants infected with influenza virus

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Abstract

Background—Breast milk-mediated protection against respiratory viruses is well established. However, protective mechanisms are unclear. Type I interferons (IFN) mediate host defence against respiratory viruses, particularly influenza virus. The relationship among type I IFN, respiratory viral infections and breastfeeding has not been explored.

Methods—Type I IFN responses were studied by ELISA and real time PCR in nasal secretions of infants experiencing their first respiratory infection. Modulation of IFN by breastfeeding and other variables affecting severity during viral infection was explored.

Results—One hundred and twenty infants were positive by RT-PCR for influenza virus (n = 24), human metapneumovirus (hMPV) (n = 30) or respiratory syncytial virus (RSV) (n = 66). Type I IFNs were detected more frequently in infants infected with influenza virus than in those infected with RSV or hMPV. Breastfeeding promoted higher rates and levels of type I IFN only in infants infected with influenza virus. No effect on IFN production was observed for age, gender or smoking.

Conclusion—Our study confirms that type I IFN production is detected more frequently in infants infected with influenza virus. Importantly, higher rates and levels of type I IFN in these infants are associated with breastfeeding. These observations suggest that breast milk can protect against respiratory viruses by activating innate antiviral mechanisms in the host.

Keywords

Breast milk; Influenza; Interferon

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INTRODUCTION

The protective role of breastfeeding against viral respiratory infections in developing countries is well established (1). Human milk modulates severity of illness in term and premature infants (1–4), and several hypotheses have been advanced to explain its protective mechanism (5–7). The most widely accepted theory attributes protection to passive transfer of secretory neutralizing IgA (sIgA) against viruses in colostrum and milk (5). However, recent evidence demonstrates that the effect of breast milk can be different for infections in girls and boys, and therefore a passive protective mechanism (i.e. sIgA passive transfer) appears less likely (2–4). Given its broad range of benefits and the differences in virus-specific symptomatology, it is likely that human milk-mediated protection is multifactorial.

Type 1 interferons (IFN) are important mediators of host defence against respiratory viral infections (8). Involved in a wide variety of pro-inflammatory, pro-apoptotic and antiviral activities, the role of type I IFN in viral pulmonary disease was first studied in influenza, where these cytokines were detected in the nasal secretions of infected children and adults (9–11). Moreover, earlier comparisons suggested that type I IFN responses may also vary according to pathogen, and in particular, may be produced more frequently in response to influenza virus than to respiratory syncytial virus (RSV) (9,10). However, the relationship between type I IFN responses and other natural molecules – like those present in breastfeeding – is unknown. In fact, whether variables detrimental for respiratory illnesses, such as second hand smoking, affect type I IFN production in infants and young children is also ignored.

Here, we investigate the production of type I IFNs in infants during their first acute respiratory infection due to influenza virus, RSV, or human metapneumovirus (hMPV) in the context of important epidemiological variables for disease severity, including breastfeeding, gender, second hand smoking, age and family history of asthma.

METHODS

Study population

Full-term infants were prospectively enrolled between June and September 2002 (12) and very low birth weight (VLBW) infants (<1500 g) at high risk for pulmonary disease between June 2003 and May 2005 (13) as part of studies to assess the impact of respiratory viruses in acute pulmonary illness. Children were evaluated during their first upper respiratory infection (URI) or lower respiratory infection (LRI). Written, witnessed informed consent was obtained from the parents or guardians of the infants. The Institutional Review Boards of all participating institutions approved the protocol.

Demographical and clinical data on eligible infants were collected by study physicians as previously described (12,13). An infant was defined as breastfeeding if he or she received breast milk at least once daily. Criteria for hospitalization included respiratory distress and/or oxygen requirement (oxygen saturation <93% when breathing room air). Study physicians monitored whether previously enrolled infants had subsequently been admitted to

the hospital. High-risk infants did not receive a humanized monoclonal antibody against RSV (palivizumab) during the respiratory season, because of cost constraints.

Detection of viruses in nasal secretions

Nasal secretions were aliquoted, snap-frozen with dry ice and stored at -80°C until diagnostic testing or cytokine determinations were performed. Detection of hMPV was performed by RT-PCR using primers against a conserved region of the hMPV nucleocapsid protein, as previously described (12). Detection of RSV groups A and B and influenza virus types A and B was conducted by using the Hexaplex assay (Prodesse, Waukesha, WI, USA). Infants with mixed infections were excluded from the study (12,13).

Interferon detection by immunoassays

Levels of IFN- α and IFN- β were measured by sandwich ELISA (PBL Interferon Source, Piscataway, NJ, USA; catalogue No: 41100-1 and 41410) in nasal washes after homogenization and centrifugation. The sensitivity of the IFN- α assay is 12.5–500 pg/mL, and that of IFN- β is 25–2000 pg/mL.

Real time polymerase chain reaction for IRF-3, IRF-7, RIG-I, IFN- α and IFN- β

RNA was extracted from randomly selected nasal washes using RNA Easy Minikit (Qiagen, Valencia, CA, USA) and the DNAses protocol (Qiagen). The relative mRNA expressions were measured by Real Time PCR (ABI 7000 Sequence Detection System; Applied Biosystems, Carlsbad, CA, USA) using the TaqMan Gene Expression Assays IRF3-Hs00155574_m1, IRF7-0024219_g1 and RIG I HS00204833_m1, IFN- α Hs00356648_s1, IFN- β Hs01077958_s1 and ACTB 4333762 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Data were analysed using GraphPad Prism 5.0 (La Jolla, CA, USA) and Stata10.1 (Stata Corp, College Station, TX, USA). Student's *t*-test, Fisher exact, chi-squared test or ANOVA were used for comparisons wherever appropriate. Multiple logistic regression analyses were used to examine the associations between IFN production and epidemiological factors. A *p*-value <0.05 was considered significant.

RESULTS

Study population

This study was conducted in infants from two previously reported prospective studies on the role of primary viral respiratory infections (12,13). Type I IFN was assayed in nasal secretions from 120 infants infected with influenza virus, RSV or hMPV. Of these 120 infants, 24 were positive by RT-PCR for influenza virus (17 for influenza virus type B and seven for influenza virus type A), 30 were positive for hMPV and 66 were positive for RSV. Nasal secretions were unavailable for assay from 13 additional patients; two of them infected with influenza virus type B, four with hMPV and seven with RSV. No significant epidemiological or clinical differences were detected between this group of patients and the studied infants (data not shown).

Among the 120 infants, those infected with influenza virus were older than those infected with hMPV or RSV (Table 1). RSV and hMPV caused greater morbidity than influenza virus. Infections confined to the upper respiratory tract were more common in infants infected with influenza virus, while lower respiratory tract infections (mainly presenting as bronchiolitis) were more frequent in RSV and hMPV infected infants (Table 1). Eight percentage of infants infected with influenza virus were hospitalized compared to 23% of those infected with hMPV and 30% infected with RSV (Table 1). No difference in the duration of symptoms prior to sampling was observed between the groups (not shown).

Detection of type I interferon in nasal secretions

Overall, type I IFN was detected more often in infants infected with influenza virus than in infants infected with RSV or hMPV (Table 2). No differences were observed between infants infected with RSV subgroups A or B (not shown). Significant differences were apparent between infants infected with different viruses for IFN- α production (Table 2).

Type I IFN upstream signal mediators in nasal secretions

To explore the expression of upstream signal mediators in patients with detectable type I IFN, we performed real time PCR in nasal secretions for interferon regulatory factor-3 (IRF-3), IRF-7 and retinoic-acid inducible genes-I (RIG-I). Two pathways in upstream signal mediators of type I IFN have been described (14) and include the TLR/IRF-3 endosomal pathway and the RIG-I/IRF-7 cytosolic pathway. Higher levels of RIG-I mRNA expression were observed in infants infected with influenza virus when compared with those infected with RSV or hMPV ($p = 0.05$; Fig. 1B). We did not detect the differences in IRF-7 (Fig. 1A) expression between groups. All three signal mediators involved in the endosomal and cytosolic pathways were detected in patients infected with the three viruses (Fig. 1, IRF-3 not shown).

Breastfeeding is associated with enhanced type I IFN production during influenza virus infection

We then examined whether human milk affected the rate of production and level of type I IFN in infants during influenza virus infection. Interestingly, type I IFN detection was more frequent in breastfed compared with formula-fed infants infected with influenza ($p = 0.01$; Table 3). In addition, breastfeeding also affected type I IFN levels: mean IFN- α levels were 47.2 ± 21.3 pg/mL for breastfed infants compared with 1.9 ± 1.9 pg/mL in those receiving formula ($p = 0.01$; Fig. 2A). Mean IFN- β levels were 139.5 ± 74.7 pg/mL in infants drinking breast milk vs. 5.29 ± 5 pg/mL in those that did not ($p = 0.04$; Fig. 2B). IFN- α mRNA expression was higher in breastfed compared with formula-fed influenza infected infants ($p = 0.03$; Fig. 3). A similar trend was observed for IFN- β mRNA expression (not shown). Type I IFN production in infants infected with influenza was unaffected by age (as a continuous variable or divided in two groups of infants younger, or 180 days and older), gender or passive smoking (Table 3). Adjustment of the breast milk effect for these variables did not alter the outcome (Table 3). No differences in type I IFN production in association with any measured epidemiological variable were observed among infants infected with RSV or hMPV (not shown).

DISCUSSION

Our study describes a novel and important association between breastfeeding and a critical paediatric illness: breastfeeding is associated with enhanced production of type I IFNs against influenza virus in the respiratory tract of infants. Type I IFN production was only affected by human milk during influenza infections, and the cytokine was detected more frequently in infants with influenza than in those infected with hMPV or RSV.

Our findings suggest that certain protective mechanisms are triggered by human milk in the host, instead of being passively transferred by the mother (3–5). This hypothesis is supported by the differences in human milk-mediated type I IFN modulation in infections caused by different viruses. Viral single-stranded and double-stranded RNA with phosphorylated 5' ends activate several transcription factors and initiate the expression of type I IFN (14). These proteins activate hundreds of genes, including some with antiviral properties. In influenza virus, the non-structural protein 1 (NS1) modulates a variety of host cell functions and inhibits production of type I IFN at different levels and in a strain dependent manner (14), including the inhibition of RIG-I signal transduction (15). Whether breast milk directly modulates the inhibitory effect of NS1 on RIG-I or promotes type I IFN production by enhancing an alternative pathway remains to be determined.

Although infants infected with influenza virus were older than those infected with other viruses, type I IFN production was independent of age. However, IFN production in breastfed infants with influenza may explain why children infected with this virus present at older ages than those infected with RSV or hMPV (12,13,16). An exuberant IFN response elicited by influenza during the first months of life in breastfed infants may lead to *formes frustes* unable to establish illness. In fact, these observations may have important implications for the development of live attenuated influenza vaccines for young breastfeeding infants (17). Previous hypotheses ascribing the difference in age at consultation in those infected with different viruses to the presence of protective transplacentally acquired maternal antibodies against influenza cannot explain similar observations in infants born before the third trimester of pregnancy (and therefore lacking maternal antibodies) (13). Furthermore, the passive transfer theory fails to account for the yearly antigenic changes that occur with influenza virus (18).

Our findings confirm previous observations suggesting a higher rate of type I IFN detection in children with influenza (9). Hall et al. examined hospitalized infants and young children infected by this virus or RSV and described lower rates of IFN detection in RSV-infected subjects [(9); a finding replicated in adults (10)]. Contrary to its effect on influenza viral shedding, IFN production was not associated with diminishing concentrations of RSV in the respiratory tract (9).

Importantly, this is also the first report to describe the type I IFN responses in children infected with hMPV. Studies in mice detected high levels of type I IFN production after hMPV infection, and type I IFN genes and interferon-inducible proteins were upregulated in human-derived airway epithelial cells infected with hMPV and examined by microarray analysis (19–21). Human metapneumovirus lacks the non-structural genes with anti-

interferon activity that are present in RSV. Yet, as with RSV, IFN- α failed to reduce hMPV replication in lung and bronchial epithelial cells (22), and hMPV appears to replicate the observations described for RSV infections in infants in our study.

Our study has certain limitations, including the absence of numerous infants with severe disease caused by influenza virus (which would have required a very large population; 23) and the obvious inability to measure cytokines in the lower respiratory tract. However, several observations are reassuring that our findings are biologically meaningful including the confirmed association between type I IFN and influenza virus infections(9), the similar findings in infants infected with RSV subgroups A and B and previous reports of correlation between cytokine production in the upper and lower respiratory tract during viral respiratory infections (24,25).

In summary, our findings identify a potential novel mechanism for breast milk-mediated protection against a critical paediatric pathogen: breastfeeding is associated with higher rates and levels of type I IFN production against influenza virus. This association appears to be specific, as it is not observed during RSV or hMPV-mediated illness. Furthermore, these observations provide further support to the existent hypothesis that breast milk protects against respiratory viruses by activating innate antiviral mechanisms in the host (4).

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Figure 1.

Detection of upstream signal mediators of type I IFN in randomly selected samples from each viral infection. Relative ratios by virus subgroup for mRNA transcripts of IRF-7 (A) and RIG-I (B) compared with β -actin ($p = 0.05$).

Figure 2. IFN- α (A) and IFN- β concentrations (B) in pg/mL by breastfeeding status in infants with detectable antiviral cytokines. Horizontal bar represents median values.

Figure 3. IFN- α mRNA expression levels by breastfeeding status in randomly selected samples of infants infected with influenza virus ($p = 0.03$). Horizontal bar represents median values.

Table 1

Epidemiological and clinical characteristics of study patients with laboratory-confirmed respiratory viral infection: influenza virus (24), RSV (66) or hMPV (30)

	Influenza	RSV	hMPV	p
Age (d), mean $\pm$ SD	225 $\pm$ 95	168 $\pm$ 108	177 $\pm$ 98	0.02
Gender (male, %)	12 (50)	45 (68)	14 (47)	0.16
Breastfeeding (%)	16 (67)	44 (67)	23 (77)	0.2
Second hand smoke (%)	13 (54)	38 (58)	18 (60)	0.81
Parental asthma (%)	5 (21)	16 (24)	5 (18)	1
VLBW (%)	5 (21)	20 (30)	7 (23)	0.65
Congenital heart disease (%)	2 (8)	6 (9)	4 (13)	0.65
URI (%)	19 (79)	24 (36)	9 (30)	0.001
LRI (%)	5 (21)	42 (64)	21 (70)	0.001
Hospitalization (%)	2 (8)	20 (30)	7 (23)	0.05

RSV = Respiratory syncytial virus; hMPV = Human metapneumovirus; LRI = Lower respiratory infection; URI = Upper respiratory infection; VLBW = Very low birth weight.

Table 2

Type I IFN production in infants infected with influenza virus, RSV or hMPV

	Influenza	RSV	HMPV	p
IFN α (%)	9 (37)	6 (9)	3 (10)	0.003
IFN β (%)	8 (33)	15 (23)	6 (20)	0.48
Type I IFN (%)	15 (62)	17 (26)	8 (27)	0.003

RSV = Respiratory syncytial virus; hMPV = Human metapneumovirus.

Table 3

Analyses of epidemiological variables potentially associated with type I IFN production in nasal secretions of infants infected with influenza virus

	<u>Univariate analysis</u>		<u>Multivariate analysis</u>	
	OR (95% CI)	p	OR (95% CI)	p
Age	5.33 (0.52–54.34)	0.16	7.36 (0.32–169.2)	0.21
Gender	0.7 (0.13–3.68)	0.67	1.79 (0.19–17.02)	0.61
Breastfeeding	24 (2.04–282)	0.01	53.7 (22–132.5)	0.02
Second hand smoke	0.53 (0.1–2.83)	0.46	0.39 (0.03–5.18)	0.48