

Lower interleukin-8 levels in airway aspirates from breastfed infants with acute bronchiolitis

Dixon D-L, Griggs KM, Forsyth KD, Bersten AD. Lower interleukin-8 levels in airway aspirates from breastfed infants with acute bronchiolitis.

Pediatr Allergy Immunol 2010.

© 2010 John Wiley & Sons A/S

Breastfeeding during the first 12 months of life confers demonstrable immunologic benefit against infective pathogens, including those of the respiratory tract. However, the mechanism by which the ingestion of human milk modifies immunologic defense against such pathogens remains elusive. Bronchiolitis, caused predominantly by respiratory syncytial virus, is the most common clinical presentation of severe upper respiratory illness requiring hospitalization in infants and remains one of the developed world's leading causes of infant mortality and morbidity over both the short and long term. The mechanism by which an early, severe case of bronchiolitis can result in the development of recurrent childhood wheeze or asthma is unclear; however, mucosal inflammation and pulmonary neutrophilia are believed to play a significant role. The aim of this study was to examine the immune response of breastfed infants hospitalized with severe bronchiolitis, compared with formula-fed controls. Nasopharyngeal aspirates (NPA) were collected from 18 infants (aged ≤ 12 months), seven breastfed and 11 formula fed and assayed by enzyme immunoassays for chemokines interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1. NPA cellular component was quantified by light microscopy. Breastfed infants had lower levels of the chemokine IL-8 in their nasal airways with a concurrent decrease in cellular infiltrate ($p \leq 0.04$). NPA cell number correlated with lactoferrin concentration ($p = 0.02$) but not with myeloperoxidase, suggesting the predominance of mature, secondary granule laden neutrophils. These findings indicate a potential mechanism of protective immune regulation during bronchiolitis in the breastfed infant.

**Dani-Louise Dixon^{1,2}, Kim M. Griggs²,
Kevin D. Forsyth³ and Andrew D.
Bersten^{1,2}**

¹Intensive and Critical Care Unit, Flinders Medical Centre, Adelaide, Australia, Departments of ²Critical Care Medicine and ³Paediatrics and Child Health, Flinders University, Adelaide, Australia

Key words: bronchiolitis; breastfeeding; chemokines; neutrophils; nasopharyngeal aspirate

Dr. Dani-Louise Dixon, Intensive and Critical Care Unit, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia

Tel.: +61 8 8204 5494

Fax: +61 8 8204 5751

E-mail: dani.dixon@flinders.edu.au

Accepted 29 January 2010

Bronchiolitis, primarily the result of infection with the respiratory syncytial virus (RSV), remains one of the developed world's leading causes of infant mortality and morbidity in both the short and long term. Despite this, neither an effective vaccine or treatment for active bronchiolitis nor a preventative for the development of recurrent childhood wheeze subsequent to infant bronchiolitis has been found (1, 2).

The etiology by which bronchiolitis predisposes infants to the development of recurrent wheeze or asthma is unclear. However, the extensive damage caused to the respiratory airways by acute inflammation is a probable causative factor. It is now recognized that initial exposure to pathogens such as RSV in the airways induces an acute mucosal inflammatory response including up-regulation of chemokines

such as interleukin (IL)-8, resulting in an influx and degranulation of polymorphonuclear granulocytes, particularly neutrophils, in the airways (3–5). Prolonged airway inflammation and tissue neutrophilia results in airway edema, sensory nerve stimulation and triggering of reflex bronchoconstriction, leading to airway hyper-responsiveness and persistent wheeze (6), as well as increased permeability of the airway mucosa to allergens (7).

Epidemiology of bronchiolitis has identified a number of risk factors including that of exclusive formula feeding. Breastfeeding, whether exclusively or partially and for variant periods of time, has been consistently demonstrated to confer benefit against both incidence and severity of bronchiolitis in infants (6–9). Human milk provides protection for the recipient infant by the transfer of a number of factors including anti-infectious agents such as immunoglobulins, lactoferrin and oligosaccharides, as well as immunomodulatory agents such as cytokines and viable leukocytes (10). However, the specific mechanisms by which human milk confers protection against bronchiolitis have yet to be elucidated.

In this study, we aimed at examining the effect of human milk feeding on chemotactic factors in the airways of infants with severe acute bronchiolitis. Therefore, nasopharyngeal aspirates (NPA), providing samples representative of the upper and lower airways (11, 12), were collected from infants hospitalized with bronchiolitis over a single winter season. NPA concentrations of the chemokines IL-8 and monocyte chemotactic protein (MCP)-1 and the neutrophil products myeloperoxidase (MPO) and lactoferrin were determined by immunoassay, and results were analyzed based on infant feeding regime.

Materials and methods

Subjects

Eighteen bronchiolitic infants provided a single NPA sample during the first 24 h of hospitalization in the pediatric ward of Flinders Medical Centre, Adelaide, South Australia. Clinical diagnosis of bronchiolitis was defined as wheezing illness in an infant (≤ 12 months of age) caused by a viral respiratory tract infection associated with hyperinflation. Information regarding current feeding regime including introduction of solids was obtained from each infant's mother at the time of enrollment, and infants were grouped as breastfed, if currently receiving any human milk, or formula fed, if only receiving formula or cow's milk, at the time of admission.

Clinical scores were calculated using the respiratory distress assessment instrument (RDAI) (13) within 6 h of sample collection. Briefly, clinical assessment of the respiratory variables wheezing and retraction was performed by a senior pediatric registrar trained in the use of this instrument, and a clinical score allocated. The maximum total clinical score was 8 (wheezing) + 9 (retractions) = 17. Demographic data and information relating to illness severity were collected *post hoc* from patient's medical records.

The study was approved by the Flinders Clinical Research Ethics Committee of Flinders Medical Centre, and infants were included in the study with the informed consent of each parent or guardian.

Collection and processing of NPA samples

All infants underwent a routine NPA for viral identification. The remaining aliquot of NPA not required for the purposes of diagnosis was made available for the study. For the nasal wash, infants were placed in the supine position, and one or two drops of sterile normal saline placed in each nostril. The nasal airway was suctioned with a fine catheter and fluid collected into a mucus trap (Busse Hospital Disposables, Hauppauge, NY, USA). About 0.5 ml of NPA from both nostrils is collected using this method. This fluid was diluted with 1–2 ml phosphate-buffered saline (PBS) and cells released from mucus by gentle pipetting prior to transfer into a centrifuge tube. Samples were made up to a final volume of 10 ml with PBS before centrifugation (800 g, 5 min) and aspiration and storage of supernatant at -80°C .

Viral assays

The NPA cell pellet, obtained as described previously, was resuspended 1:2 in PBS and an aliquot taken for standard diagnostic immunofluorescence for viral detection (RSV, human metapneumovirus [hMPV], influenza virus A and B, adenovirus, para-influenza viruses 1–3; South-Path, Adelaide, South Australia). A further aliquot was stored at 4°C prior to cell count by direct light microscopy. The remaining cells were snap frozen in liquid nitrogen and stored at -80°C until assay by standard diagnostic viral PCR for the viral panel, as above, with the addition of *Mycoplasma pneumoniae* (Microbiology and Infectious Diseases, Women's & Children's Hospital, North Adelaide, South Australia).

Immunoassays

NPA supernatant was assayed for total soluble protein (BioRad DC Protein Assay; BioRad Laboratories, Hercules, CA, USA) and for IL-8 and MCP-1 by enzyme linked immunosorbent assays (ELISA) developed in our laboratory using matched antibody pairs at concentrations recommended by the manufacturer (R&D Systems Inc., Minneapolis, MN, USA), as described previously (14). Subsequent assay for MPO and lactoferrin was performed on NPA samples by commercially available ELISA kits (R&D Systems Inc.).

Statistical analysis

Samples below the detectable limit for MPO (250 pg/ml) were assigned a value of half that level for statistical analysis. Because of the small sample size and divergent group sizes, difference between breastfed and formula-fed groups was determined by Mann–Whitney *U* tests with data presented as median (25th, 75th percentiles) or Spearman rank tests with data presented as percent of group (yes/total), as appropriate. Relationships between variables were assessed by Pearson correlation. *Post hoc* analysis of statistical power found a level of 84% for the primary outcome measure IL-8 with an alpha of 5%. A probability level of ≤ 0.05 was considered statistically significant, and all analyses were performed using SPSS for Windows 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Despite demographic similarity (Table 1), formula-fed infants tended toward greater disease severity, indicated by significantly longer total

Table 1. Demographic characteristics of infants with bronchiolitis by feeding type (median [25th–75th percentiles] or percentage [yes/no])

	Breastfed	Formula fed	p
n	7	11	
Age (months)	7 (4–9)	6 (4–6)	0.3*
Gestation (weeks)	40 (38–40)	39 (37–40)	0.4*
Male gender	71% (5/7)	82% (9/11)	0.6†
Weight (percentile)	75th (20th–90th)	78th (60th–95th)	0.6*
Family history of atopy‡	71% (5/7)	55% (6/11)	0.5†
Length of breastfeeding (months)§	7 (4–9)	0 (0–1)	<0.001*

*Mann–Whitney *U* test.

†Spearman rank test.

‡Immediate family member with asthma or allergic rhinoconjunctivitis.

§At the time of sample collection.

length of illness (first symptoms to discharge) than breastfed infants, with a trend toward a more common requirement for supplemental oxygen and intravenous therapy (IVT), and longer hospital admission (Table 2). While 72% (13/18) of all infants required some period of supplemental oxygen via nasal prongs, none required any form of mechanical ventilation. There was no difference in tachypnoea, oxygen saturation (SpO₂) or temperature between feeding groups at presentation in the emergency department (data not shown, $p > 0.3$) or in RDAI score at sample collection (Table 2).

Length of breastfeeding demonstrated a negative relationship with total length of illness ($r^2 = -0.376$, $p = 0.003$). Infants in the formula-fed group had ceased breastfeeding 1–5 months prior to onset of illness or had never received any breast milk (45%, 5/11). Four infants in the breastfed group were exclusively breastfed, receiving no other forms of milk, and three infants were partially breastfed, receiving either formula or cow's milk complimentary feeds. Solids were introduced to all infants over the age of 6 months (61%, 11/18) and two infants (11%) under 6 months.

As expected, the most common causal virus detected was RSV (Table 2). Two infants were positive for multiple viruses; RSV + hMPV and RSV + adenovirus, respectively. However, over a quarter (28%, 5/18) of infants had no detectable virus by either immunofluorescence or PCR.

Table 2. Infection status and severity measures in infants with bronchiolitis by feeding type (median [25th–75th percentiles] or percentage [yes/no])

	Breastfed	Formula fed	p
n	7	11	
Previous admission with URTI	43% (3/7)	55% (6/11)	0.6*
Duration of illness (days)	5 (4–6)	8 (6–11)	0.003†
Length of admission (days)	3 (2–4)	4 (3–5)	0.1†
Severity (RDAI score 1–17)	10 (7–12)	9 (6–12)	0.7†
Oxygen saturation (SpO ₂)	97 (91–98)	96 (93–96)	0.3†
Tachypnea‡	71% (5/7)	82% (9/11)	0.6*
Fever (>38°C)	43% (3/7)	18% (2/11)	0.3*
Supplemental oxygen >24 h	29% (2/5)	73% (8/11)	0.07*
Intravenous therapy	NIL	27% (3/11)	0.1*
Virus			
RSV+	57% (4/7)§	36% (4/11)	0.4*
MPV+	14% (1/7)	27% (3/11)	ND
Other¶	29% (2/7)	9% (1/11)	ND
All viruses negative	29% (2/7)	27% (3/11)	ND

RDAI, respiratory distress assessment instrument; RSV, respiratory syncytial virus.

*Spearman rank test.

†Mann–Whitney *U* test.

‡Respiratory rate > 50 breaths/min at >1 observation.

§One RSV+ also MPV+, one RSV+ also adenovirus+. ND – not done as $n < 3$.

¶Other – parainfluenza 3, adenovirus.

Those infants who were negative for all viruses had significantly lower RDAI score (6 [4–7] vs. 11 [9–12], $p \leq 0.001$) and were less likely to require IVT (0% [0/5] vs. 44% [4/9]), than their positive counterparts.

Inflammatory mediators in NPA

Total soluble protein was unrelated to feeding group (breastfed 321 [234–514] vs. formula fed 168 [54–574] $\mu\text{g/ml}$, $p = 0.4$) or any subject variable listed in Table 1 or Table 2 ($p > 0.09$) and was therefore used for normalization of soluble mediators to account for potential variability in sample dilution.

The neutrophil chemokine IL-8 was significantly decreased in NPA from breastfed infants when compared with formula-fed infants (Fig. 1a). However, the monocyte/macrophage chemoattractant MCP-1 was not different between breastfed and formula-fed groups (Fig. 1b). NPA IL-8 concentration was inversely related to degree of breastfeeding when the breastfed group was divided into exclusively and partially breastfed groups (exclusively BF 135 [24–206], partially BF 218 [176–218], formula fed 304 [270–388] pg/ml ; $p = 0.003$), while length of breastfeeding tended toward a negative correlation with IL-8 concentration ($r^2 = 0.178$, $p = 0.08$).

Cell counts were decreased in NPA from breastfed versus formula-fed infants (Fig. 2a). However, there was no correlation between IL-8 concentration and cell count ($r^2 = 0.02$, $p = 0.6$). NPA were then assayed for neutrophil products of degranulation, primary (azurophilic) granules containing MPO and secondary (specific) granules containing lactoferrin. Cell counts were not related to NPA MPO concentration ($r^2 = 0.05$, $p = 0.9$) but were to lactoferrin (Fig. 2b), indicating a predominance of mature, secondary granule laden neutrophils in the cellular infiltrate.

Discussion

Neutrophils are the primary leukocyte present in NPA from bronchiolitic infants (4, 5), with a contribution of sloughed epithelial cells in those with most severe infection (15). This pattern of cell prevalence in severe bronchiolitis concurs with our previous findings *in vitro* of the extensive epithelial damage resultant from neutrophil activation with RSV infection (16) and *in vivo* of markedly increased levels of plasma surfactant protein-B, a marker of alveolar epithelial damage (17). Therefore, it is not remarkable that the benefit of breastfeeding on infant bronchiolitis may be elicited via decreases in the neutrophil chemoattractant IL-8 and in airway cellular infiltrate and, or epithelial damage.

Both respiratory epithelial cells and alveolar macrophages are primary sources of IL-8 in RSV infection (18, 19), with subsequent addition by infiltrating neutrophils (20). Human milk contains many factors acting on both the innate and acquired immune responses which may regulate the production of IL-8 and subsequent neutrophil chemotaxis, as well as neutrophil activity (21). Milk-derived lactoferrin, present in quantities over 1000-fold greater than plasma (22), not only decreases epithelial production of IL-8 by inhibition of NF- κ B activation (23, 24) but may also decrease neutrophil response to this potent chemokine (25). Similarly, milk-derived oligosaccharides and procathepsin D alter the expression of neutrophil adhesion molecules (CD11b and L-selectin), thereby decreasing neutrophil traffic (26–28). In addition to these innate factors, we, and others, have demonstrated the presence of large amounts of immunomodulatory cytokines and functional, cytokine-producing leukocytes in human milk, some of which are altered in response to maternal RSV exposure (14, 22), and which can modify epithelial IL-8 production (23).

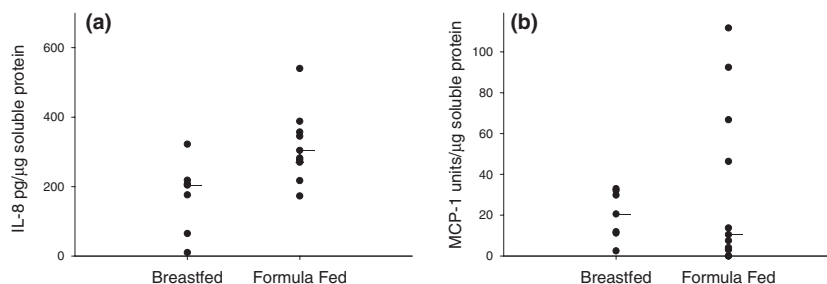


Fig. 1. Chemokines in nasopharyngeal aspirates (NPA) samples from bronchiolitic infants by feeding type. (a) NPA interleukin (IL)-8 was significantly decreased in infants who were breastfed when compared with those who were formula fed ($p = 0.01$). (b) NPA monocyte chemotactic protein (MCP)-1 was not different between the groups ($p = 0.8$). The median is represented by a black line, and data were compared with Mann-Whitney U non-parametric analysis.

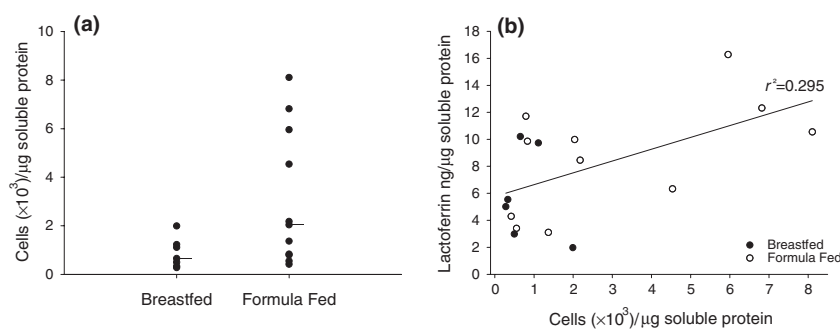


Fig. 2. Cellular infiltrate in nasopharyngeal aspirates (NPA) samples from bronchiolitic infants was decreased with breast-feeding and positively associated with concentration of lactoferrin, a product of neutrophil degranulation (a). NPA cell count was significantly decreased in infants who were breastfed when compared with those who were formula fed ($p = 0.04$). The median is represented by a black line, and data were compared with Mann–Whitney U non-parametric analysis. (b) NPA cellular infiltrate was directly related to the concentration of the neutrophil secondary granule mediator lactoferrin ($p = 0.02$). The relationship was examined with Pearson correlation.

Because of small sample size, a thorough analysis of the effect of exclusivity of breastfeeding and of various potential environmental confounders is not possible. Nishimura and colleagues (9) found that exclusive breastfeeding reduced the duration of hospital admission as well as the risk of respiratory failure, demonstrated via decreased requirement for supplemental oxygen because of hypoxemia (low SpO_2), while partial breastfeeding only partially conferred benefit. A similar pattern with degree of breastfeeding and IL-8 concentration, as well as requirement for oxygen (exclusively BF 25%, 1/4; partially BF 33%, 1/3; formula-fed (FF) 73%, 7/11; $p = 0.07$), was reflected in our smaller study group, adding to the proposition that exclusivity of breastfeeding relates to degree of protection. It should be noted that while there was only a trend toward lesser oxygen requirement in the breastfed groups for our study, supplemental oxygen was not administered solely in response to low SpO_2 but also to reduce work of breathing. Roine and colleagues (29) similarly found that exclusive breastfeeding decreased concentration of the activation marker sCD25 in association with RSV disease severity beyond that of partial breastfeeding. However, a number of environmental contributors such as parental smoking and respiratory illness in sibs were also identified.

Although a potent neutrophil chemoattractant, the concentration of IL-8 did not correlate with cells in NPA. Noah and colleagues (2002) demonstrated a weak correlation between IL-8 and neutrophil counts, but not epithelial counts, in NPA of bronchiolitic infants (15). Unfortunately, because of diagnostic protocols, differential cell counts were not obtained in our study; however, based on these data, we may hypothesize a contribution of epithelial cells to our total

NPA cells. As these two cell types are recognized as predominant in bronchiolitic airway aspirates and the presence of substantive numbers of epithelial cells is subsequent to increasing neutrophil infiltration and degranulation, a reduction in total cells relating to decreasing disease severity remains plausible.

Although no statistical difference was found in length of admission between feeding groups, the total length of illness was greater in formula-fed infants. This raises the possibility that IL-8 concentration is related to stage of illness at sample collection rather than feeding type. However, despite formula-fed infants presenting at the emergency department following longer duration of symptoms, there was no difference in disease severity by any clinical marker at this time. While it may be suggested that parents of breastfed infants attended the emergency department with their infant at an earlier stage of their bronchiolitic illness, similarity in both disease severity resulting in admission as well as in length of subsequent hospitalization does not support this. Therefore, the clinical parameters available, tachypnoea, SpO_2 , temperature and RDAI score, all suggest that sample collection occurred at the same point of acute disease in this group of infants.

This study provides the first clinical evidence for a potential link between breastfeeding and protection against neutrophil-mediated damage in infant bronchiolitis. Future studies utilizing larger cohorts will enable further examination of these findings, as well as investigation into the specific mechanisms involved.

Acknowledgments

The authors thank the Financial Markets Fund for Children, the Channel 7 Children's Research Foundation and

the Flinders Medical Centre Foundation which funded this research. The authors gratefully acknowledge the infants and parents who participated in the study. We also thank Dr. Victoria Atkinson for performing the RDAI scoring and Ms. Malgosia Krupa for technical assistance.

References

- OPENSHAW PJ, DEAN GS, CULLEY FJ. Links between respiratory syncytial virus bronchiolitis and childhood asthma: clinical and research approaches. *Pediatr Infect Dis J* 2003; 22: S58–64; discussion S64–5.
- HENDERSON J, HILLIARD TN, SHERRIFF A, STALKER D, AL SHAMMARI N, THOMAS HM. Hospitalization for RSV bronchiolitis before 12 months of age and subsequent asthma, atopy and wheeze: a longitudinal birth cohort study. *Pediatr Allergy Immunol* 2005; 16: 386–92.
- GAROFALO RP, PATTI J, HINTZ KA, HILL V, OGRA PL, WELLIVER RC. Macrophage inflammatory protein-1 α (not T helper type 2 cytokines) is associated with severe forms of respiratory syncytial virus bronchiolitis. *J Infect Dis* 2001; 184: 393–9.
- SMITH PK, WANG SZ, DOWLING KD, FORSYTH KD. Leucocyte populations in respiratory syncytial virus-induced bronchiolitis. *J Paediatr Child Health* 2001; 37: 146–51.
- BATAKI EL, EVANS GS, EVERARD ML. Respiratory syncytial virus and neutrophil activation. *Clin Exp Immunol* 2005; 140: 470–7.
- SIMÕES EA. Environmental and demographic risk factors for respiratory syncytial virus lower respiratory tract disease. *J Pediatr* 2003; 143: S118–26.
- KOEHOORN M, KARR CJ, DEMERS PA, LENCAR C, TAMBURIC L, BRAUER M. Descriptive epidemiological features of bronchiolitis in a population-based cohort. *Pediatrics* 2008; 122: 1196–203.
- ODDY WH, SLY PD, DE KLERK NH, et al. Breastfeeding, respiratory illness and infection in infancy: a prospective birth cohort study. *Arch Dis Child* 2003; 88: 224–8.
- NISHIMURA T, SUZUE J, KAJI H. Breastfeeding reduces the severity of respiratory syncytial virus infection among young infants. *Pediatr Int* 2009 [Epub ahead of print].
- RAUTAVA S, WALKER WA. Academy of Breastfeeding Medicine founder's lecture 2008: breastfeeding – an extrauterine link between mother and child. *Breastfeed Med* 2009; 4: 3–10.
- HALL CB, DOUGLAS RG JR, GEIMAN JM. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. *J Pediatr* 1976; 89: 11–5.
- BONT L, HEIJNEN CJ, KAVELAARS A, et al. Local interferon-gamma levels during respiratory syncytial virus lower respiratory tract infection are associated with disease severity. *J Infect Dis* 2001; 184: 355–8.
- LOWELL DI, LISTER G, VON KOSS H, MCCARTHY P. Wheezing in infants: the response to epinephrine. *Pediatrics* 1987; 79: 939–45.
- BRYAN DL, HART PH, FORSYTH KD, GIBSON RA. Immunomodulatory constituents of human milk change in response to infant bronchiolitis. *Pediatr Allergy Immunol* 2007; 18: 495–502.
- NOAH TL, IVINS SS, MURPHY P, KAZACHKOVA I, MOATS-STAATS B, HENDERSON FW. Chemokines and inflammation in the nasal passages of infants with respiratory syncytial virus bronchiolitis. *Clin Immunol* 2002; 104: 86–95.
- WANG SZ, XU H, WRAITH A, BOWDEN JJ, ALPERS JH, FORSYTH KD. Neutrophils induce damage to respiratory epithelial cells infected with respiratory syncytial virus. *Eur Respir J* 1998; 12: 612–8.
- WANG SZ, DOYLE IR, NICHOLAS TE, FORSYTH KD. Plasma surfactant protein-B is elevated in infants with respiratory syncytial virus-induced bronchiolitis. *Pediatr Res* 1999; 46: 731–4.
- NOAH TL, HENDERSON FW, WORTMAN IA, et al. Nasal cytokine production in viral acute upper respiratory infection of childhood. *J Infect Dis* 1995; 171: 584–92.
- BECKER S, QUAY J, SOUKUP J. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. *J Immunol* 1991; 147: 4307–12.
- JAOVISIDHA P, PEEPLES ME, BRES AA, CARPENTER LR, MOY JN. Respiratory syncytial virus stimulates neutrophil degranulation and chemokine release. *J Immunol* 1999; 163: 2816–20.
- GRAZIOSO CF, BUESCHER ES. Inhibition of neutrophil function by human milk. *Cell Immunol* 1996; 168: 125–32.
- LONNERDAL B. Breast milk: a truly functional food. *Nutrition* 2000; 16: 509–11.
- CLAUD EC, SAVIDGE T, WALKER WA. Modulation of human intestinal epithelial cell IL-8 secretion by human milk factors. *Pediatr Res* 2003; 53: 419–25.
- MULLIGAN P, WHITE NR, MONTELEONE G, et al. Breast milk lactoferrin regulates gene expression by binding bacterial DNA CpG motifs but not genomic DNA promoters in model intestinal cells. *Pediatr Res* 2006; 59: 656–61.
- CHACON-CRUZ E, OELBERG DG, BUESCHER ES. Human milk effects on neutrophil calcium metabolism: blockade of calcium influx after agonist stimulation. *Pediatr Res* 1999; 46: 200–7.
- KEENEY SE, SCHMALSTIEG FC, PALKOWETZ KH, RUDLOFF HE, LE BM, GOLDMAN AS. Activated neutrophils and neutrophil activators in human milk: increased expression of CD1 and decreased expression of L-selectin. *J Leukoc Biol* 1993; 54: 97–104.
- VETVICKA V, FUSEK M. Activation of peripheral blood neutrophils and lymphocytes by human procathepsin D and insulin-like growth factor II. *Cell Immunol* 1994; 156: 332–41.
- BODE L, RUDLOFF S, KUNZ C, STROBEL S, KLEIN N. Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil beta 2 integrin expression. *J Leukoc Biol* 2004; 76: 820–6.
- ROINE I, FERNANDEZ JA, VASQUEZ A, CANEO M. Breastfeeding reduces immune activation in primary respiratory syncytial virus infection. *Eur Cytokine Netw* 2005; 16: 206–10.