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Immunity to respiratory syncytial virus infection

A CLINICAL PERSPECTIVE

Afweer tegen respiratoir syncytieel virus infectie
een klinisch perspectief

(met een samenvatting in het Nederlands)

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Tina Elvira Faber

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te Leeuwarden

PROMOTOR:
Prof. dr. L.J. Bont

PARANIMFEN:
Dr. D.A.M. Perquin
Dr. J.S. de Graaf

BEOORDELINGSCOMMISSIE:
Prof. dr. M.J.N.L. Benders
Prof. dr. A.M. van Furth
Prof. dr. F.J.M. van Kuppeveld
Prof. dr. E.A.M. Sanders
Prof. dr. N.M. Wulffraat

CONTENTS

GENERAL INTRODUCTION

GENERAL INTRODUCTION

A mother presents her previously healthy 6 week old boy to the emergency department. He had a stuffy nose for 2 days with a low grade fever, but is now progressively coughing and breastfeeding less than normal. The pediatrician can see clear signs of respiratory distress with tachypnea, nasal flaring, wheezing and retractions. Although there are reassuring signs, he is a well-fed term baby and his oxygen saturation is 96%, he is young and she cannot predict his course of disease. She explains to he mother that she would like to admit him to the pediatric ward for careful observation.

This is the classic picture of primary Respiratory Syncytial Virus (RSV) bronchiolitis, a disease so common that it has affected all infants by the time they are 3 years old [1]. Globally, RSV is the most common cause of respiratory infection in young children leading to more than 3 million hospital admissions every year [2]. RSV is characterized by a seasonal and epidemic pattern. In The Netherlands, it causes approximately 2000 hospital admissions and 200 admissions to pediatric intensive care units every winter season [3,4]. Mortality rates in developed countries are low, but RSV causes more than 66,000 deaths worldwide each year [2,5]. Since its discovery 60 years ago [6,7], the virus itself has been largely unraveled (figure 1). Yet, there is no cure, and exact mechanisms of disease and immune responses remain a mystery. This research is vitally important to enable development of treatment or vaccines and reduce worldwide morbidity and mortality.

Many young patients with RSV remain asymptomatic or develop mild upper respiratory symptoms. In approximately one third of patients, the infection progresses to the lower respiratory tract causing bronchiolitis. Overall, 10% of patients require hospitalization and of those 10% are admitted to the pediatric intensive care[2]. (figure 2). It remains difficult to predict who will develop serious disease and why. Some risk factors are well established, they include prematurity and pre-existing medical conditions such as bronchopulmonary dysplasia (BPD), congenital heart disease (CHD), immunodeficiency such as HIV, and Down syndrome [5,8-11]. Other host factors associated with severe disease include male sex, small for gestational age, and a family history of atopy or asthma [9]. However, the largest risk factor for severe bronchiolitis is young age and the majority of infants hospitalized with RSV bronchiolitis are previously healthy without known risk factors [5,12-14].

Part of this can be explained by waning maternal antibodies, immature adaptive T-cel responses, direct cytopathological effects and in utero influences. Specific IgG antibody to RSV is passed through the placenta and colostrum and declines rapidly in the following 2-3 months [15,16]. The titer of maternal antibody correlates with protection against infection, severe disease, and hospitalization [16-20]. On the other hand, protection is short-lived and incomplete. Infants can be re-infected with the same strain of virus in the same season [21]. Even in adults, antibody produced after natural infection is poorly protective against reinfection. Another factor is that young infants have immature immune systems that lack adaptive cytotoxic T-cell responses reducing their ability to clear the virus. Indeed, many studies have shown a relationship between higher viral loads and serious disease[22-27]. By predominately infecting and completing its life cycle in the epithelium of airway cells, RSV can evade systemic immunity and cause direct cellular damage. In utero risk factors associated with more severe bronchiolitis such as smoking, diet and low cord blood vitamine D, may exert influences by altering the infant's developing immune system [15-17]. Alternatively, exposure of the fetal lung to pro-inflammatory signals such as high IL-8 en TNFα may induce protection to RSV [28].

Genetic susceptibility to RSV bronchiolitis is predominantly associated with innate immune genes. Polymorphisms in immune genes, such as variants in the 5q31 Th2 cytokine locus and interleukins such as IL-8, IL-10, and IL-4R, are associated with severe disease in infants [29-35]. Indeed, there is much evidence supporting a role for immune mediated pathology to RSV. Innate immune responses to RSV involve detection of the virus by pattern recognition receptors (PRR's) leading to upregulation of pro-inflammatory cytokines, chemokines, and anti-viral factors such as interferon. Neutrophils [36,37], dendritic cells [38] and natural killer (NK) cells [39] are then recruited into the airways contributing to further mediator production and a cascade of inflammation, destruction of infected cells and activation of T-cell responses. Regulatory T-cells, lower in children with bronchiolitis, help to balance T-cell response to RSV [40]. This cascade of inflammation in the neonate is a difficult area to study. Evidence from animal and in vitro studies may not necessarily reflect responses that occur during natural RSV infection in infants. The whole of the neonatal immune response is likely bigger than the sum of its parts. Early mediator responses from neutrophils, dendritic cells and NK-cells display complex interactions and are known to exert protective as well as pathogenic effects. Furthermore, most human studies focus on children hospitalized with severe disease making it difficult to ascertain whether the observed effects are protective or constitute exaggerated inflammation leading to severe disease.

Keeping in mind these obstacles, combined results from many dedicated researchers in the field have enabled a glimpse into the innate immune responses to RSV, in particular those potentially leading to severe disease. Host detection of RSV virus particles is essential for initiation of this response. Pattern recognition receptors important for recognition of RSV are TLR-4, TLR-3 and TLR-7 [41-43]. Neonatal PRR responses have been shown to be immature [44-46] potentially leading to exacerbated pathology as is seen in animal knock-out models [47-49]. PRR's, through NFkβ and IRF, lead to upregulation of pro-inflammatory mediators and chemotaxis of innate immune cells. Increased levels of IL-6, IL-8, IL-9 and TNFα have been found in lavage fluids in infants with RSV bronchiolitis [50,51]. IL-8 attracts neutrophils that destroy infected cells and can damage surrounding tissue and are believed to be the major recruited leucocyte in the early stages of RSV bronchiolitis [36,37]. Interferons are also important in the initiation of the early immune response to RSV by induction of an anti-viral state of cells. Low IFNγ in nasal lavage in infants with RSV has been associated with more severe disease [52-55]. Neonatal dendritic cells and NK-cells are immature and less able to produce IFNα and IFNγ in response to RSV [56,57]. Interestingly, RSV itself appears equipped to influence IFN signalling. Nonstructural NS1 and NS2 proteins can inhibit IFN signaling [58-62] and RSV G-protein can inhibit chemokine attraction for NK-cells [63]. NK-cells produce IFNγ that help activate dendritic cells, prime appropriate T-cell responses and cause cytotoxic destruction of infected cells [64,65]. Taken together, there is evidence that the immaturity of dendritic and NK-cells in the neonate and/or altered cytokine and chemokine responses, such as low IFNγ and IL-12 [66] and high IL-6 and IL-23 [45,67], can lead to skewed T-cell responses promoting Th17 and Th2 rather than Th1, resulting in more severe disease [68]. IL-17 is produced by various cells, such as Th17 cells, dendritic cells, and NK-cells, and its family represents a distinct signaling system with pro-inflammatory responses and induction of neutrophils [69-74]. RSV infected mice produce Th17

cytokines and subsequent responses include neutrophil migration, lung inflammation, IL-13 production, mucus production and inhibited cytotoxic T-lymphocyte responses [75-77]. However, IL-17 responses to RSV in neonates and exact effector mechanisms are not completely understood. We studied chemokine and cytokine responses, and in particular IL-17 responses, during course of disease in young children hospitalized with RSV infection (Section 1, Chapter 1).

Within several hours after hospital admission, the baby deteriorates. His oxygen saturation falls below 90% and he is not able to feed. The pediatrician prescribes oxygen therapy and nasal gastric tube feeding to ensure adequate fluid intake and prevent aspiration. By doing this she adheres to the current international guidelines for the management of bronchiolitis, but a helpless feeling remains. She decides to try nebulization with hypertonic saline solution as it may improve mucociliary clearance.

Prevention of RSV-related hospitalization in high-risk groups can be achieved with prophylactic palivizumab [78,79], but there is no treatment for acute bronchiolitis. Despite ongoing widespread use of systemic corticosteroids, ribavirin, antibiotics and epinephrine, there is no evidence for efficacy of these interventions [80,81] and current guidelines do not recommend their use [82-85]. The American Association for Pediatrics advised the use of inhaled bronchodilators on a trial and error basis in individual patients [86], but recently withdrew these recommendations on the basis of a large meta-analysis [82,87]. Current therapy remains supportive and infants in respiratory distress are managed by nasogastric or intravenous fluids, supplemental oxygen, and non-invasive or invasive ventilatory support. The American Association of Pediatrics weakly recommends the use of hypertonic saline for hospitalized children [82]. Nebulized hypertonic saline improves mucociliary clearance [88] and has been reported to reduce length of hospital stay and provide symptomatic relief in patients with bronchiolitis [89-93], but its use remains controversial [91,94-98]. Studies often rely on subjective measurements of wheeze by auscultation. We used a respiratory acoustic monitor that digitally quantifies lung sounds to assess the effects of nebulization with hypertonic saline solution in children admitted for RSV bronchiolitis (Section 2, Chapter 6, 7, 8).

Immunocompromised patients, in particular hematopoietic cell transplant (HCT) recipients with impaired T-cell immunity, are generally regarded as high risk for developing severe RSV disease [99-103], but population based studies on incidence or severity of disease in children are lacking. In the past decade, small retrospective studies have found mortality rates varying from 0-7% among pediatric HCT recipients with RSV infection [104-108], but RSV attributability is difficult to confirm. Much higher rates of up to 50% have been reported in adult HCT patients with RSV infection [109]. Graft source, timing of RSV diagnosis and graft-versus-host disease (GVHD) are associated with disease severity [109], but morbidity and mortality are not always correlated with the degree of immune suppression [110]. This indicates an incomplete understanding of the immune response to viral respiratory infections in an immunocompromised host. Considering evidence supporting immune mediated pathology to RSV, it is conceivable that pediatric HCT patients whom are severely immunocompromised, may not necessarily progress to serious RSV disease after infection. Ribavirin, the only antiviral approved by the Food and Drug Administration for treatment of RSV infections in high-risk populations, is now widely used in adult HCT patients with RSV infection. A recent systematic review, based on retrospective and prospective observational studies, reported variable success for preventing RSV-associated morbidity and mortality, yet it recommends prompt and aggressive monotherapy or combination therapy with intravenous immunoglobuline (IVIG) or palivizumab in those patients with additional risk factors such as lymphopenia [109]. With lack of data on efficacy in the pediatric population and possible side effects, treatment with ribavirin in pediatric HCT patients with symptomatic RSV infection remains controversial. We performed an observational study on RSV incidence and disease outcome in pediatric HCT patients that do not standardly receive ribavirin (Section 1, Chapter 3).

RSV is not an exclusive childhood disease. Clinical symptoms in adults are similar to those in children with more severe disease occurring in the elderly and patients with underlying cardiopulmonary disease. But, as is the case in the pediatric population, RSV can cause considerable disease in those that are healthy and without risk factors. Reported incidences for healthy adult patients presenting with influenza-like illness to the general practice reach 20% [111]. In adults, respiratory failure occurs in 8-15% of patients and mortality rates of up to 9% have been reported [112-119]. The social, economic and health impact of RSV in adults appears to be substantial and comparable to that of influenza [118-121]. Routine testing for viral infections, including RSV, is not usually performed in adult patients. Therefore, not much is known about the incidence and clinical picture of RSV disease in hospitalized adult patients. Yet, it is recognized that RSV may play a significant role in the outcome of intensive care unit (ICU) patients 122. We performed a multi-center observational study of RSV incidence and outcome in adults admitted to the ICU (Section 2, Chapter 5).

The baby boy received oxygen therapy for 2 days and slowly improves. He recovers completely within 5 days and is discharged from the hospital. One month later his mother returns to the emergency department. She is very worried because, although less severe, her baby is showing similar signs of respiratory distress as before. He has a mild tachypnea and is clearly wheezing, symptoms comparable to asthma.

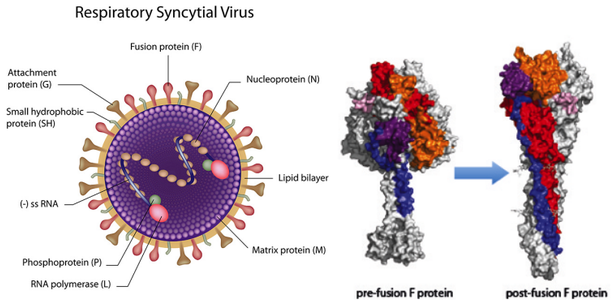
Unfortunately, RSV has long-term implications for respiratory health due to the strong association between RSV infection and persistent wheeze [123-135]. Almost 80% of children hospitalized for bronchiolitis go through episodes of wheezing in the first 2 years of life and symptoms of asthma may persist through childhood and adolescence [129,133,136,137]. Polymorphisms in the IL-10 family and IL-13 family, and population based twin studies, suggest a genetic link between RSV and asthma [33,138-143]. Predisposition to bronchial hyperresponsiveness may exist before RSV infection [144,145], but recent evidence suggests a causal link as prevention of RSV by administration of palivizumab also prevents post-bronchiolitis wheeze, both during and beyond infancy [146-148]. RSV, post-bronchiolitis wheeze and asthma have common pathophysiological mechanisms of disease displayed by airway inflammation, airway hyperresponsivenss, and mucus production. This link can potentially offer clues to RSV pathogenesis of disease, genetic predisposition and targets for intervention. Clinical and genetic studies indicate that IL1RL1 plays an important role in the development and exacerbations of asthma [149-154]. Human IL1RL1 encodes the receptor for IL-33 and has 3 isoforms that can modify T-helper responses by inhibition of IL-33 signaling [153,155-158]. Soluble IL1RL1 plays a role in the inflammatory response to epithelial damage and appears to be essential for the normal function of T-helper cells [156-158]. In mice, blocking IL1RL1a signaling diminished RSV induced eosinophil recruitment and the production of IL-33 and Th17 type cytokines indicating that IL-33 signaling is involved in

RSV induced Th2-associated airway inflammation [159]. We studied IL1RL1 polymorhisms for association with RSV disease and IL1RL1 nasopharyngeal concentrations in RSV bronchiolitis for association with RSV disease severity (Section 1, Chapter 2).

RSV causes an overwhelmingly complex immune response, but its effect on nasopharyngeal colonizing bacteria is not known. There is an association between RSV and subsequent pneumococcal infection, probably through increased adherence of pneumococci to epithelial cells by the RSV-G protein acting as a receptor [160-163], but this relationship is not entirely clear. Rates of serious bacterial infections in infants with RSV bronchiolitis are low and around 1% [164-167]. Recent evidence suggests existence of specific viral-microbial associations and children with RSV are distinctly characterized by a high abundance of colonizing streptococci [168,169]. However, directionality is not known; do viral infections increase bacterial populations, or does the microbial community create an environment suitable for specific viruses? RSV infects infants through a respiratory tract that is colonized with bacteria [170,171]. It is not known whether the acquisition and clearance of common viral infections during childhood, such as bronchiolitis, affects this process. The composition of this microbiome may have a major influence on the immune response. Considering future developments that may succesfully prevent RSV disease in young children, this is important to consider. Young children display high rates of nasopharyngeal pneumococcal colonization that decrease with age as their immune systems mature. We studied the dynamics of pneumococcocal carriage in young infants during the course of RSV bronchiolitis (Section 1, Chapter 4).

The aim of this thesis is to further understand immune responses and pathophysiological mechanisms of RSV disease from a clinical perspective. What innate immune responses play a role during course of disease? What is the natural course of disease in high-risk patients? Does the strong association between RSV and childhood asthma provide clues to genetic predisposition and development of severe disease? Furthermore, in light of current progress in development of vaccine candidates, it is important to study the potential interaction between with RSV and colonizing bacteria in the nasopharynx.

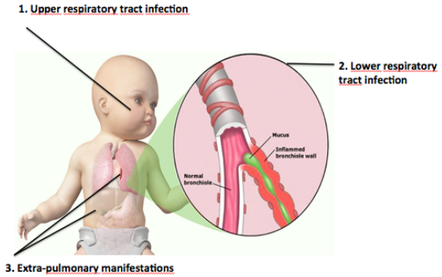
FIGURE 1 [68]



RSV is a single stranded RNA paramyxovirus with two circulating antigenic subgroups A en B. The virus particle consists of two major surface proteins: the fusion glycoprotein (F) and the attachment glycoprotein (G). RSV F is essential for entry, as it facilitates fusion of the viral membrane with the host cell plasma membrane, and its expression can also cause fusion with neighbouring cells [172]. The RSV F and G proteins are frequent targets for therapeutic intervention and vaccine development [173]. The viral envelope is made up of a small hydrophobic (SH) ion channel and an inner matrix (M) protein. Inside are several proteins that are involved in RNA binding (nucleoprotein N), RNA replication (phosphoprotein P, polymerase L, M2-1 and M2-2) and non-structural proteins that inhibit interferon responses (NS1 and NS2) [174].

Recently it was discovered that a compact folded prefusion RSV F is triggered to refold into an extremely stable six-helix postfusion conformation that brings the viral and host membranes together [173,175]. Both prefusion and postfusion conformations are present on the virion surface [176]. Palivizumab, a humanised monoclonal antibody that protect infants from developing severe disease, binds with postfusion RSV F antigenic sites II and prevents entry and subsequent infection of the host cell. It was approved by the FDA and is currently used for for immunoprophylaxis in high-risk infants [78,79]. Prefusion-specific antibodies have now been isolated that are much more potent than palivizumab and could have a major impact on prevention [177]. Surface epitopes of prefusion RSV F are also under investigation as targets for subunit vaccines intended for maternal immunisation [174,178].

FIGURE 2 [1,2,11,179]



1. Upper respiratory tract infection: The RSV virion infects the respiratory epithelium through eyes, nose and mouth causing upper respiratory symptoms including otitis media.

2. Lower respiratory tract infection: Bronchiolitis is caused by infiltration of inflammatory cells into the airspaces, mucus hyper-production, shedding of necrotic airway epithelial cells, and edema of the airway wall. These processes lead to a narrowing of the airway lumen, airflow obstruction, overinflation, and impaired gas exchange. Clinically, more severe RSV disease is characterized by crackles and wheezing, labored breathing, tachypnea, and hypoxia.

3. Extra-pulmonary manifestations: Seizures, cardiac failure and hepatitis are rare but have been described

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CHAPTER 1

SPECIFIC INCREASE IN LOCAL IL-17 PRODUCTION DURING RECOVERY FROM PRIMARY RSV BRONCHIOLITIS

Faber TE
Groen H
Welfing M
Jansen KJG
Bont LJ

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ABSTRACT

Although Respiratory syncytial virus (RSV) bronchiolitis is the most important cause of hospital admission for infants during the winter season, the pathogenesis is largely unknown. Interleukin-17 (IL-17) concentrations were studied in nasopharyngeal aspirates from 21 non-ventilated and 17 ventilated infants admitted to hospital with RSV bronchiolitis at time of admission and discharge from the hospital. On admission, nasopharyngeal concentrations of most cytokines and chemokines were lower in non-ventilated infants than in ventilated infants, reaching statistical significance for Eotaxin, IL-1α, and IL-6. During course of disease, nasopharyngeal concentrations of most cytokines and chemokines decreased, reaching statistical significance for IL-6 and IP-10. However, nasopharyngeal IL-17 concentrations were higher at discharge than at admission in children with non-ventilated RSV disease (209-101 pg/ml, P = 0.008), a response pattern not observed in ventilated RSV patients nor for other cytokines or chemokines. It is speculated that local IL-17 production may be involved during convalescence from RSV bronchiolitis in non-ventilated patients by facilitating innate and adaptive antiviral immune responses. The role of IL-17 in the pathogenesis of RSV bronchiolitis is to be explored further.

INTRODUCTION

Human respiratory syncytial virus (RSV) is a species in the genus Pneumovirus, family Paramyxoviridae. RSV bronchiolitis is the most common cause of hospitalization for infants during the winter season. Prematurity, chronic lung disease, congenital heart disease, Down's syndrome and neuromuscular disease are conditions associated with severe course of disease. [1-3] However, most children hospitalized for RSV bronchiolitis were healthy until infected. Viral loads have been associated with disease severity [4,5]. The precise mechanisms underlying human RSV bronchiolitis are largely unknown and most likely multi-factorial, in which both immunological and non-immunological responses, as well as genetic susceptibility play a role.

Interleukin-17 (IL-17) or IL-17A is a member of a family of cytokines with pro-inflammatory effector function. The IL-17 family, including IL-17A to IL-17F, represents a distinct signaling system and has been linked to many immune and auto-immune related diseases such as rheumatoid arthritis, lupus, allograft rejection and anti-tumor immunity, and specifically for IL-17F, allergic airway inflammation and asthma [6,7]. It was originally described as a product of Th17 cells, a distinct CD4+ T-cell subset bearing the IL-23 receptor [8]. It has now become clear that IL-17 is produced by various cells from the adaptive and innate immune system, such as γδ-T-cells, Tc17 cells and invariant natural killer T-cells (iNKT)-cells [8-10]. Although effector functions are incompletely understood, it is appreciated that IL-17 is capable of inducing a specific pro-inflammatory immune response through the production of many other cytokines, chemokines and prostaglandins. During infection, IL-17 induces a tissue response required for clearance of bacteria, viruses and fungi, possibly through induction of a neutrophil response [11].

There are only limited data on the role of IL-17 in the defense against viral infections, in particular against RSV infection.

In the current study, it was hypothesized that IL-17 production plays a role during convalescence of RSV disease. IL-17 was therefore studied during the acute phase and at recovery of disease in a cohort of infants hospitalized for RSV bronchiolitis. Differences in severity of disease was differentiated by need for mechanical ventilation. It was shown that local IL-17 production increases during recovery in children with hospitalized non-ventilated RSV bronchiolitis, suggesting that IL-17 may play a protective role against the development of severe RSV disease requiring mechanical ventilation.

METHODS

Patients: Children aged under 13 months with symptoms of lower respiratory tract infection admitted to the pediatric ward of two hospitals in The Netherlands were included during two consecutive winter seasons. Symptoms of lower respiratory tract infection were severe chest cough, wheezing, hoarseness, stridor, shortness of breath, cyanosis and apnea. Children were included after nasopharyngeal aspirates were found positive for RSV by direct immunofluorescence, diagnosis was later confirmed by culture and polymerase chain reaction (PCR). Infants born prematurely with congenital heart disease or chronic lung disease, infants with wheezing illness before RSV bronchiolitis was diagnosed, and patients using corticosteroids or bronchodilators were not included. Differences in severity of RSV disease was differentiated by need for mechanical ventilation. All infants admitted to the pediatric intensive care unit were intubated and mechanically ventilated, therefore stay at the intensive care unit was identical to the requirement of mechanical ventilation.

The study was performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki and approved by the local Medical Ethics Committee at the Medical Center Leeuwarden. All parents or guardians gave written consent to participate in this study.

Nasopharyngeal aspirates: Undiluted nasopharyngeal aspirates were taken within 24 hours after admission and at discharge from the hospital. Nasopharyngeal secretions were gently aspirated by an experienced physician with a 3.3 mm suction catheter, placed on ice immediately and stored at -80 degrees for later analysis. Xylomethazolin or NaCl 0.9% nasal spray was not given in the 6 hours prior to aspiration. Cytokine and chemokine concentrations were measured in all aspirates using a highly specific multiplex assay [12]. Sample collection and processing has been described previously [13,14]. Samples were weighed, diluted as necessary, sonicated, centrifuged, and duplicate assays were performed on each. In addition to IL-17 (detection limit 10-5,000 pg/ml), the following cytokine and chemokine concentrations were measured: Eotaxin, IL-1α, IL-6, interferon (IFN), inducible protein 10 (IP-10), IL-1β, monokine induced by IFN-γ (MIG), macrophage inflammatory protein 1α (MIP-1α), soluble intercellular adhesion molecule 1 (sICAM1), and IFN-γ (detection limits 5-5,000 pg/ml).

Statistical analysis: Paired cytokine and chemokine concentrations at time of admission in non-ventilated and ventilated infants were compared by Mann-Whitney U test. Mann-Whitney U test was also used to compare paired cytokine and chemokine concentrations at time of admission versus time of discharge from the hospital in both non-ventilated and ventilated patients. All tests of significance were two-sided. P-values < 0.05 were considered to be statistically significant.

RESULTS

The patients investigated consisted of 21 non-mechanically ventilated infants and 17 mechanically ventilated infants. Except for length of hospital stay, no significant differences were found in baseline characteristics between infants with or without need for mechanical ventilation. There was an equal distribution of male infants in both groups (43% versus 59%). The median age at time of admission and the percentage of infants born prematurely before 37 weeks gestation was similar in non-ventilated and ventilated infants (14 versus 9 weeks, and 14% versus 29% respectively). In both groups, a similar number of children had been ill ≥ 3 days before admission (76% versus 53%). Median length of hospital stay was significantly longer in children with severe RSV disease requiring mechanical ventilation (6 days versus 10 days; Table I).

Concentrations of IFN-γ were below the level of detection in all samples. For each inflammatory mediator, analyses were made comparing time of admission to discharge in non-ventilated as well as ventilated patients. Furthermore, cytokine and chemokine concentrations at time of admission were compared in both groups. Finally, an attempt was made to analyze emerging patterns during course of disease. At time of admission, there was no difference in IL-17 concentration between non-ventilated and ventilated infants (101 versus 72 pg/ml; not significant). Median nasopharyngeal concentrations of Eotaxin, IL-1α, and IL-6 were lower in non-ventilated patients (18 vs 118 pg/ml, 368 vs 4001 pg/ml, 362 vs 12500 pg/ml respectively; $p<0.001$). IL-17 concentrations were significantly higher at discharge than at admission (209 versus 101 pg/ml; $p=0.0083$) in non-ventilated infants. IL-17 concentrations did not increase during course of disease in ventilated infants (Figure I). Concentrations of other cytokines and chemokines followed equal patterns in both groups, and either decreased ($p< 0.05$ for IL-6 and IP-10) or remained stable ($p\geq 0.05$ for Eotaxin, IL-1α, IL-1β, MIG, MIP-1α and sICAM1) during hospitalization

(table II). In both ventilated and non-ventilated infants, length of hospital stay was not correlated to IL-17 concentration at discharge. Furthermore, length of hospital stay was not correlated to the difference in IL-17 concentration between admission and discharge. Age at admission was not correlated to IL-17 concentration at time of admission nor at time of discharge.

DISCUSSION

In this study, local IL-17 production increased during the course of disease in non-ventilated infants with RSV bronchiolitis. Other inflammatory mediators either decreased or remained stable during course of disease in both ventilated and non-ventilated infants. These results could suggest that local IL-17 may play a protective role against the development of severe RSV bronchiolitis requiring mechanical ventilation. Furthermore, it implies that mechanisms that determine disease severity are apparently different than those that are involved in resolution of disease.

At time of admission, children requiring mechanical ventilation for RSV disease had higher levels of Eotaxin, IL-1α and IL-6. Although it is not known whether cytokine concentrations are effected by mechanical ventilation, these results are in accordance with previous research showing high initial concentrations of pro-inflammatory mediators in children with most severe disease [15-19]. Therefore, this study confirms that development of more severe RSV bronchiolitis is associated with a more robust cascade of inflammatory responses. Because most of these mediators can be produced by airway epithelium in response to RSV infection, this observation suggests, as is generally accepted in the case of RSV, a critical role for the epithelium as a first line of defence [20].

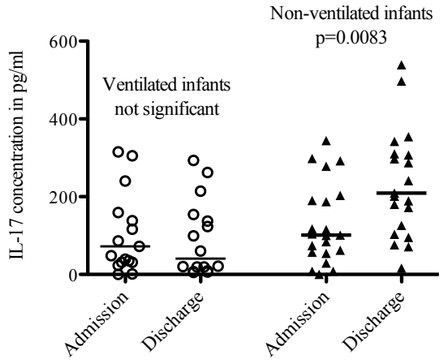
IL-17 is a pleiotropic cytokine, originally thought to be produced only by T-cells, but is now known to be produced by innate immune cells, such as dendritic cells, iNKT and as γδ-T-cells. The IL-17 receptor is expressed ubiquitously in many tissues, including the lungs. [10] The role of IL-17 in host defence against viruses is understood incompletely. In general, antiviral defence depends heavily on type I IFNs, which are not known to be regulated by IL-17. Nevertheless, during experimental influenza infection, IL-17 production by CD8+ T-cells protects mice against disease and death. [9,11,21] In this study, IL-17 increases during the course of disease in children with less severe RSV disease. This is in accordance with a recent study in which plasma IL-17 could not be detected in healthy children, and the highest concentration was found in moderately ill patients with RSV bronchiolitis when compared with severe cases [22]. The source of IL-17 and the precise mechanism underlying local IL-17 production are not known and it remains unclear why the IL-17 response does not occur in ventilated children. It can be considered that adaptive immune cells, such as γδ-T-cells or Tc17 cells, are the source of increased IL-17 production. Another study did show increased IL-17 levels in tracheal aspirate samples from severely ill infants with RSV infection [23]. The power of our study may have been too small to detect differences in this subpopulation. In addition, these children are generally younger and they may have different mechanisms of viral clearance. Also, it is not known whether intubation and mechanical ventilation itself have an effect on production of IL-17.

This study is the first attempt to measure local cytokine profiles during the course of disease in ventilated and non-ventilated patients with RSV bronchiolitis. A unique response pattern of IL-17 was identified which contrasts with other pro-inflammatory cytokines and chemokines that are associated with initial disease severity and generally decrease during the course of disease. In addition, the use of nasopharyngeal aspirate allowed for a measurement of absolute cytokine concentrations, which is not possible using other techniques, such as nasal washes. And although the association between nasopharyngeal aspirate cytokine concentrations and endotracheal cytokine concentrations is high [13,24], there are unknown variables to be kept in mind when interpreting results that may influence mucus secretion, such as mechanical ventilation and respiration rate. Also, as the time between admission and discharge and hence the time between measurements is shorter in non-ventilated patients when compared to ventilated patients, this may potentially introduce a systemic error. Cytokine and chemokine response is a dynamic process and differences in sampling time may have a distorting effect. A further study limitation is the absence of proof that the increase in local IL-17 levels during the course of RSV bronchiolitis is causally related to less severe disease. In addition, the relatively small study population did not allow for extensive regression analysis to study potential confounders, in particular age, premature birth, length of hospital stay, and mechanical ventilation. Yet it is reassuring that length of hospital stay is not correlated to IL-17 concentration at discharge, nor to differences in concentration between admission and discharge. And although age at admission is an important variable to consider, it was not correlated to IL-17 concentration at time of admission nor at time of discharge. Another limitation in this study is not to have considered the quantification of viral load from respiratory secretions that would have allowed correlation of clinical and immunologic data.

CONCLUSION

IL-17 levels in the airways of RSV bronchiolitis patients increase during the course of disease in hospitalized non-ventilated patients, suggesting a role in controlling of the disease. Further identification of the role of IL-17 in recovery from RSV bronchiolitis may lead to further insights into pathogenesis of disease.

FIGURE I: Local IL-17 concentration increases during course of disease in non-ventilated infants with RSV bronchiolitis.



IL-17 concentration increases from time of admission to discharge from the hospital in 19 out of 21 (90%) non-ventilated children hospitalized for RSV bronchiolitis (median 101 to 209 pg/ml; p=0.0083) and remains stable in ventilated infants (median 72 to 41 pg/ml; not significant).

TABLE I: Subject characteristics

	Non-ventilated infants (n=21)	Ventilated infants (n=17)	Non-ventilated vs ventilated infants
Number of male infants	9 (43%)	10 (59%)	NS #
Prematurity (amenorrhoe <37 weeks of gestation)	3 (14%)	5 (29%)	NS #
Age at admission (median in weeks after birth (CI))	14 (13-26)	9 (6-29)	NS *
Symptoms ≥ 3 days before admission	16 (76%)	9 (53%)	NS #
Length of hospital stay (median in days (min-max))	6 (3-13)	10 (5-28)	P< 0.001 *
Length of ventilation (median in days (min-max))	NA	9 (5-16)	

NS = Not significant. NA = Not applicable to non-ventilated infants.
# = Fisher's exact test. * = Mann-Whitney U test *

TABLE II: Chemokine and cytokine analysis of nasopharyngeal aspirates of ventilated and non-ventilated infants with RSV bronchiolitis during course of disease

	Non-ventilated infants		Ventilated infants		Analyses			Pattern
	Admission (1)	Discharge (2)	Admission (3)	Discharge (4)	P (1) vs (2)	P (3) vs (4)	P (1) vs (3)	
IL-17	101 *	209	72	41	<0.05	NS	NS	↑
	56-197 #	115-326	31-200	12-150				
IL-6	362	47	12500	6666	<0.001	<0.05	<0.001	↓
	107-2021	26-97	6666-24750	2130-6666				
IP10	325	132	221	121	<0.05	<0.05	NS	↓
	201-453	1-278	142-201	65-251				
Eotaxin	18	22	118	137	NS	NS	<0.001	→
	11-50	15-56	56-277	73-338				
IL-1α	368	248	4001	3091	NS	NS	<0.001	→
	94-868	49-1629	2080-9703	1519-7048				
IL-1β	132	130	203	111	NS	NS	NS	→
	62-176	64-282	79-254	68-177				
MIG	1173	574	1463	1018	NS	NS	NS	→
	545-2229	213-1935	788-2272	477-1618				
MIP-1α	2243	2382	2325	2499	NS	NS	NS	→
	1097-3163	845-4201	1949-3218	1172-4065				
sICAM	40459	40986	46820	32982	NS	NS	NS	→
	27889-49966	27117-59873	34314-59871	19303-52253				

NS = Not significant.
Cytokine concentrations in median* and quartiles# in pg/ml.

In contrast to local IL-17 concentrations which increase (↑) during course of disease in non-ventilated children with RSV (specifically p=0.0083), other inflammatory mediators show the same pattern in non-ventilated and ventilated children and either decrease (↓) during course of disease, such as IL-6 and IP-10, or remain stable (→) such as Eotaxin, IL-1α, IL-1β, MIG, MIP-1α and sICAM.

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CHAPTER 2

IL1RL1 GENE VARIANTS AND NASOPHARYNGEAL IL1RL1-A LEVELS ARE ASSOCIATED WITH SEVERE RSV BRONCHIOLITIS: A MULTICENTER COHORT STUDY

Faber TE
Schuurhof A
Vonk A
Koppelman GH
Hennus MP
Kimpen JL
Janssen R
Bont LJ

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ABSTRACT

Targets for intervention are required for respiratory syncytial virus (RSV) bronchiolitis, a common disease during infancy for which no effective treatment exists. Clinical and genetic studies indicate that IL1RL1 plays an important role in the development and exacerbations of asthma. Human IL1RL1 encodes three isoforms, including soluble IL1RL1-a, that can influence IL-33 signalling by modifying inflammatory responses to epithelial damage. We hypothesized that IL1RL1 gene variants and soluble IL1RL1-a are associated with severe RSV bronchiolitis. We studied the association between RSV and 3 selected IL1RL1 single-nucleotide polymorphisms rs1921622, rs11685480 or rs1420101 in 81 ventilated and 384 non-ventilated children under 1 year of age hospitalized with primary RSV bronchiolitis in comparison to 930 healthy controls. Severe RSV infection was defined by need for mechanical ventilation. Furthermore, we examined soluble IL1RL1-a concentration in nasopharyngeal aspirates from children hospitalized with primary RSV bronchiolitis. An association between SNP rs1921622 and disease severity was found at the allele and genotype level (p=0.011 and p=0.040, respectively). In hospitalized non-ventilated patients, RSV bronchiolitis was not associated with IL1RL1 genotypes. Median concentrations of soluble IL1RL1-a in nasopharyngeal aspirates were >20-fold higher in ventilated infants when compared to non-ventilated infants with RSV (median [and quartiles] 9,357 [936-15,528] pg/ml vs. 405 [112-1,193] pg/ml respectively; p<0.001). We found a genetic link between rs1921622 IL1RL1 polymorphism and disease severity in RSV bronchiolitis. The potential biological role of IL1RL1 in the pathogenesis of severe RSV bronchiolitis was further supported by high local concentrations of IL1RL1 in children with most severe disease. We speculate that IL1RL1a modifies epithelial damage mediated inflammatory responses during RSV bronchiolitis and thus may serve as a novel target for intervention to control disease severity.

INTRODUCTION

Respiratory syncytial virus (RSV) bronchiolitis is the most common cause of hospitalization for infants during the winter season. About 1-2% of all children are hospitalized for RSV bronchiolitis, mechanical ventilation is required in 10% of hospitalized cases [1]. Approximately half of the infants with RSV lower respiratory tract infection (LRTI) go on to have recurrent wheezing episodes until they reach school age [2-4]. The overall risk of concurrent bacterial infection is low, yet the reported incidence of bacterial pneumonia in children with severe RSV infection requiring ventilation ranges from 9%-44% [5-13]. Mechanisms underlying severe respiratory syncytial virus bronchiolitis are incompletely understood. There is increasing evidence, from both clinical and genetic studies, that IL1RL1 plays an important role in the development of childhood asthma [14-18]. IL1RL1 gene cluster polymorphisms were found to be associated with asthma and atopy [14,15,18]. Human IL1RL1 encodes the receptor for interleukin-33 (IL-33) and has three isoforms, including soluble IL1RL1 (IL1RL1-a), that can modify T helper cell responses by inhibition of IL-33 signalling [17,19-23]. IL1RL1-a can be induced by pro-inflammatory stimuli and appears to be essential for the normal function of T helper cells [20-22]. Clinically, IL1RL1 genetic polymorphisms and IL1RL1-a serum levels have been associated with severe arthritis, acute heart disease, and airway disease such as asthma [24-28].

METHODS

Ethics Statement: The study protocol was approved by the institutional review board "RTPO" at the Medical Center Leeuwarden in and the medical ethics committee "METC" at the University Medical Center Utrecht in The Netherlands. All parents of hospitalized infants agreed to participate and gave written informed consent.
Objectives: We hypothesized that IL1RL1 plays a role in the pathogenesis of severe RSV bronchiolitis. To this end, we analyzed IL1RL1 single-nucleotide polymorphisms (SNPs) for association with RSV disease. Furthermore, we examined the association between local IL1RL1-a concentrations and RSV disease severity.
Participants: In a multicenter cohort study, previously healthy infants under 1 year of age hospitalized with a first episode of RSV bronchiolitis were included from October 2007 until March 2009 in fifteen large urban hospitals in The Netherlands. Infants with Down syndrome, a history of wheezing, or cardiac or pulmonary pathology were excluded. RSV infection was confirmed by positive immunofluorescence in epithelial cells from nasopharyngeal aspirates (NPAs) as described previously [29,30]. Severity of RSV illness was distinguished by need for mechanical ventilation, apparent by intubation and admission to a Pediatric Intensive Care Unit (PICU). Indication for mechanical ventilation in all centers was: severe respiratory distress or exhaustion, apnea's, respiratory acidosis (pH < 7.25), or hypoxia (oxygen saturation < 92% despite oxygen therapy). For the genetic cohort study, only children of Dutch ethnicity were selected. The control population consisted of 930 healthy Dutch children that were randomly taken from the Regenboog study, a large Dutch population health examination survey [31].
In a subgroup of hospitalized patients (n=207) with primary RSV disease, IL1RL1-a concentrations on the day of admission were measured in NPAs.
Investigations: Three SNPs were chosen on the basis of their potential functionality or their association with asthma in previous association studies [14,15,18,32,33]. For each SNP 1.5 µl of genomic DNA was used at 7 ng/µl. SNPs rs1921622, rs11685480 and rs1420101 present in intronic and 5'near-gene regions of the IL1RL1 were selected for genotyping by SNP Genotyping Services at KBioscience (Hoddesdon, United Kingdom) with the KASPar technology and compared to healthy controls in the population. IL1RL1-a concentrations on the day of admission were measured in NPAs using ELISA (R&D systems, Abingdon, United Kingdom).
Statistical Methods: For the genetic study, a convenient genetic cohort of 465 patients and 930 controls was used. Because IL1RL1 SNPs were specifically tested, no multiple testing was required. Genotyping data were viewed graphically as a scatter plot with SNPviewer2. All SNPs were analyzed for association with RSV disease, both at the allele level (df = 1) and at the genotype level (df = 2) by Kruskal-Wallis test. Furthermore, a subanalysis was performed for association between SNPs and severe RSV disease characterized by need for mechanical ventilation. Association between IL1RL1-a concentration and RSV genotype was examined by Kruskal-Wallis test. The clinical study including 20 ventilated and 187 non-ventilated patients was designed to detect an arbitrarily chosen difference in IL1RL1-a concentrations of 180 pg/ml with a standard deviation of 200 and a power and significance of 0.8 and 0.05 respectively for a two-sided test. Mann-Whitney U test was used to compare IL1RL1-a concentrations on the day of admission in non-ventilated versus ventilated infants. All tests of significance were two-sided. P-values < 0.05 were considered to be statistically significant.

RESULTS

In the genetic cohort study, a total of 465 Dutch children from 0-12 months of age hospitalized with primary RSV bronchiolitis were included and compared to 930 healthy Dutch controls in the population. Polymorphisms tested were in Hardy-Weinberg equilibrium. RSV bronchiolitis was not associated with selected genotypes rs1921622, rs11685480 or rs1420101 (p>0.05, table 1). Further analysis of the hospitalized infants showed an association between disease severity for the IL1RL1 SNP rs1921622 at the allele level (p=0.011) and the genotype level (p=0.04) (table 2).
We further investigated our genetic finding of a potential role of IL1RL1 in severe RSV bronchiolitis by analyzing the relationship between local IL1RL1-a concentration and disease severity. In 207 hospitalized infants with RSV bronchiolitis, 20 ventilated and 187 non-ventilated, NPA was available to measure local IL1RL1-a levels. As expected, gestational age was lower, and length of hospital stay was longer, in children requiring mechanical ventilation (38.7 weeks vs. 39.7 weeks, p=0.032; 14 days vs. 4 days, p<0.001). All other variables, such as age at admission, male gender, breastfeeding, older siblings, smoking in the household or during pregnancy, born during RSV season, daycare attendance and atopy did not differ between groups. (table 3) Median concentrations of IL1RL1-a in nasopharyngeal aspirates were >20-fold higher in ventilated infants when compared to non-ventilated infants with RSV (median [and quartiles] 9,357 [936-15,528] pg/ml vs. 405 [112-1,193] pg/ml respectively; p<0.001, figure 1). Regression analysis showed that this effect was independent from gestational age. IL1RL1-a concentrations were not associated with any of the 3 IL1RL1 SNPs. 3/20 (15%) of ventilated patients had evidence of concurrent bacterial pneumonia by definition of a positive bacterial culture on tracheal aspirate with single growth >100 per visual field on the day of intubation (2 patients with Haemophilus influenzae and 1 patient with Moraxella catarrhalis).

DISCUSSION

This study shows an association between the intron SNP rs1921622 and severe RSV disease requiring mechanical ventilation. The association between local IL1RL1-a levels and disease severity found in this study, warrants speculation on a potential role of IL1RL1 in modifying RSV disease severity. IL1RL1 is a member of the Toll-like receptor (TLR) superfamily and can affect Th2 responses by influencing Toll-like receptor pathway signalling. [23,34-36]. IL1RL1 is located on chromosome 2q12. IL1RL1 translation results in 3 isoforms, of which IL1RL1-a is soluble [37]. The IL1RL1 gene encodes the receptor for interleukine (IL) 33, and is located on mast cells, T helper type 2 (Th2) cells, regulatory T cells, and macrophages [38-40]. IL-33 stimulates Th2 cytokine responses, such as IL-4, IL-5 and IL-13, that induce eosinophilic influx, airway inflammation, airway hyperresponsiveness, and mucus production [23]. IL1RL1-a may act as a decoy receptor for IL-33 thus affecting the inflammatory response to epithelial damage [17].

There is increasing evidence that IL1RL1 plays an important role in the development of asthma. IL1RL1 gene cluster polymorphisms and SNPs such as rs3771166, rs1420101 and rs1041973 have been associated with childhood asthma [14-18,32]. The results of this study suggest that IL1RL1 polymorphisms also play a role in affecting the severity of RSV disease. This is not surprising considering the common pathophysiological mechanisms of disease between asthma and RSV displayed by airway inflammation, airway hyperresponsiveness and mucus production as a result of epithelial damage.

Higher IL1RL1-a concentrations were found in children with more severe disease requiring ventilation. This is consistent with previous studies showing that higher IL1RL1-a serum concentrations were correlated with other clinically severe diseases such as acute heart disease and asthma [24,26,27]. IL1RL1-a in serum is also elevated in patients with acute pulmonary disease and prognostic for death within one year [28]. The results of the current study suggest that, in children with severe RSV disease, IL1RL1-a inhibits IL-33 signaling and thus affects the endogenous "danger signal" that is normally stimulated by epithelial damage. The role of IL1RL1-a in RSV disease has not otherwise been studied in humans, but our findings are supported by studies in RSV sensitized mice in which the administration of anti-IL1RL1 antibodies resulted in attenuated Th2-type cytokine-associated eosinophilic airway inflammation [41].

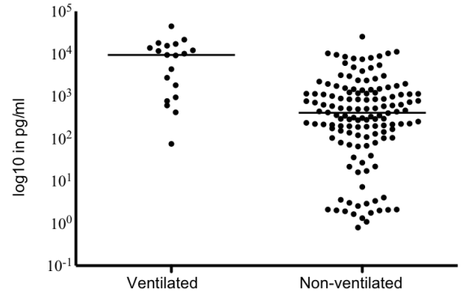
A limitation of this part of the study is that the control group did not include a group of non-RSV infected, hospitalized patients affected by a different respiratory disease. Furthermore, there may be potential confounders, such as secondary bacterial infection. In general, rates of bacterial infection in hospitalized, febrile infants with RSV bronchiolitis is low, but rates as high as 30-40% are reported in ventilated patients [5-8,42]. In the 20 ventilated infants in this study, only 3 (15%) had a positive bacterial culture with single growth >100 per visual field on the day of intubation. Haemophilus influenzae was found in 2 patients and Moraxella catarrhalis was found in 1 patient.

Local IL1RL1-a concentration in RSV disease is not associated with selected genotypes. Thus, although both RS 1921622 IL1RL1 polymorphisms and higher local IL1RL1-a concentrations are associated with severe RSV disease, the suggestion of a gain-of-function polymorphism could not be confirmed. These results are similar to asthma studies in which associations between IL1RL1 genotypes and acute asthma or severe asthma could be demonstrated, but not between serum IL1RL1-a and studied genotypes [15]. In contrast, one recent study showed an association between IL1RL1 polymorphisms and serum IL1RL1-a [32]. A recent large scale genome-wide association study of asthma found associations between asthma and SNP rs3771166 on chromosome 2 implicating a role for IL1RL1, but also variants at different loci associated with different types of asthma, such as childhood asthma [17]. Intron 10 SNPs are in linkage disequilibrium with IL1RL1 and IL18R1. Although we attribute the effect at this locus to IL1RL1, as in previous studies [17], an IL18R1 effect cannot be excluded since SNP rs1921622 is in linkage disequilibrium with SNPs in IL18R1.

CONCLUSION

Our data demonstrate for the first time a genetic association between IL1RL1 and disease severity of RSV bronchiolitis. High IL1RL1-a production in the airways of children with severe RSV bronchiolitis suggests this molecule plays a role in modifying the inflammatory response to epithelial damage, as it does in severe asthma.

FIGURE 1: Local IL1RL1-a is associated with RSV disease severity



Median concentration 9357 pg/ml vs 405 pg/ml; Mann-Whitney U test p<0.0001

Median concentrations of IL1RL1-a in nasopharyngeal aspirates of hospitalized infants with RSV were >20-fold higher in mechanically ventilated infants at the Pediatric Intensive Care Unit (n=19) when compared to non-ventilated infants admitted to the general pediatric ward (n=135) (median [and quartiles] 9,357 [936-15,528] pg/ml vs 405 [112-1,193] pg/ml respectively; Mann-Whitney U test p<0.0001).

TABLE I: IL1RL1 SNPs not associated with RSV bronchiolitis

refSNP ID	RSV hospitalized infants[1] (n=465)			Population controls[1] (n=930)			Missing values	P-value[2]
Allele	A	G		A	G			
rs1921622	498	422		1002	826		42	0.734
rs11685480	468	454		925	915		28	0.809
rs1420101	343	571		706	1130		40	0.638
Genotype	AA	GA	GG	AA	GA	GG		
rs1921622	138	222	100	287	428	199	42	0.849
rs11685480	117	234	110	219	487	214	28	0.727
rs1420101	62	219	176	129	448	341	40	0.882

IL1RL1 selected genotypes rs1921622, rs11685480 and rs1420101 are not associated with RSV bronchiolitis in hospitalized infants when compared to healthy controls in the population.
RefSNP ID is the Reference SNP (rs) Number; SNP, single-nucleotide polymorphism.
[1] Number of alleles and genotypes.
[2] According to $\chi 2$ distribution of a 2 x 2 table on allele or genotype frequencies.
[3] Reference allele is the major allele.

TABLE II: IL1RL1 SNP rs1921622 associated with severe RSV bronchiolitis.

refSNP ID	RSV ventilated infants[1] (n=81)			RSV non-ventilated infants[1] (n=384)			Population controls[1] (n=930)			Missing values	P-value[2]
Allele	A	G		A	G		A	G			
rs1921622	73	89		425	333					10	**0.011**
rs1921622	73	89					1002	826		32	**0.017**
Genotype	AA	GA	GG	AA	GA	GG	AA	GA	GG		
rs1921622	17	39	25	121	183	75	287	428	199	5	**0.040**

Subgroup analysis showed an association between severe RSV disease characterized by need for mechanical ventilation and IL1RL1 SNP rs1921622 at both the allele level, and at the genotype level (p=0.040).
RefSNP ID is the Reference SNP (rs) Number; SNP, single-nucleotide polymorphism. [1] Number of alleles and genotypes. [2] According to $\chi 2$ distribution of a 2 x 2 table on allele or genotype frequencies. [3]

TABLE III: Subject characteristics of infants hospitalized for RSV bronchiolitis with IL1RL1-a measured in nasopharyngeal aspirate.

	Mechanically ventilated infants (n=20)	Non-mechanically ventilated infants (n=187)	P-value
Male gender (n (%))	16 (80%)	105 (56%)	0.055 #
Gestational age (median weeks (quartiles))	38.7 (37.0-39.9)	39.7 (38.1-40.6)	**0.032 ***
Born during RSV season (n (%))	17 (85%)	129 (70%)	0.197 #
Breastfeeding (n (%))	12 (60%)	111 (60%)	1.000 #
One or more older siblings (n (%)	18 (90%)	146 (78%)	0.261 #
Daycare attendance ≥ 1 day per week (n (%))	3 (15%)	48 (26%)	0.415 #
Atopy in the 1st degree family (n (%))	11 (55%)	129 (69%)	0.216 #
Smoking during pregnancy (n (%))	6 (30%)	34 (18%)	0.233 #
Smoking in the household (n (%))	3 (15%)	15 (8%)	0.392 #
Age at admission (median days (quartiles))	70 (41-137)	51 (35-75)	0.094 *
Length of hospital stay (median days (quartiles))	14 (12-19)	4 (2-6)	**<0.001 ***
Duration of ventilation (median days (quartiles))	9 (7-15)	n.a.	
IL1RL1-a measured in nasopharyngeal aspirate (n (%))	19 (95)°	135 (72)°	
Genotyping for 3 selected *IL1RL1* SNPs (n (%))	10 (50)°	120 (64)°	

Fisher's exact test
* Mann-Whitney U test
n.a.=not applicable

Analyses were performed in nasopharyngeal aspirates of hospitalized, non-ventilated infants with respiratory syncytial virus (RSV) infection and ventilated infants at the Pediatric Intensive Care Unit with RSV.
∞Excluded samples of poor quality had too little material to perform genotyping or an IL1RL1-a measurement.

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CHAPTER 3

NON-TREATED RSV INFECTION IN PEDIATRIC HEMATOPOIETIC CELL TRANSPLANT PATIENTS

Faber TE
Versluys B
Boelens JJ
Bont LJ

submitted

ABSTRACT

Not much is known about the natural course of respiratory syncytial virus (RSV) infection in pediatric hematopoietic cell transplant (HCT) patients. During a twelve year period at the transplantation unit of Wilhelmina Children's Hospital in Utrecht, 310 pediatric HCT recipients were actively screened for respiratory virus (RV) infection, before and after transplant. Eight patients (3%) developed RSV infection, all were community acquired. None of the patients received antiviral treatment nor palivizumab. Despite the presence of previously published risk factors such as young age and severe neutropenia, none of the patients had severe RSV related disease and there were no RSV attributable deaths. The results from this study suggest a more favourable outcome of non-treated RSV infection in pediatric HCT recipients than previously suggested.

INTRODUCTION

Over 25 years ago, reports indicated that children with immunodeficiency are at high risk for complicated RSV disease [1]. Hematopoietic cell transplant (HCT) recipients are considered especially vulnerable [2,3]. Progression to lower respiratory tract infection occurs in 50% of patients and mortality rates of 12-55 % have been reported [4-6]. Factors associated with progression to serious disease are: cord blood donors, infection early after HCT, neutropenia and lymphocytopenia, graft versus host disease (GVHD), myeloablative regimens, concomitant immunosuppression, bacterial coinfection, and age under 2 years [6-12]. In adult HCT patients with RSV infection, the use of ribavirin is associated with better outcomes and lower mortality rates [2,9]. Contrary to adult literature, there is a lack of well-designed controlled trials on the efficacy of ribavirin in pediatric HCT patients with symptomatic RSV infection and treatment remains controversial. Furthermore, not much is known about the natural course of RSV disease in pediatric HCT patients, especially when infection occurs pre-transplant. At the pediatric HCT unit of Wilhelmina Children's Hospital, UMCU, in The Netherlands, all patients are screened for respiratory viruses (RV) before transplantation. This study describes the incidence and outcome of non-treated RSV infection in these patients that are generally regarded high risk for development of serious disease.

METHODS

All pediatric patients undergoing their first HCT between 2004 and 2015 at the transplantation unit of Wilhelmina Children's Hospital, were included. According to our local pre-HCT screening policy [13], all patients were routinely screened for RV by polymerase chain reaction (PCR) in nasal pharyngeal aspirate (NPA) and/or bronchoalveolar lavage (BAL) prior to HCT. The respiratory panel includes RSV, bocavirus, human herpes virus 6 (HHV-6), influenzavirus A and B, parainfluenzavirus 1–4, rhinoviruses, adenoviruses, human coronavirus OC43, NL63 and 229E and human metapneumovirus (hMPV). Methods used for the real-time PCR assays specific for RSV were described before [13]. CT values ≤ 40 were considered positive. From august 2006 until may 2008 we performed a surveillance study on our ward with weekly NPA sampling for RV as long as the patient was admitted. Otherwise, PCR testing for respiratory viruses was repeated in patients that developed (new) respiratory symptoms. Patients were defined as asymptomatic (AS); showing signs of upper respiratory infection (URTI) such as rhinitis, congestion, otitis media, pharyngitis and/or cough; or lower respiratory infection (LRTI) such as tachypnea, wheezing, rales, use of accessory muscle and/or hypoxia [14]. RSV positive patients were scored using the immunodeficiency scoring index for RSV infection (ISI-RSV) in HCT recipients [15]. This score combines neutrophil count, lymphocyte count, age, myeloablative regimen, GVHD, corticosteroids and recent engraftment or pre-engraftment, to predict the risk of progression to lower respiratory tract infection and RSV related mortality. A score of 0-2 is considered low risk, 3-6 moderate risk and 7-12 high risk. Patients that develop RSV infection within 5 days of hospitalization were considered community acquired [16]. In elective HCT procedures, HCT was postponed for two weeks, aiming for clearance of the virus. In cases when underlying disease did not allow treatment delay, immune suppression was continued for a longer period of time post-HCT. RSV positive patients did not receive antivirals. Further monitoring and supportive care included weekly cultures of stools and nose/throat to monitor for bacterial colonization. Weekly galactomannan testing (Platelia Aspergillus enzyme immunoassay; Bio-Rad, Hercules, CA) was performed to screen for Aspergillus infection. Antimicrobial prophylaxis involved daily ciprofloxacin and fluconazole during neutropenia, with additional prophylaxis against Streptococcus viridans with cefazolin in the mucositis phase. Pneumocystis jirovecii pneumonia prohylaxis was given as co-trimoxazole 3 times a week. In case of positive serology for herpes simplex virus or varicella zoster virus, prophylaxis with acyclovir was given. In high-risk patients for invasive fungal infection (IFI) Aspergillus prophylaxis was given with daily voriconazole or twice weekly amphotericin B. Immunoglobulins were suppleted intravenously when IgG level was below 4 gr/L.

RESULTS

During the twelve-year study period, 310 pediatric patients received their first allogenic HCT (table 1). The total number of patients with RSV infection was eight (3%) (table 2). The median age of RSV infected patients was 61 months (2 months – 11 years), two patients were under 2 years of age. All infections were community acquired. Seven patients contracted RSV before the conditioning stage of treatment (range day -21 to -9) and one girl became RSV infected more than 50 days post-transplantation. At time of diagnosis, six patients were immunocompromised, two patients were asymptomatic, five patients had signs of upper respiratory tract infection and one patient had signs of lower respiratory tract involvement with tachypnea and fever. The average CT value was 25 (range 17-40). According to ISI-RSV, 7 patients were considered high risk for progression to develop serious RSV disease. All patients were monitored closely for course of disease. Four patients (patient 3,4,5,6) with URTI did not progress to develop RSV-LRTI. One patient (patient 1) with initial signs of rhinitis 2 weeks prior to transplantation, progressed to develop LRTI with tachypnea 37 days later. The BAL was positive for RSV with a CT value of 18 (versus CT value 23 pre-HCT) and negative for other viruses and/or pathogenic bacteria. At that time there were early signs of neutrophil engraftment. The patient was not hypoxemic and recovered spontaneously within several days. One patient (patient 6) was RSV positive (CT-value 27) prior to HCT. There were mild symptoms of rhinorrhea and cough that did not progress to LRTI in the first weeks following myeloablative conditioning. Three weeks after transplantation, he developed symptoms of dyspnea and hypoxia. The BAL was still positive for RSV (CT value 15), but also positive for galactomannan indicating aspergillosis infection. Subsequent high resolution computed tomography (HRCT) scan showed multiple consolidations in the peripheral lung fields without air trapping, suspect for fungal infection rather than viral bronchiolitis. After initial recovery under antifungal treatment, the patient died 3 weeks later from progressive respiratory disease most likely from a combination of invasive Aspergillosis and immune mediated lung disease, based on findings on HRCT scan showing ground glass lesions and pneumomediastinum. Although a causal role for RSV cannot be excluded, positive galactomannan testing and radiographic signs of Aspergillosis at time of occurrence of respiratory symptoms, together with initial recovery upon antifungal treatment with further deterioration at time of immune reconstitution would render progression of RSV disease unlikely. The patient with RSV acquisition after HCT (patient 7) had signs of LRT involvement at time of diagnosis with tachypnea and fever. Her symptoms progressed mildly in the following 3 days and she was admitted with symptoms of dyspnoea, cough and hypoxia. At that time she had a normal neutrophil count and only mild lymphopenia. She received oxygen, steroids, and antibiotics to prevensecondary bacterial infection and showed an uneventful recovery. In three patients we had serial NPA samples, taken routinely during admission on the ward. In these patients we saw prolonged RSV shedding for 2-7 months. Overall, none of the patients received antiviral treatment, none of the patients had severe RSV related disease and there were no RSV attributable deaths.

DISCUSSION

A major strength of this prospective study is universal RSV testing in all children. During the twelve year study period, the overall incidence of PCR confirmed RSV positivity in our routinely monitored cohort of pediatric transplant patients was 3%, the incidence of symptomatic RSV infection was 2%. Although none of the children received antiviral treatment, none of the patients required intensive care treatment and there were no RSV attributable deaths. Seven patients contracted RSV before conditioning, HCT was not postponed but tapering of immunosuppression was delayed in 5 patients. Five patients did not progress to develop serious disease, three patients showed signs of (mild) LRTI. Three patients received steroids and/or antimicrobial treatment.

The small number of RSV positive patients does not allow for further analysis of associated risk factors for development of serious disease such as cord blood donor, myeloablative regimen, concomitant immunosuppression and age < 2 years [12,15,17]. However, it is interesting that 7 children were considered high risk for development of LRTI or RSV-associated mortality according to the ISI-RSV score, 6 of the patients received cord blood donor transplantation, two of the patients were under 2 years of age and all but one patient were infected pre-transplant, but subsequent myeloablative regimens and concomitant immunosuppression did not lead to development of severe disease. In a Canadian surveillance study, early infection was also found to have a relatively favourable outcome [16].

Up until now, reports on the outcome of RSV infection in pediatric HCT recipients were gloomy with high incidence rates of 7 to 21% [18,19], progression to LRTI in half of patients and mortality rates of 12-55% [4,5,20]. The results of our study are more favourable with a 2% incidence of symptomatic infection, 25% progression to LRTI and no RSV attributable mortality. To the best of our knowledge, there is only one other study on the outcome of RSV infection in pediatric HCT patients without ribavirin treatment and results are comparable to our study. In this retrospective report from Cincinnati Children's Hospital Medical Center, the incidence of symptomatic RSV infection in 450 patients was 7% [21]. Of the 32 patients with RSV, 19% progressed to develop LRTI and there was no RSV attributable mortality [21]. The favourable outcome cannot be explained by absence of known high risk factors. The median age in this study was 7 years and the authors conclude that severe neutropenia and young age were not associated with development of severe disease [21]. All RSV infected patients in the Cincinnati study received IVIG and five received palivizumab. It was hypothesized that administration of antibodies offered passive protection from development of serious disease.

In healthy infants, there is evidence supporting an important role of immune mediated pathology to RSV disease. Upregulation of pro-inflammatory cytokines and chemokines and recruitment of neutrophils [22-27], results in a cascade of inflammation that can inherently exert pathogenic as well as protective effects. There is an incomplete understanding of the immune response to RSV in an immune compromised host and recent data suggests that morbidity and mortality are not correlated to the degree of immune suppression [12,21]. The absence of immunity may in fact aid to prevent neutrophil exerted epithelial damage when acute RSV infection occurs pre or early post transplant. In a previous study we showed that infection with respiratory viruses early post transplant is not synonymous with progression to serious disease, but strongly associated with later development of life-threatening alloimmune lung syndromes (allo-LS) during the immune reconstitution phase [28]. Prolonged immunosuppressive therapy had a protective effect against the evolvement of inflammatory mediated allo-LS [28]. In this regard, the severe immunosuppression and the paradoxal policy to prolong immunosuppression post-HCT in patients that are RSV positive may aid to prevent both progression of disease and development of allo-LS .

In pediatric HCT patients, there is no proof of efficacy for the use of ribavirin in patients with RSV infection and its use remains controversial. The results from our study and the Cincinnati study that pediatric HCT recipients with RSV without antiviral therapy do not have worse outcomes, despite infections pre or early post-transplant and/or with concurrent severe neutropenia and/or young age, should lead to very restrictive criteria for the use of ribavirin. Considering evidence supporting immune mediated pathology in RSV infection, future research should focus on targeting the immune system rather than antivirals.

The results from this study are limited because of the small number of patients. Furthermore, we have no data on possible long-term respiratory RSV complications such as development of post-bronchiolitis wheeze, lung damage and bronchiolitis obliterans. However, the results are important as there is little data on the incidence and outcome of non-treated RSV infection in pediatric HCT recipients. The strongest part of our study is that we performed pre-transplant screening for respiratory viruses, routine monitoring post transplant with occurrence of respiratory symptoms and the inclusion of patients with assumed high risk factors such as cord-blood transplant, young age and severe neutropenia.

CONCLUSION

In this large cohort of pediatric HCT recipients routinely screened for respiratory viruses, the incidence of RSV is much lower than previously reported. In a setting with presumable high risk factors for development of serious disease such as young age, early infection and neutropenia, non-treated patients do not have worse outcomes. Larger studies in young patients are urgently needed to unravel the role of inflammation and immunity in RSV infection, especially in the allogenic transplant setting.

TABLE I: Patient characteristics of HCT patients (n= 310)

Number of patients	> 2 years (percentage)	242	(78%)
	< 2 years (percentage)	68	(22%)
Age at time of transplant	In years (range)	7,3	(0,1-22,7)
Sex	Male (percentage)	186	(60%)
	Female (percentage)	124	(40%)
Diagnosis	Malignant disease	163	
	Bone marrow failure	34	
	Inborn error of metabolism	54	
	Immune deficiency	59	
Conditioning regimen	Chemotherapy based	265	
	Total body irradiation based	45	
Type of donor	Matched related	74	
	Matched unrelated	68	
	Unrelated cord blood	168	

TABLE II: Patient characteristics of RSV positive patients

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Age at HCT (in months)	31	120	62	134	31	92	2	16
Diagnosis [1]	ALL	ALL	ALL	ALL	MPS III	X-CGD	HLH	MPS I
Type of donor [2]	10/10 MUD	5/6 uCB	10/10 MUD	6/6 uCB	6/6 uCB	6/6 uCB	6/6 uCB	5/6 uCB
Conditioning regimen[3]	Bu/Cy/VP16/ ATG	TBI/VP16/ ATG	TBI/VP16/ ATG	TBI/VP16/ ATG	Bu/Cy/ ATG	Treo/Flu/ Campath	Bu/Flu/ ATG	BU/Flu/ ATG
GVHD prophylaxis [4]	CsA, MTX	CsA, Pred	CsA, MTX	CsA, Pred	CsA, Pred	CsA, pred	CsA, Pred	CsA, Pred
RSV diagnosis	BAL+ NPA+	BAL+ NPA+	BAL- NPA+	BAL+ NPA+	NPA+	BAL+ NPA+	NPA+	BAL+ NPA-
Time after HCT (in days)	-14	-14	-14	-11	-21	-17	+ 52	-9
ISI-RSV [5]	8	9	8	10	8	10	1	9
Lowest RSV CT-value	17	20	42	17	40	15	22	27
Initial RSV symptoms	URTI	AS	URTI	URTI	URTI	URTI	LRTI	AS
Progression of symptoms	YES Day + 23 Tachypnea	NO	NO	NO	NO	YES Day + 21 Dyspnea, oxygen	YES Day + 55 Tachypnea, oxygen	NO
Treatment	Steroids	NO	NO	NO	NO	Antifungals, Steroids	Antibiotics, Steroids	NO
Outcome	Died + 7 mnths due to relapsed disease	Alive and well	Alive and well	Died + 27 mnths due to cGVHD, pulmonary aspergillosis, organ failure	Alive, progressive disease	Died day + 53 due to Aspergillosis, immune mediated lung disease	Alive and well	Alive and well
RSV-attributable death	NO	N/A	N/A	NO	N/A	NO	N/A	N/A

[1] Diagnosis: ALL = acute lymphoblastic leukemia, MPS I = mucopolysacharridose type I = hurlers disease, MPS III = mucopolysacharridose type III = San Filippos disease, X-CGD = X linked chronic granulomatous disease, HLH = Hereditary Hemofagocytic Lymphohistiocytosis.

[2] Type of donor: 10/10 MUD = fully matched unrelated donor, uCB = unrelated cord blood, 5/6 and 6/6 indicates HLA match.

[3] Conditioning regimen: Bu = Busulfan, Cy = Cyclofosfamide, VP 16 = Etoposide, ATG = anti thymocyte globulin, TBI = total body irradiation, Treo = Treosulfan, Flu = Fludarabine.

[4] GVHD profylaxis: CsA = Ciclosporine A, MTX = Methotrexate

[5] ISI-RSV: Immunodeficiency scoring index for RSV infection [15], in a pediatric cohort max 10, low risk 0-2, moderate risk 3-6, high risk 7-10

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CHAPTER 4

DYNAMICS OF NASOPHARYNGEAL PNEUMOCOCCAL CARRIAGE DURING THE COURSE OF VIRAL BRONCHIOLITIS

Faber TE
Schuurs TA
Veeger NJ
Hennus MP
Bont LJ

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ABSTRACT

The effect of viral infection on nasopharyngeal carriage of Streptococcus pneumoniae during childhood is not well known. We studied dynamics of pneumococcal colonization by quantitative PCR during the natural course of viral bronchiolitis. At time of admission, 47 (47%) of 100 patients with bronchiolitis carried pneumococci. In patients with viral bronchiolitis who did not receive antibiotics, pneumococcal load decreased from time of admission to discharge (n = 35, cycle threshold 23 vs. 25, P = 0.0017) and from discharge to follow-up (n = 22, cycle threshold 25 vs. 40, P = 0.003). We conclude that viral respiratory infection is negatively associated with pneumococcal colonization of the upper airways.

INTRODUCTION

The nasopharynx is the major niche of Streptococcus pneumoniae and carriage rates of up to 45% have been reported for children under 2 years of age [1]. Since 2006, children in the Netherlands are vaccinated against seven serotypes of pneumococci to prevent invasive disease such as meningitis, sepsis and pneumonia. Pneumococcal conjugate vaccine against 13 serotypes was introduced in 2011. It is thought that pneumococcal carriage is a risk factor for invasive pneumococcal disease, but this relationship is not entirely clear. Pneumococcal carriage decreases with age due to maturing of the immune system. Interaction between respiratory viruses and bacteria have well been documented, yet it is not known whether the acquisition and clearance of common viral infections during childhood affects this process. Bronchiolitis is one of the most common viral infections during infancy, almost all children have serological evidence of respiratory syncytial virus (RSV) infection by the time they are 2 years of age. In this prospective observational study, we studied the dynamics of pneumococcal carriage in young infants during bronchiolitis.

METHODS

Patients: Infants under 13 months of age admitted to the pediatric ward of two hospitals in the Netherlands, with a clinical diagnosis of bronchiolitis were included during two consecutive winter seasons. Symptoms of bronchiolitis were chest cough, wheezing, rales, use of accessory muscles, and/or nasal flaring [2]. Infants born prematurely with congenital heart disease or chronic lung disease, infants with previous wheezing illness, and patients using corticosteroids, antibiotics or bronchodilators were not included. The following patient characteristics were recorded: gender, gestational age, birth weight, age at admission, length of hospital stay, and need for mechanical ventilation. At time of admission, all patients were assigned a modified respiratory distress assessment index (RDAI) score evaluating respiratory rate, use of accessory muscle, color and auscultation finding (range 0-12), specifically validated for children suffering from bronchiolitis [3,4].

Samples: Undiluted nasopharyngeal aspirates were taken at the emergency department at time of admission to the hospital, at time of discharge and during a routine follow-up visit. All patients were analyzed at time of discharge, 58 (58%) of patients at follow-up. Nasopharyngeal secretions were placed on ice immediately and stored at -80 degrees for later analysis. By design, xylomethazolin or normal saline nasal spray were not given in the 6 hours prior to aspiration. Samples were weighed, diluted as necessary, sonicated, centrifuged, and duplicate assays were performed on each.

Cycle threshold (CT) values for RSV and S. pneumoniae were analyzed in the repeated aspirates by target-specific real-time polymerase chain reaction (PCR) assays, as previously described [5,6]. CT values below 40 were considered positive. A sensitivity analysis was performed on the subgroup of patients whom were positive for RSV.

Statistical analysis: Due to expected inhibitory effects of antibiotics on pneumococcal colonization, samples from patients that were prescribed antibiotics by the attending pediatrician (Group AB) were analyzed separately from those that did not (Group NoAB). For statistical purposes, CT values of 40 were used for values “undetected” by PCR at time of discharge in patients whom were colonized with pneumococci at time of admission. In cases where more than 2 aspirates were taken during hospitalization, only those at time of admission and discharge were used for analysis. Wilcoxon matched-pairs signed rank test was used to compare pneumococcal CT-values at time of admission (t=1) versus time of discharge (t=2) from the hospital, and from time of discharge (t=2) versus out-patient follow-up (t=3). In a similar manner, a secondary analysis was performed on the subgroup of patients whom were positive for RSV. Values are reported as medians with interquartile range. All tests of significance were two-sided. P-values < 0.05 were considered to be statistically significant.

Ethics Statement: The study protocol was approved by the Medical ethics committee of the Medical Center Leeuwarden and the University Medical Center Utrecht (NL 12722.099.06-TPO 427). All parents agreed to participate and gave written informed consent.

RESULTS

A total of 100 patients with bronchiolitis were included in the study. Median length of stay was 7 days (IQR 4-11 days), median number of days from discharge to follow-up was 7 days (range 4-16 days). Group NoAB consisted of 72 patients who did not receive antibiotics. All 72 patients were seen at admission (t=1) and discharge (t=2), 53% (38/72) of patients were seen at follow-up (t=3). Group AB consisted of 28 patients who received antibiotics, either amoxicillin or amoxicillin/clavulanic acid. All 28 patients were seen at admission (t=1) and discharge (t=2), 71% (20/28) of patients were seen at follow-up (t=3). Baseline characteristics as well as clinical characteristics were similar in both groups, except a higher percentage of boys and a higher percentage of ventilation was found in the AB group. Disease severity, indicated by RDAI score, did not differ between the NoAB and AB groups (median 5.0 vs 6.5, p=0.1180). Pneumococcal bacterial load at time of admission was not correlated to disease severity (Spearman r=0.0550, p=0.7134). In both groups, the majority of patients were RSV positive (85% and 79%). (Table I).

The overall rate of pneumococcal carriage was 47% (47/100). At time of admission, AB and NoAB groups had similar carriage rates and bacterial load as measured by CT values (rates 49% vs 43%, p=0.6598; median 23.43 vs 25.82, p=0.5339) (Table I). In patients receiving antibiotics, a decrease in pneumococcal carriage was observed during hospitalisation, especially at time of follow-up. Of the 12 patients that carried pneumococci at time of admission, 8 visited the outpatient clinic at which time 4/8 (50%) had become negative. Remarkably, this pattern was also seen in patients that did not receive antibiotics. Of the 35 patients that carried pneumococci at time of admission, 22 visited the outpatient clinic at which time 13/22 (60%) had become negative. Three patients in the NoAB group and four patients in the AB group became colonized during hospitalization. The 47 bronchiolitis patients who carried pneumococci at time of admission were further analyzed by examining bacterial load during course of disease. In both groups, pneumococcal bacterial loads decreased from time of admission to discharge, and from time of discharge to follow-up (t=1 vs t=2 and t=2 vs t=3). In the non-AB group, median pneumococcal CT values increased from 23.43 to 25.21 (n=35, t=1 vs t=2, p=0.0017), and from 25.21 to 40.00 (n=22, t=2 vs t=3, p=0.0003) (Figure I). In the AB group, median pneumococcal CT values increased from 25.82 to 32.71 (n=12, t=1 vs t=2, p=0.0068), and from 32.71 to 37.91 (n=8, t=2 vs t=3, p=0.1563). In a sensitivity analysis in RSV positive patients not receiving antibiotics, results were comparable. Median pneumococcal CT values increased from 23.89 to 24.78 (n=31, t=1 vs t=2, p=0.0032), and from 24.78 to 40.00, (t=2 vs t=3, p=0.0005).

DISCUSSION

Studies on bacterial co-infection and viral respiratory tract disease are not unique, yet this is the first attempt to prospectively study the dynamics of nasopharyngeal pneumococcal carriage in children hospitalized for bronchiolitis. A high degree of pneumococcal carriage (47%) was found at time of admission. This is in accordance with a large Dutch cohort study of healthy young children in the general population [7]. The present study showed that in half of patients, pneumococci were cleared from the nasopharynx during the course of infection. From a longitudinal study of S. pneumoniae carriage in a cohort of infants from the general population in Thailand we know that the average duration of a carriage episode is 63 days. A total of 234 infants were swabbed monthly from delivery up to the age of 24 months. Carriage rates for the most common serotypes 19F and 23F were even longer, 213 days and 184 days respectively [8]. These results suggest that the approximate 2-week clearance of pneumococci in children with bronchiolitis, as observed in our study, would not be expected to occur in children without bronchiolitis.

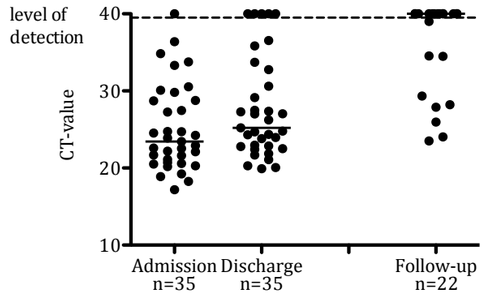
In patients who did not receive antibiotics, pneumococcal load decreased during the course of viral bronchiolitis. This is in apparent contrast with evidence suggesting that, in general, preceding respiratory viral infection contributes to bacterial superinfection [9]. In the case of viral bronchiolitis, in vitro and mice studies have shown that RSV challenge, followed by the introduction of pneumococci, resulted in decreased bacterial clearance and higher levels of bacteriaemia [10]. Proposed mechanisms of action are RSV-glycoproteins that act as bacterial receptors and direct binding in which RSV-pneumococcal complexes show enhanced adherence to uninfected epithelial cells [10]. Clinically, there is a report showing that recent hospitalization for viral bronchiolitis results in a five- to sevenfold increased risk of invasive pneumococcal disease [11]. Furthermore, viral co-infections are commonly seen in children with invasive pneumococcal disease [12]. However, the emphasis of this study was on bacterial colonization, rather than bacterial coinfection. It is, therefore, important to characterize better the impact of respiratory viral coinfections in the occurrence and outcome of pneumococcal colonization and disease. Perhaps the increased risk of pneumococcal disease is limited to patients who do not clear their pneumococci during viral infection. This represents a small number of patients as rates of serious bacterial infections in infants with RSV bronchiolitis are low and around 1% [13,14,15]. Our study points to the possibility of viral interference with bacterial colonization of the upper respiratory tract. In a previous study, we showed that recovery from RSV bronchiolitis is also associated with T-helper-17 cell responses with a 90% increase in interleukine-17 concentrations [16]. This coincides with an abundant neutrophil influx during viral bronchiolitis [17]. In addition, respiratory viral infection may induce neutrophil extracellular traps (NETs) which kill bacteria extracellularly [18,19]. Together, these results indicate that nasopharyngeal viral infection induces a range of immune responses which, conceivably, may lead to enhanced neutrophil killing of pneumococci from the nasopharyngeal cavity.

Strengths of this study are the combination of bacterial carriage and viral respiratory infection as it occurs in a natural setting, and the repeated measurement design. The repeated measurement design allows insight into bacterial load during hospitalization, and after discharge. Limitations of this study also require discussion. First, the patient population was relatively small. Nevertheless, the study population was large enough to allow for sensitivity analysis in RSV bronchiolitis patients not receiving antibiotics. Second, we included patients hospitalized for viral bronchiolitis. It is not certain whether results can be extrapolated to patients with less severe viral respiratory tract infection. Furthermore, the nature of this design did not allow for sampling before the acute episode of bronchiolitis. Third, it could be argued that there is a selection bias due to the smaller size in the follow-up group. However, there were no baseline differences between children analyzed at the outpatient clinic and those who were not. Fourth, individual data on immunization status was not recorded, but >95% of children in the Netherlands adhere to the national immunization program, which, during the study period, included the seven-valent pneumococcal conjugate vaccine. Fifth, future studies should distinguish pneumococcal serotypes as they differ in potential for colonization and virulence. For example, certain serotypes such as 19A displays high propensities for both nasopharyngeal colonization and invasive pneumococcal disease [20]. A recent study in children with RSV and radiographically confirmed community acquired pneumonia found pneumococcal carriage rates of 52% with almost exclusively serotype 19A [21]. It is conceivable that other serotypes have protective propensities when it comes to subsequent viral respiratory infection and invasive disease, or that viral respiratory infection may have specific inhibitory or synergistic effects on pneumococci depending on serotype. Finally, the emphasis of this study was on S. pneumoniae, hence other bacterial pathogens that are known to colonize the upper respiratory tract of young children were not studied. But pneumococcal colonization is known to correlate with the presence of Haemophilus influenzae and Staphylococcus aureus in the nasopharynx [22], and to potentially modify clinical outcome of subsequent metapneumovirus infection [23], thus it is important to consider these potential synergistic and competitive interactions on a larger scale.

CONCLUSION

Viral bronchiolitis appears to impact colonization of pneumococci in the upper airways of previously healthy infants. Therefore, it is crucial to study the fluctuations in non-invasive bacterial colonization of the nasopharynx that occur during childhood in relation to viral infection and to recognize the complex dynamics of microbial communities in the upper respiratory tract, especially in the development and evaluation of candidate viral vaccines.

FIGURE I: Pneumococcal CT values of bronchiolitis patients that did not receive antibiotics (NoAB)



In 72 bronchiolitis patients that did not receive antibiotics (NoAB), 35 (49%) carried pneumococci at time of admission (t=1), 34 (89%) of them were RSV positive. Pneumococcal CT values increased from admission to discharge (n=35, median 23.43 (IQR 20.70-28.75) vs n=35, median 25.21 (IQR 22.80-32.75), t=1 vs t=2, p=0.0017), and from discharge to follow-up (n=35, median 25.21 (IQR 22.80-32.75) vs n=22, median 40.00 (IQR 29.05-40.00), t=2 vs t=3, p=0.0003), by Wilcoxon matched-pairs signed rank test.

TABLE I: Subject characteristics of infants hospitalized for bronchiolitis

	Group NoAB (n=72)		**Group AB (n=28)**		**P-value**
Clinical characteristics					
Male gender (n (%))	33	(46)	20	(71)	0.0263 #
Gestational age (median weeks (quartiles))	39.6	(38.0-41.0)	39.0	(37.0-40.3)	0.2393 *
Birth weight (median grams (quartiles))	3500	(3000-3758)	3500	(2619-3663)	0.5836 *
Age at admission (median weeks (quartiles))	14	(8-27)	15	(5-29)	0.6951 *
Disease characteristics					
Length of hospital stay (median days (quartiles))	7	(4-11)	7	(5-11)	0.3028 *
Mechanical ventilation (n (%))	5	(8)	11	(28)	0.0114 #
RDAI score at admission (median score (quartiles))	5.0	(3-7)	6.5	(4-12)	0.1180 *
RSV positive (n (%))	61	(85)	22	(79)	0.5546 #
Pneumococcal carriage at time of admission (n(%))	35	(49)	12	(43)	0.6598 #

Fisher's exact test
* Mann Whitney test

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CHAPTER 5

RESPIRATORY SYNCYTIAL VIRUS IN CRITICALLY ILL ADULT PATIENTS WITH COMMUNITY ACQUIRED RESPIRATORY FAILURE: A PROSPECTIVE OBSERVATIONAL STUDY

Ong DSY
Faber TE
Klein Klouwenberg PMC
Cremer OL
Boerma CE
Sietses M
van Loon AM
Bonten MJM
Bont LJ

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ABSTRACT

The incidence of respiratory syncytial virus (RSV) and influenza virus infection was determined during three RSV seasons in 158 adult patients consecutively admitted to the intensive care unit with community-acquired respiratory failure. Nasopharyngeal swabs were tested for the presence of RSV and influenza virus by real-time polymerase chain reaction. Six patients (4%) were positive for RSV and all recovered. This finding was in sharp contrast to influenza (23 (15%) patients, 4 (17%) deaths). In conclusion, even in the midst of the RSV season, RSV is an infrequent cause of respiratory failure in adults admitted to the intensive care unit.

INTRODUCTION

Respiratory syncytial virus (RSV) is the most important cause of bronchiolitis and pneumonia in young children worldwide [1]. Disease severity depends on the patients' age and health status, whereby premature infants are typically at greatest risk. However, RSV is not an exclusive pathogen for the pediatric population. Reported incidence proportions in the United Kingdom for adult patients presenting with influenza-like illness to the general practice have been as high as 20% [2]. RSV illness in adults is often mild, but can cause significant morbidity in the elderly and in high-risk adults with mortality rates around 8% [3-6]. In a population based cohort study in the United States RSV infection, independent of bacterial co-infection, was associated with poor outcome [7]. In a Dutch study, seasonal mortality in the elderly was attributable to multiple viruses, including RSV [8]. It has been suggested that RSV infection is a trigger for acute exacerbation of chronic obstructive pulmonary disease (COPD) [9], and thus may play an important, yet unrecognized role, in etiology and outcome of patients admitted to the intensive care unit (ICU) with respiratory failure. In contrast to the pediatric age group, routine testing for viral infections, including RSV, is not usually performed in adult patients.

METHODS

In this multi-center observational study the incidence of RSV infection was compared to influenza infection in patients over 18 years of age admitted to the ICU with community-acquired respiratory failure. Participating centers in The Netherlands were the mixed ICUs at the University Medical Center Utrecht and the Medical Center Leeuwarden. Patients were prospectively included during the three winter seasons of 2010 until 2013. Community-acquired respiratory failure was defined by symptoms of dyspnea, cough and/or wheezing present before or within 48 hours after hospital admission and the presence of respiratory failure requiring ICU admission. The Ethics Committee at the Medical Center Leeuwarden approved the study as part of a prospective cohort study. The Ethics Committee at the University Medical Center Utrecht approved this study and waived the need for informed consent. Brushed nasopharyngeal swabs (Copan®) were taken upon ICU admission, stored in viral transportation medium, and transported to the diagnostic virology laboratory. Real-time polymerase chain reaction (PCR) was performed for the detection of RSV and influenza virus by the MagNA Pure LC extraction system (Roche Diagnostics, Mannheim, Germany) and the Taqman 7500 amplification platform (Applied Biosystems, Foster City, CA) 10. All data were analyzed with SAS 9.2 (Cary, NC).

RESULTS

A total of 158 patients were included. Data from the National Institute for Public Health and the Environment (RIVM) in The Netherlands confirmed that the study periods concurred with the RSV seasons (Figure 1). In the majority of cases, diagnosis of community-acquired pneumonia was confirmed (Table 1). There were no differences in baseline characteristics between patients with influenza and those with RSV infection. Six patients (4%; 95% CI 2%–8%) tested positive for RSV, all required mechanical ventilation within the first 24 hours after ICU admission and did not receive antiviral therapy before or during ICU admission. The median duration of ICU admission was 7 (IQR 6–18) days. One of the RSV infected patients was an immunocompetent 61-year-old female with severe COPD disease. Two other patients with RSV infection developed acute kidney injury, one of whom required renal replacement therapy. The remaining three patients had a relatively uncomplicated course of disease. Sputum and blood cultures failed to identify bacterial or viral co-pathogens in the RSV positive patients. Twenty-three patients (15%; 95% CI 10%-21%) had influenza, of whom four (17%) died. The median duration of ICU admission was 7 (IQR 2–10) days in patients with influenza infection.

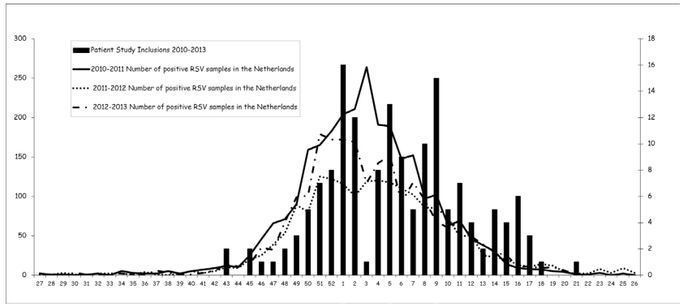
DISCUSSION

This is the largest prospective study defining the incidence of RSV in adult patients with community-acquired respiratory failure admitted to either an academic or non-academic ICU, including patients with or without chronic conditions. The incidence of RSV infection was 4% and lower than the observed 15% for influenza. Only three previous studies have evaluated RSV infection in ICU patients. One study included a select group of 122 ICU patients with chronic cardiac or pulmonary disorders, and found a comparable incidence rate of 5% [9]. The other study assessed 64 ICU patients with severe community-acquired and 134 ICU patients with healthcare-associated pneumonia, and found RSV incidence rates of 11% and 2% respectively [11]. The higher incidence found in the community-acquired group suggested that RSV infection is primarily found in patients with community-acquired respiratory failure. The most recent study included 70 non-immunocompromised patients with acute respiratory failure and found an incidence rate of 6% [12]. Based on the limited amount of published data, publication bias cannot be ruled out, perhaps resulting in an overestimation of the incidence of RSV in adult ICU patients. Recently, Lee et al showed high morbidity and mortality in adults hospitalized for RSV infections of whom 10% required mechanical ventilation [4]. Patients with RSV infection more frequently had underlying chronic lung diseases and major systemic comorbidities compared to patients with influenza, but both groups had comparable clinical outcomes. Rapid recognition of RSV as a cause of infection is becoming more important because of the recent progression in development of vaccines and antiviral therapeutics, several agents have now entered clinical trials. Antisense anti-RSV drugs, fusion inhibitors and nucleoside analogues show promising results [13,14]. However, beneficial cost-effectiveness of such new treatment and prevention modalities will most likely depend on targeting high-risk populations. Our study demonstrates that patients admitted to the ICU with community- acquired respiratory failure are not part of the high-risk population. More detailed studies are needed to provide estimates of the disease prevention and health gains that could be achieved in more specific patient populations and settings. The absence of RSV in the nasopharynx does not exclude the presence of RSV in the lower respiratory tract. Although availability of lower respiratory tract specimens might have increased our diagnostic yield, it has been well established that the nasopharynx is the porte d'entree for RSV and it is unlikely that patients with RSV infection were missed in our study [15]. This study did not investigate the presence of other respiratory viruses, due to practical reasons and the specific aim of comparing RSV with influenza.

CONCLUSION

RSV infection is uncommon among adults with acute community-acquired respiratory failure leading to ICU admission. Further research is necessary to identify high-risk adult patients who may possibly benefit from antiviral therapy and vaccination against RSV in the future.

FIGURE 1: RSV season in the Netherlands and patient inclusion



The lines represent the number of RSV positive tests per week in the Netherlands during the three years of the study period (left vertical axis) according to The National Institute for Public Health and the Environment (RIVM). The bars represent the number of patients included in each week number during this study (right vertical axis).

TABLE 1: Patient characteristics

Variable	RSV (n=6)		Influenza (n=23)		Neither RSV nor Influenza (n=129)	
Age (years)	62	(60-68)	60	(50-67)	66	(51-73)
Gender (male)	3	(50)	15	(65)	80	(62)
Apache IV score	68	(52-81)	72	(54-95)	84	(63-105)
Mechanical ventilation within 24 h after ICU admission	6	(100)	14	(65)	100	(78)
Corticosteroid or other immunosuppressive therapy	3	(50)	3	(13)	26	(20)
Congestive heart failure	0	(0)	0	(0)	13	(10)
COPD	1	(17)	6	(26)	37	(29)
Diagnosis at time of admission						
Pneumonia	5	(83)	22	(96)	91	(71)
Congestive heart failure	0	(0)	1	(4)	11	(29)
Other/unknown	1	(17)	0	(0)	25	(20)
ICU length of stay (days)	7	(6-18)	7	(2-10)	6	(3-15)
ICU mortality	0	(0)	4	(17)	19	(15)

Apache, acute physiology and chronic health evaluation; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; RSV, respiratory syncytial virus.
Numbers are expressed in median (IQR) and number (%).

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CHAPTER 6

MANAGEMENT OF RESPIRATORY SYNCYTIAL VIRUS INFECTION

Faber TE
Kimpen JL
Bont LJ

European Infectious Disease 2007;1:55-57

ABSTRACT

Respiratory syncytial virus (RSV) is considered a pathogen of early childhood with a peak incidence below 12 months of age [1]. More than 90% of infants are infected by the age of two years, increasing to almost 100% by the age of three years. Infection usually results in symptoms of acute mild to moderate upper respiratory illness, but clinical manifestations depend on the person's age and health status. In very young and premature infants, disease typically progresses to more severe infection of the lower respiratory tract, causing bronchiolitis and/or pneumonia. In this age group, patients may present with atypical symptoms such as lethargy, irritability, poor feeding and/or apnoea. RSV is the most common viral cause of lower respiratory tract infection (LRTI) in infants and young children worldwide. Approximately 0.5–1% of all children are hospitalised for RSV LRTI during the first year of life. Of these children, 10% will develop respiratory insufficiency necessitating mechanical ventilation. Currently, no effective treatment exists for this significant childhood disease. This article aims to provide an overview of RSV LRTI with special emphasis on prevention and treatment of RSV infection.

VIROLOGY

RSV is a member of the Paramyxoviridae family. It is a single-stranded, negative-sense RNA virus. The genome encodes for 11 proteins. The F (fusion) and G (attachment) glycoproteins are the major surface antigenic determinants. Two antigenic subtypes of RSV (A and B) are distinguished based on polymorphisms of the G protein [2]. Non-structural proteins NS1 and NS2 are thought to contribute to the immuno-suppressive effects of RSV [3]. During yearly winter epidemics, both strains usually circulate within the community. Although several studies have shown that RSV type A is associated with more severe disease, others have not been able to confirm this finding[4]. Infection usually occurs through direct contact, but aerosol transmission is possible. RSV replicates in the nasopharynx and spreads to the lung, giving rise to lower respiratory tract disease several days later. The incubation period ranges from two to eight days, and viral shedding may last up to four weeks in young infants.

IMMUNOPATHOLOGY

The pathogenesis of RSV LRTI is not fully understood (see Figure 1). Post-mortem data from children with RSV LRTI are sparsely available. The general pathological changes in the lung include oedema, macrophage and lymphocyte infiltration, necrosis of epithelium and mucus plugs in the small bronchioles [5]. Although higher RSV titres are found in nasopharyngeal samples from mechanically ventilated infants than in those from hospitalised, non-ventilated infants [6], a high viral load is not required for severe RSV bronchiolitis, suggesting that the immune response is at least partially responsible for the severity of disease. The most convincing evidence that RSV LRTI is an immune-mediated disease comes from animal experiments. It has been shown by several investigators that T-cell depletion protects mice from disease in case of RSV infection, despite the presence of high viral replication in the airways of these mice [7]. In apparent contrast, adaptive immunity also plays a protective role in the pathogenesis of RSV LRTI in infants [8]. Initially, RSV-specific antibodies were thought to contribute to disease severity in cases of RSV LRTI, partially because re-infection occurs even when significant specific antibodies are present. However, it is now clear that mucosal and serum antibodies contribute to protection against RSV infection or re-infection. High maternal antibodies may prevent severe RSV LRTI in newborns [9]. Immature innate and adaptive immune responses could have an important role in controlling viral replication in case of RSV LRTI. In fact, evidence exists that adaptive immunity during RSV LRTI is immature, characterised by low monocyte IL-12 production [8]. These data suggest that mature immunity might protect against the development of severe infection, potentially by controlling viral replication.

RISK FACTORS FOR SEVERE COURSE OF DISEASE

Hospitalisation rates for prematures with and without chronic lung disease are increased. There is an inverse relationship between gestational age and risk of severe disease in cases of RSV LRTI. Also, dual infection with human metapneumovirus has been associated with severe bronchiolitis leading to a 10-fold increase in relative risk of admission to a paediatric intensive care unit for mechanical ventilation 10. Other infants at high risk for developing serious disease are those with underlying lung disease and congenital heart disease. The mortality rate in healthy infants is nil, but may be up to 3% in high-risk infants and up to 70% in immune-compromised patients [11].

Although RSV bronchiolitis is the leading cause of paediatric hospitalisation during the winter season, it is not only a childhood disease. Previous infection does not offer protection and re-infection – although often with milder symptoms – is common. Recent studies indicate that young healthy adults tend to have more severe symptoms than the average cold resulting in work absence, with manifestations similar to those of influenza [12]. In addition, RSV causes significant morbidity and mortality in elderly bone marrow transplant and lung transplant patients. In a prospective cohort analysing 2,514 acute respiratory illnesses, 3–7%of healthy elderly patients 65 years or older and 4–10% of high-risk adults with chronic obstructive pulmonary disease or heart disease were found to develop RSV infection annually [13]. Again, the disease burden in terms of length of hospital stay, rates of use of intensive care (12–15%) and mortality (7–8%) are similar to that of influenza.

LONG-TERM CONSEQUENCES OF RSV LOWER RESPIRATORY TRACT INFECTION

Not only is the overall disease burden of acute RSV infection high in terms of morbidity and economic costs, long-term morbidity is considerable. There is a strong association with post-bronchiolitis wheezing. Approximately half of infants with RSV LRTI go on to have recurrent wheezing episodes until they reach school age [14]. Multi-factorial mechanisms – both immunological and non-immunological responses – appear to play a role. With regard to similarities between post-bronchiolitis wheeze and asthma, allergic mechanisms have been considered. However, post-bronchiolitis symptoms cannot be fully explained by allergic risk factors. Several mechanisms have been suggested to underlie airway inflammation and hyper-reactivity resulting in both RSV LRTI and the subsequent development of recurrent wheeze. Genetic susceptibility polymorphisms of airway inflammation (IL-8), airway hyper-responsiveness (IL-10) and Th2 responses (IL-4, IL-4Ra) have been associated with severe RSV LRTI [15]. Whether these genes are also involved in post-bronchiolitis wheezing is not known. In addition, congenitally abnormal airway function has been mentioned to play a role in the pathogenesis of recurrent airway disease, in which RSV LRTI may only be the first indication of chronic airway symptoms. Because airway morbidity following RSV LRTI has a seasonal pattern, viral upper respiratory tract infections seem to be the predominant trigger for wheezing [16].

TREATMENT

Although RSV was discovered half a century ago, there is currently no effective treatment available, except for oxygen therapy in the presence of hypoxaemia [17]. The primary treatment is supportive. Antibiotics are rarely indicated because bacterial superinfection is uncommon in children hospitalised with RSV bronchiolitis or pneumonia. Because the majority of hospitalised infants with RSV LRTI develop bronchospasm, bronchodilators are commonly used. In patients with RSV-induced respiratory failure, approximately 50% will respond to inhaled β-agonists [18]. However, a large meta-analysis of 22 randomised controlled trials (RCTs) found only a modest short-term improvement in the overall average clinical scores when comparing bronchodilators with placebo in infants with mild to moderate disease. No significant differences were seen in oxygen saturation or duration of hospitalisation. Local epinephrine treatment was shown to be ineffective [19]. Taken together, bronchodilators can therefore not be recommended for routine use, but may be warranted on a 'trial-and-error' basis. Corticosteroids potentially decrease bronchiolar swelling and airway obstruction through anti-inflammatory effects. The amount of research on this treatment modality is not surprising considering the similarity between the clinical syndromes of childhood asthma and RSV bronchiolitis and post-bronchiolitis wheezing. An initial meta-analysis

showed a statistically significant improvement in clinical symptoms, hospital length of stay and duration of symptoms in patients hospitalised with RSV bronchiolitis when comparing systemic corticosteroids with placebo [20]. A significant reduction in the duration of hospitalisation stay was found with the use of systemic corticosteroids in ventilated patients with RSV bronchiolitis [21]. However, later meta-analyses and systematic reviews found no evidence in favour of corticosteroids [22]. The general consensus is that systemic corticosteroids are not indicated. Data on the prevention of post-bronchiolitis wheezing are conflicting. Although most RCTs concluded that treatment with corticosteroids during RSV bronchiolitis did not prevent recurrent wheezing, there may be benefits if treatment is continued beyond the acute infection. Aerosolised ribavirin, a guanosine analogue, has good anti-viral in vitro activity against RSV and was approved for treatment of RSV LRTI in children. Early study results on efficacy were conflicting and there are concerns about occupational exposure, possible teratogenicity and high costs. A large meta-analysis found an overall lack of sufficient power to provide reliable estimates of the effects when comparing ribavirin with placebo in infants and children below the age of six months with RSV LRTI [23]. Early use has been shown to reduce morbidity and mortality in adult and paediatric bone marrow transplantation patients. It is currently believed that antiviral therapy should be instituted within the first 36 hours of disease, because later symptoms are largely caused by the inflammatory host response. In practice, we believe there is no place for the use of ribavirin in the treatment of RSV LRTI. Future studies may attempt to evaluate the effect of combined antiviral-steroid treatment to treat RSV LRTI. Taken together, it can be concluded that there is sufficient evidence that there is no effective treatment for RSV LRTI.

PREVENTION

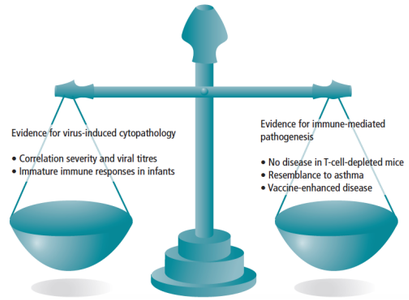
Avoiding RSV infection at an early age, especially for high-risk patients, is probably the best preventative strategy, but it is challenging to implement. Significant risk factors for RSV hospitalisation – such as male sex, age below six months at acquisition, birth during the first half of the RSV season and crowding/multiple siblings – cannot be influenced, and RSV is ubiquitously present in the community during the winter season. With lack of adequate treatment, research has focused on vaccine development for high-risk groups.

Currently, no vaccine for RSV is available. Vaccine development for a very young patient population against a disease with antigenically divergent strains, causing recurrent infections throughout life, faces many challenges. In the 1960s, a formalin-inactivated vaccine was used in infants [24], but it did not offer protection against naturally acquired RSV. In fact, enhanced disease and increased mortality were observed during RSV infection following vaccination. It was recently shown that this disastrous effect can be attributed to reactive carbonyl groups due to formalin inactivation of the virus [25]. Several new strategies for safe and effective vaccination, including immunisation with attenuated strains and sub-unit vaccines, are currently under investigation [26]. Recombinant NS1-or NS2-gene-deleted RSV vaccines are interesting new candidates. RSV polyclonal hyperimmune globulin, prepared from donors with high serum titres of RSV-neutralising antibody, was licensed in 1996 and recommended by the American Academy of Pediatrics (AAP) on the basis of initial positive study results [27]. Due to increased morbidity and mortality associated with its use in infants with congenital heart disease, theoretical risk of transmission of blood-borne pathogens, interference with childhood vaccination and theoretical cost–benefit analyses, prophylaxis with palivizumab is preferred over RSV immunoglobulin. Palivizumab is an immunoglobin G (IgG)-1 humanised monoclonal antibody against the RSV F protein, which neutralises RSV and prevents viral binding to cells. It does not interfere with immune responses to live attenuated vaccines. Monthly administration resulted in a 45–55% reduction of RSV LRTI hospitalisation in infants with bronchopulmonary dysplasia and/or prematurity and infants with congenital heart disease [28,29]. It was licensed for use and recommended by the AAP in selected infants and children under 24 months of age with chronic lung disease, preterm birth <35 weeks or haemodynamically significant congenital heart disase. Whether palivizumab prevents RSV LRTI in other high-risk populations is not known. There is much debate about the prophylactic use of palivizumab due to the associated high costs and epidemiological differences between countries further influencing the cost–benefit ratio. Even in subgroups with the most favourable cost–benefit ratio –prematures <32 weeks who required oxygen for 28 days or more in the intensive care unit and who were discharged during the RSV season – the number needed to treat to avoid one hospitalisation is 7.4 [30].

CONCLUSION

After four decades of study, supportive treatment remains the general mode of therapy for RSV bronchiolitis. Despite evidence showing no clear beneficial effect, bronchodilators, corticosteroids and ribavirin are still used. Even with proper therapeutic agents, morbidity during acute infections may be diminished, but the long-term consequences are not. Currently, reduction of RSV-related hospitalisation in high-risk groups can be achieved with prophylactic palivizumab and, although mortality rates from RSV in affluent countries have fallen dramatically in recent years, it has been estimated that 463,000 die annually, mostly in the developing world. Prevention will be the key to controlling RSV and post-bronchiolitis wheezing. Future research should focus on the development of vaccines, potent anti-virals, improved antibody prophylaxis and immuno-modulants, in which studies aimed at deciphering immunological mechanisms of disease and genetic predispositions will hold the answers.

FIGURE I: Double Role of Adaptive Immune Responses in the Pathogenesis of RSV LRTI



Immature adaptive immune responses during infancy allow for high viral titres, resulting in direct cytopathology. In apparent contrast, aberrant T-cell-mediated immune responses appear to augment disease. RSV-infected T-cell-depleted mice have high viral titres, but no disease.

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CHAPTER 7

RESPIRATORY SYNCYTIAL VIRUS BRONCHIOLITIS: PREVENTION AND TREATMENT

Faber TE
Kimpen JLL
Bont LJ

Expert Opinion on Pharmacotherapy 2008;9:2451-2458

ABSTRACT

Acute respiratory illness due to respiratory syncytial virus is one of the most common causes of hospitalization in very young children worldwide. The virus was discovered half a century ago, yet the underlying mechanisms of the disease are not fully understood and treatment remains supportive. This review article discusses therapeutic and preventive strategies, past, present and future, in the battle against respiratory syncytial virus. Prevention of severe respiratory syncytial virus infection in high-risk children can be achieved by the administration of specific monoclonal antibodies. Current issues include the management of respiratory syncytial virus infection in those with underlying immunological disease, the prevention of long-term airway morbidity and the development of innovative vaccines.

INTRODUCTION

Respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) is the most common viral cause of hospitalization in very young children worldwide. The symptoms range from mild upper respiratory disease such as rhinitis, to severe and life-threatening dyspnea and apnea requiring mechanical ventilation. The disease's severity depends on the patient's age and health status, whereby typically premature infants are at greatest risk. In chis age group the symptoms of acuce RSV infection can be atypical and carers muse be aware when patients present with signs such as lethargy, irritability and poor feeding. Other patients ac risk for severe disease are chose with chronic lung disease, congenital heart disease or Down's syndrome [1]. In addition, dual infection with human metapneumovirus and possibly other respiratory viruses has been associated with a more severe course of bronchiolitis [2]. However, these known risk factors for severe RSV disease cannot fully explain the differences in the disease's severity. Genetic susceptibility, such as RANTES and TLR-4 polymorphisms, has been implicated in severe bronchiolitis following RSV infection [3,4]. The incidence of RSV is similar in developed and developing countries and malnutrition does not appear co increase the risk of severe infection [5,6]. However, the mortality rates due co acute respiratory infections are 10 - 50 times higher in developing countries, with RSV causing an estimated 500,000 deaths in children each year globally [7,8].

More than 90% of children are infected with RSV by the age of 2 years, increasing to almost 100% by the age of 3 years. Although RSV bronchiolitis is the leading cause of pediatric hospitalization during the winter season and at least 0.5 - 1% of all children require hospitalization during the first year of life, RSV is not only a childhood disease. The disease burden in young healthy adults is more severe than the common cold and can be compared to that of influenza [9-11]. RSV causes significant morbidity, particularly in high-risk groups, such as the elderly and organ transplant patients, with 12 -15% rates of admission or use of intensive care and mortality rates of up to 8% [12,13]. Considering these staggering epidemiological characteristics and the fact that RSV was discovered half a century ago, it is frustrating that supportive care is the only current treatment available. The prevention of severe RSV infection in high-risk children can be achieved by the administration of monoclonal antibodies. This article discusses the therapeutic and preventive strategies most commonly implemented or extensively researched, past, present and future, in the battle against RSV.

TREATMENT OF RESPIRATORY SYNCYTIAL VIRUS

Antiviral therapy

Background: Human RSV is a negative-sense, single-stranded, enveloped RNA virus belonging to the Paramyxoviridae family, subfamily Pneumovirinae. Spread occurs from respiratory secretions through dose contact with infected persons or contact with contaminated surfaces, on which it survives for up to several hours. Aerosol transmission is rare. Respiratory syncytial virus disease partly occurs from a direct cytotoxic effect of the virus on the airway epithelium.

Pharmacology: Ribavirin is an antiviral drug that is active in vitro against a number of DNA and RNA viruses such as influenzas, flavivimses, hepatitis C and many viral hemorrhagic fevers. It is only registered in nebulized form for RSV infection. Ribavirin is a synthetic nucleoside analog that is converted through cellular kinases into the 5' triphosphate nucleotide. In this form it is thought to interfere with RNA metabolism and viral reproduction. Its mechanisms of action are thought to include hypermutation and inhibition of RNA polymerases, but the exact mechanism of action is unknown. The primary serious adverse effect of ribavirin is hemolytic anemia through erythrocyte trapping and it is teratogenic in all non-primate animal species on which it has been tested at higher levels than administered to people. Consequently, there is theoretical concern about repeated occupational exposure of pregnant nurses to aerosolized drugs.

Efficacy: Initial clinical intervention studies during the 1980s reported a positive effect of nebulized ribavirin when compared to placebo treatment [14-19], but there were major methodological flaws, thereby preventing aggregation of the data for meta-analyses. Although aerosolized ribavirin has good antiviral in vitro activity against RSV, subsequent double-blind placebo-controlled smdies in mechanically ventilated RSV patients have failed to show positive effects and even found prolongation of mechanical ventilation and the duration of hospital stays [20-23]. A large meta-analysis found an overall lack of sufficient power for providing reliable estimates of the effects when comparing ribavirin with placebo in infants and children with RSV LRTI below the age of 6 months [2-4].

The American Academy of Pediatrics (AAP) has recommended that ribavirin 'may be considered' in high-risk children for serious RSV disease since 1996 [25]. Early use has been shown to reduce the morbidity and mortality in high-risk patients such as adult and pediatric bone marrow transplantation patients. Recently, a randomized controlled multicenter trial using pre-emptive aerosolized ribavirin in hematopoietic cell transplant patients with upper respiratory tract RSV infection found a decreasing viral load over time, but proof of its efficacy in terms of progression to pneumonia remained elusive [26]. Combining ribavirin with glucocorticosteroids or specific monoclonal antibodies, although only in retrospective and non-controlled studies, was recently determined safe and associated with a decreased mortality in high-risk pediatric patients and in patients with severe hematological immunodeficiency [27,28].

Conclusion: When considering the lack of uniquivocal evidence, the price of the product, the difficult administration of the product and the fear of adverse events, the authors believe that there is no place for the routine use of ribavirin in the treatment of either ventilated or non-ventilated patients with RSV LRTI. The use of ribavirin in highest risk patients, such as bone marrow transplant recipients, remains controversial. Future studies with high-risk immunodeficient patients may attempt to evaluate the effect of combined antiviral-steroid or antiviral-immunoglobulin or antiviral-palivizumab treatment.

Bronchodilator therapy

Background: The typical RSV patient presents with signs of bronchiolitis: cough, wheeze/bronchospasm and shortness of breach. Airway obstruction secondary to inflammation and constriction of the smaller airways plays an important role in the pathogenesis of serious RSV disease. There is also a strong association with RSV and postbronchiolitis wheezing and asthma: approximately half of the infants with RSV LRTI go on to have recurrent wheezing episodes until they reach school age [29,30]. Multifactorial mechanisms, both immunological and non-immunological responses, as well as genetic susceptibility, appear to play a role. Considering the similarities between bronchiolitis, postbronchiolitis wheezing and asthma, it is not surprising that research has focused on bronchodilator therapy in the treatment of RSV infection.

Pharmacology: Bronchodilators or β2 mimetics act on the β2 receptors in bronchial smooth muscle and bronchial mucous membranes and dilate the bronchi and bronchioles, hence increasing airflow. Bronchodilators may produce refractory bronchospasm.

Efficacy: Short-acting β2 agonists such as albuterol/salbutamol and anticholinergics such as ipratropium bromide and theophylline have all been evaluated in the treatment of RSV bronchiolitis, but the results are conflicting. Within the studies there is often great variation in the proportion of laboratory-proven RSV-infected patients and between studies there is great variation in the severity of disease. Overall, in patients with RSV-induced respiratory failure, 50% will respond to inhaled β-agonists [31]. However, large metaanalyses have either shown no beneficial effects or only a modest short-term improvement in the overall average clinical scores when comparing bronchodilators to placebo in infants with mild-to-moderate disease [32,33]. The effect of ipratropium bromide, either alone or in combination with albuterol, as well as local adrenaline treatment was shown to be ineffective [34]. A randomized double-blind controlled trial in infants admitted to hospital with bronchiolitis found no positive acute effects on respiratory effort and no reduction in the length of hospital stay with the use of nebulized adrenaline when compared to placebo [35]. Another randomized double-blind controlled trial compared racemic adrenaline to salbutamol and found improvements in respiratory distress in hospitalized infants with bronchiolitis, but it did not abbreviate their hospital stay [36]. A recent study evaluated the effect of heliox, a 79% helium-21 % oxygen mixture used for alleviating the symptoms of upper airway obstruction through a reduction in airway resistance, on young infants admitted to the pediatric intensive care unit with moderate-to-severe acute RSV bronchiolitis. Heliox breathing induced a rapid reduction in accessory muscle use and expiratory wheezing [37].
Conclusion: Taken together, the routine use of nebulized bronchodilator therapy in children with RSV bronchiolitis is no longer recommended. The individual patient may have some short-term benefit and therapy may thus be used on a 'trail-and-error' basis. Further studies are required in order to determine the effect of adrenaline and heliox.

Corticosteroids

Background: There is a large body of evidence in favor of the notion that RSV is an immune-mediated disease. Early evidence for a role of the cellular immune system in the pathogenesis of RSV disease comes from experimental studies demonstrating less morbidity in immunodeficient, for example CD-4- and CD-8-deficient, mice [38,39]. T cell depletion protects mice from disease, despite the presence of high viral replication in the airways of these mice [40]. In infants higher RSV titers are found in mechanically ventilated infants [41], but a high viral load is not required for severe RSV bronchiolitis. The peak of disease coincides with the development of specific T and B cell responses, rather than with the peak of viral replication [42]. High maternal antibodies prevent severe RSV LRTI in the newborn [43], but re-infection can occur even in the presence of significant specific RSV antibodies. These results, taken together with the association between RSV and postbronchiolitis wheezing and asthma, suggest that not so much viral cytotoxicity but rather the immune response and excessive airway inflammation play an extensive role in the pathogenesis of serious RSV disease. There is convincing evidence that T helper 1- and T helper 2-type cycokine patterns determine the type of immune response to RSV infection and that the spectrum of cytokine expression affects the control mechanisms involved in the regulation of disease pathogenesis and chronicity [44]. It has been hypothesized that the infection and concomitant inflammatory reaction during acute RSV infection leads to airway epithelium injury resulting in long-term obstructive airway disease and postbronchiolitis wheezing.
Pharmacology: Steroid hormones are synthesized from cholesterol in the adrenal cortex and produce an anti-inflammatory response through leukocyte adhesion and the production of prostaglandins and leukotrienes, hence potentially decreasing bronchiolar swelling and airway obstruction. Common side effects of systemic immune-modulating drugs, such as dexamethasone and prednisolone, include Cushing's syndrome, hypertension, hypokalemia, hypernatremia and central serous retinopathy.
Efficacy: Initial studies comparing systemic corticosteroids to placebo in infants hospitalized with RSV bronchiolitis were promising. A large meta-analysis showed a significant improvement in the clinical symptoms, length of stay and duration of symptoms [45]. However, later meta-analyses and systemic reviews found no evidence in favor of corticosteroids [46]. A recent systematic review evaluated the effect of inhaled corticosteroids, started during the acute phase of bronchiolitis, on the prevention of postbronchiolitis wheezing. Due to the small number of patients included and the inability to pool all clinical outcomes, strong recommendations could not be made [47].
Conclusion: The general consensus is that systemic corticosteroids are not indicated in therapy against RSV LRTI. Further studies are required in order to evaluate the effect of inhaled corticosteroids on the prevention of postbronchiolitis wheezing. Because the disease process produced by RSV infection is primarily inflammatory, other therapeutic approaches that target this inflammatory process may be successful.

Antibiotics

Background: Although RSV disease is of viral origin, nearly half of all hospitalized infants with RSV LRTI are treated with antibiotics [48-50]. Although an apparent contradiction, antibiotics are often initiated upon initial presentation of a distressed infant, with consolidation on a chest radiograph or during the course of the disease when concurrent bacterial infection is suspected.
Pharmacology: An antibiotic is a chemotherapeutic agent that inhibits or abolishes the growth of microorganisms such as bacteria. Some antibacterial antibiotics destroy bacteria (bactericidal), whereas others prevent bacteria from multiplying (bacteriostatic). Different classes of antibiotics used in the therapy of respiratory disease include penicillins, such as amoxicillin and macrolides, such as clarithromycin. The adverse effects are variable, but commonly include diarrhea and, less commonly, allergic reactions. Inappropriate antibiotic treatment may cause the development of resistance. Macrolides, through their ability for preventing the production of pro-inflammatory mediators and cytokines, may exert therapeutic effects independently of their antibacterial activity. Clarithromycin, one of the newer macrolides, suppresses cytokine production via inhibition of nuclear factor-κβ inhibition [51]. Immunomodulation further results from inhibition of neutrophil migration, an increase in phagocytosis and natural killer cell activity and induction of eosinophil apoptosis [52].
Efficacy: Pulmonary bacterial co-infection is common in children ventilated for severe RSV bronchiolitis. The rates of positive bacterial culture from broncho-alveolar lavage and endotracheal aspirates in infants admitted to the intensive care unit with laboratory-confirmed RSV infection vary from 33 to 44% [53-56]. However, it is difficult to distinguish between bacterial colonization and co-infection in these patients. The occurrence of secondary bacterial infection in all hospitalized children with RSV LRTI, including those non-ventilated, is < 1 % [57]. In a double-blind randomized placebo-controlled trial, clarithromycin reduced the length of hospital stay, duration of need for supplemental oxygen and plasma cytokine levels and readmission rates to the hospital after discharge [52]. However, there were major methodological flaws [58,59], thereby making the results unreliable and the recommendations premature. A recent randomized controlled trial did not show a beneficial effect of azithromy-

cin in children hospitalized for RSV LRTI [60].
Conclusion: Unjustified use of antibiotics must be avoided because of concern about the development of antimicrobial resistance. Although RSV LRTI can mimic bacterial pneumonia, antibiotics should not be used in non-ventilated children with RSV unless concomitant bacterial infection is proven. Empirical antibiotic therapy may be justified in children admitted to the pediatric intensive care unit with probable signs of bacterial co-infection, pending the results of definitive culcures. The regular use of macrolides in children with RSV lower respiratory trace infection does not appear justified.

PREVENTION OF RESPIRATORY SYNCYTIAL VIRUS

Vaccine

Background: Avoiding RSV infection at an early age, especially for high-risk infants, is probably the best preventive strategy but impossible to implement. Significant risk factors for serious RSV disease, such as male sex, age below 6 months at acquisition, birth during the first half of the RSV season and crowding/multiple siblings, cannot be influenced. Furthermore, RSV is ubiquitously present in the community during the winter season. With lack of adequate treatment, research focused on vaccine development as early as the 1960s.
Pharmacology: A vaccine is an antigenic preparation that is used for establishing immunity to a disease. After vaccination, the body recognizes the protein coat on the virus and responds by neutralizing the agent before it can enter cells and by destroying infected cells before they can multiply. Further efficacy is often obtained through herd immunity. Vaccines are traditionally developed from inactivated, live attenuated or fragments of microorganisms.
Efficacy: Early research with an RSV formalin-inactivated vaccine was disastrous. It offered no protection against naturally acquired RSV, but rather led to enhanced disease and increased mortality when patients were subsequently infected with RSV in the next season [61]. The mechanism responsible for this vaccine-associated enhanced disease was not completely understood until reactive carbonyl groups due to formalin inactivation of the virus were recently found to be responsible [62]. Naturally, studies on RSV vaccination were put aside until the recent development of innovative conjugate, recombinant and DNA vaccines. These new strategies for safe and effective vaccination have shown tremendous progress in the last decade. Several candidate vaccines are currently under investigation [63-68], but it will probably be at least several years before routine immunization against RSV becomes available.
Conclusion: At present, no vaccine against RSV is available. Considering its history and the criteria that an RSV vaccine must meet, such as efficacy against antigenically divergent strains in a very young population, vaccine development against RSV is a challenging field. The RSV non-structural proteins NS1 and NS2 are thought to contribute to the immunosuppressive effects of RSV. Recombinant NS1 or NS2 gene-deleted RSV vaccines, subunit vaccines and nasal peptide vaccines are interesting new candidates for future research.

Immunoglobulins

Background: Due to the lack of a safe RSV vaccine, much research has focused on immunoglobulins in the prevention of RSV infection.
Pharmacology: Immunoglobulins or antibodies are glycoproteins that bind to a specific antigen and neutralize it. The RSV genome encodes for 11 proteins. The F (fusion) and G (attachment) glycoprotcins are the major surface antigenic determinants. Respiratory syncytial virus polyclonal hyperimmune globulin (RSVIG) was prepared from donors with high serum titers of RSV-neutralizing antibody. Palivizumab, which is produced by recombinant DNA technology, is an IgGl humanized monoclonal antibody against an epitope in the A antigenic site of the RSV F protein that neutralizes RSV and prevents viral binding to cells. It does not interfere with immune responses to live attenuated childhood vaccines such as measles, mumps and rubella.
Efficacy: Standard intravenous immune globulin was neither protective nor therapeutic in patients with RSV bronchiolitis due to insufficient titers of antibodies [69]. Respiratory syncycial virus polyclonal hyperimmune globulin overcame this problem and was licensed and recommended by the AAP in 1996 on the basis of initial positive study results [70,71]. Respiratory syncytial virus polyclonal hyperimmune globulin given as treatment to infants with severe RSV has a positive effect on lowering viral titers, but this was not correlated with improved clinical outcomes [72]. Passive immunization with RSVIG is only effective as long as enough neutralizing antibody titer is achieved and monthly administration of this product is cumbersome. Furthermore, due to the increased morbidity and mortality associated with its use in infants with congenital heart disease, the theoretical risk of transmission of blood-borne pathogens, interference with childhood vaccination and theoretical case-benefit analyses, prophylaxis with palivizumab is preferred over RSVIG. Respiratory syncytial virus polyclonal hyperimmune globulin is no longer commercially available. Palivizumab, in contrast co monthly intravenous RSVIG infusions, can be given with monthly intramuscular injections during the RSV season. The results show a 45-55% reduction in RSV LRTI hospitalization in high-risk infants (bronchopulmonary dysplasia and/or prematurity and infants with congenital heart disease) [73,74]. The AAP recommends the use of palivizumab in high-risk infants and children under 24 months of age with chronic lung disease, preterm birth < 35 weeks or hemodynamically significant congenital heart disease [75]. So far, there is insufficient data for recommending palivizumab in other high-risk groups such as the elderly, immunodeficient patients and those with cystic fibrosis. There is much debate about the prophylactic use of palivizumab due to the associated high costs. The number needed to treat to avoid one hospitalization is at best 7.4 [76], However, a recent population-based study found a 7.3% reduction in hospitalizations with palivizumab prophylaxis in high-risk infants when comparing similar health regions in Canada with different palivizumab strategies [77]. This is the first 'real life' evidence favoring palivizumab prophylaxis. More promising results come from a recent study suggesting that palivizumab may reduce recurrent wheezing in premature infants [78].
Conclusion: Immunoglobulins have no place in the treatment of RSV LRTI, but prophylaxis with palivizumab can decrease hospitalization rates in infants born prematurely or those with bronchopulmonary dysplasia or hemodynamically significant congenital heart disease, but not the incidence. Implementing this strategy has resulted in a dramatic decrease in RSV mortality races in affluent countries. Further studies are required in order to evaluate the effect of palivizumab in other high-risk groups. Whether the cost-benefit ratios for the use of palivizumab will be more favorably influenced when positive long-term effects on recurrent wheezing can be taken into consideration remains to be seen. Further analyses of cost-effectiveness compared to other biological agents might also help in assessing the financial burden of palivizumab. A second-generation monoclonal antibody, motavizumab, is currently under study in Phase III clinical trials and has enhanced neutralizing activity against RSV when compared to palivizumab [79]. Prophylactic administration in mice was associated with signifi-

cant reduction in both RSV replication and cytokine concentrations [80]. Such antibody engineering technology may prove to be of great value in preventing severe RSV disease in infants in the near future.

EXPERT OPINION

Despite more than four decades of study, the treatment of RSV remains supportive. Bronchodilators, corticosteroids and antivirals have all failed to demonstrate significant efficacy in this disease. The role of antivirals is limited at present, with no new agents near availability. Most patients reach medical attention at a time when the viral load is declining and the symptoms are generated by the inflammatory imuune response. The introduction of palivizumab has so far constituted the only advance in controlling the impact of RSV infection in high-risk children, but it is very limited in indication based on the cost and limited production capacity of such a biological product. Even with improved antibody prophylaxis or proper therapeutic agents for acute RSV disease, the long-term airway morbidity may not be diminished. Prevention should be the key road to follow. Yet this field is particularly challenging, because RSV appears to have a complex interplay with its host. Respiratory syncytial virus glycoprotein G and glycoprotein F enhance binding to human cells and upregulate Toll-like receptor 4, facilitating viral entry. In particular, glycoprotein G is associated with an enhanced immune response. Nonstructural proteins modify the immune response by causing resistance to cytokines, such as type 1 interferons, by which RSV can evade the immune response. Vaccine development is focusing on genetically modified vaccines, such as recombinant viruses lacking glycoprotein G, which will likely result in a weaker inflammatory response. Maternal vaccination may be an interesting strategy and has the potential for preventing RSV disease in early infancy. However, for safety reasons, researchers have intuitively been reluctant to administer an RSV vaccine to pregnant women.

Considering the overwhelming epidemiological figures and high mortality rates among high-risk groups and those in Third World countries, there is no question that RSV research remains urgent. New insights into its pathogenesis and genetic predispositions should lead to the development of innovative vaccines and effective immunomodulants, thereby enabling control of RSV disease worldwide.

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CHAPTER 8

COMPUTERIZED ASSESSMENT OF WHEEZE IN CHILDREN WITH RSV BRONCHIOLITIS BEFORE AND AFTER NEBULIZATION WITH HYPERTONIC SALINE

Faber TE
Kamps AW
Sjoerdsma MH
Vermeulen S
Veeger NJ
Bont LJ

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ABSTRACT

Studies suggest an effect from nebulization with hypertonic saline solution on airflow limitation in patients with respiratory syncytial virus (RSV) bronchiolitis, but results are based on subjective scores of clinical severity and results are not clear. In this observational study, we used a non-invasive computerized tool to quantify wheeze before and after nebulization with hypertonic saline in children admitted for RSV infection. Twenty-seven children aged 0 to 24 months admitted to the pediatric ward at the Medical Center Leeuwarden with polymerase chain reaction confirmed RSV bronchiolitis were included. Subjects were simultaneously assessed both clinically and by computerized acoustic monitoring before and 15 minutes after nebulization with hypertonic saline solution. Clinical assessment, defined by the respiratory distress assessment index (RDAI) score, did not change after nebulization (n=27; 5.0 vs 4.7, P=0.17). Computerized acoustic monitoring showed no improvement in wheezing or inspiration/expiration (I/E) ratio's after nebulization (n=27; 3.4% vs 2.0%, P=0.05 and 0.850 vs 0.846, P=0.93 respectively). Hypertonic salt nebulization does not improve airflow, as assessed by both clinical and computerized acoustic scores, in children admitted for RSV.

INTRODUCTION

Treatment of respiratory syncytial virus (RSV) bronchiolitis, a disease that affects almost all children by the time they reach their second year of life, remains supportive. Approximately 40% to 50% of infected infants develop infection of the lower respiratory tract, leading to hospitalization in 1% to 2% of cases [1]. Numerous therapies have been tried, such as bronchodilators, nebulized adrenaline, inhaled or systemic steroids, cromoglycate, immunoglobulins and antiviral treatment, but (meta-)analyses have failed to show evidence of effect [2-5]. Several studies suggest a beneficial effect from nebulization with hypertonic saline solution on clinical severity scores, but results are not entirely clear [2,6,7]. The main rationale for hypertonic saline treatment in bronchiolitis is rehydration of the airway surface liquid leading to increased airway surface thickness, decreased epithelial edema, improved mucus rheologic properties such as elasticity and viscosity and acceleration of mucus transport rates thus increasing mucociliary clearance [8]. Due to possible beneficial effect, the lack of side effects and the limited cost, pediatricians often use this mode of therapy on a trial and error basis [2].

The PulmotrackTM (Karmelsonix Ltd. Israel), a respiratory acoustic monitor, is a validated tool to digitally quantify lung sounds such as wheezing and cough 9. Computerized wheeze detection has been shown to be feasible in young children 10. In healthy adult volunteers, specificity and sensitivity for detection of cough events are high (94% and 96%, respectively) [11]. In children with asthma, this method has been used to provide quantitative information about wheezing and efficacy of treatment, and correlates well with conventional indices of bronchus obstruction such as forced expiratory volume and forced vital capacity [12]. In the pediatric intensive care unit, the PulmotrackTM was significantly more sensitive than physicians in detecting wheeze [13]. Respiratory sonography was successfully used before in children with RSV bronchiolitis to evaluate wheeze patterns [14] and to assess treatment efficacy of nebulized salbutamol and epinephrine [9,15]. The authors conclude that it provides a noninvasive method for objective clinical assessment of young, wheezy children.

There are no objective diagnostics assessing direct effects of any treatment on airflow limitation in children with RSV bronchiolitis. Results of previous studies are based on subjective measurements such as clinical severity scores, and results are not clear. We therefore performed an observational study and used the PulmotrackTM to quantify bronchus obstruction in children admitted for RSV bronchiolitis before and after nebulization with hypertonic saline solution.

METHODS

The study protocol was approved by the institutional review board “RTPO” at the Medical Center Leeuwarden in The Netherlands (TPO787a). In this observational study, infants aged 0-24 months, admitted to the pediatric ward at the Medical Center Leeuwarden, with a polymerase chain reaction (PCR) confirmed diagnosis of RSV bronchiolitis were eligible to enroll. Symptoms of bronchiolitis were chest cough, wheezing, rales, use of accessory muscles, and/or nasal flaring [16]. Subjects with preexisting lung disease, such as asthma, cystic fibrosis or laryngomalacia, patients using bronchodilators or corticosteroids, and severely ill children requiring mechanical ventilation were excluded from the study. The following subject characteristics were recorded: gender, gestational age, age at admission, antibiotic use before admission, temperature and oxygen saturation at time of admission, and (nonrespiratory) comorbidity.

After parents agreed to participate and gave written informed consent, the PulmotrackTM was put into place.

The study was performed on the day of admission, all included subjects received hypertonic saline administrated via jet nebulizers with continuous flow of 100% oxygen. Similar to previous studies, a concentration of 3% was used with 4 ml of volume [9,17-20]. The PulmotrackTM system consists of two acoustic sensors attached to the skin over the trachea and chest with disposable insulating adhesive pads, a pneumograph belt sensor for documenting breathing activity, and an ambient microphone to filter environmental noises, all attached to a laptop. Wheeze detection is carried out by the Karmelsonix software using advanced algorithms that apply to strict criteria to determine the presence of wheezing. Wheeze, as defined by Computerized Respiratory Sound Analysis guidelines, is characterized by periodic waveforms with a dominant frequency over 100 Hz and with a duration of ≥100 ms [21]. The wheeze rate (percent of time wheezing of total breath time), respiratory rate, inspiration/expiration (I/E) ratio’s and cough events (frequency content between 50 and 3000 Hz) were measured by the PulmotrackTM in all participating subjects for a period of 5 minutes, both before and 15 minutes after single nebulization with 4 ml of hypertonic (3%) saline solution. Considering the duration time of nebulization of approximately 10 minutes, actual assessment was performed at 25-35 minutes after start of nebulization. Simultaneously during both 5 minute intervals of PulmotrackTM recording, disease severity was clinically assessed by the modified respiratory distress assessment index (RDAI) score, once before and once 15 minutes after nebulization. The RDAI score has a range of 0 to 12 and evaluates respiratory rate, use of accessory muscle, colour and auscultation findings [22,23]. Heart rate and respiratory frequency before and after nebulization was also noted. Care was taken to ensure that all participating infants were relatively settled and not crying during the assessment. According to standard care in our hospital in patients with bronchiolitis, laboratory testing and x-rays were not routinely performed. In the statistical analysis, Wilcoxon signed rank test for paired samples was used. P-values <0.05 were considered statistically significant.

RESULTS

A total of 30 infants with bronchiolitis were eligible to enroll in the study during the winter season of 2012-2013. All parents of eligible subjects agreed to participate. Three subjects were excluded for being RSV negative. The average age at time of admission was 4.9 months. All but one child was older than 12 months. There was a larger percentage of male infants (n=18 (67%)), and most were term babies (n=23 (85%)). Almost half of the infants presented with fever and need for oxygen therapy. (Table 1).

Clinical assessment before and after nebulization with hypertonic saline showed no improvement in total RDAI score. Analysis of the individual parameters within the RDAI score showed slight improvement in use of accessory muscles, but no improvement in respiratory rate, colour, or auscultation findings. Heart rate did not differ after nebulization. Digital acoustic monitoring showed large individual differences in efficacy of nebulization on wheezing; 9/27 (33%) of subjects showed a clear decrease in wheezing and 5/27 (18%) showed a clear increase in wheezing. Overall, no improvement in wheezing, respiratory rate or I/E ratio was found after nebulization. Percentage of cough increased after nebulization. No other side effects occurred. In a subgroup analysis of subjects with more severe disease (RDAI score≥5, n=15), results were comparable. (Table 2).

DISCUSSION

This is the first study that uses computerized acoustic monitoring to assess the acute effect of hypertonic saline nebulization in children with RSV bronchiolitis. Clinical assessment by RDAI score showed no effect from nebulization. Results were confirmed by digital acoustic monitoring showing that hypertonic saline nebulization has no short term effect on wheezing, respiratory rate, or inspiration/expiration ratio's in children admitted for RSV. The number of cough episodes increases after nebulization, but the clinical implications of this result are not clear. Increased cough reflex is due either to direct stimulation of the cough receptors by hypertonic saline or to increased mucociliary clearance. Increased cough may cause increased dyspnea in some subjects, whereas others may benefit from loosening of mucus plugs [8,20].

Previous studies have used respirosonography for assessment of treatment modalities, other than hypertonic saline, in children with bronchiolitis. In a study including 16 infants with bronchiolitis, 7 showed a decrease in proportion of wheeze (time spent wheezing/total respiratory time) after nebulization with salbutamol [9]. Another study including 27 children with RSV bronchiolitis found no significant change in objective quantification of wheeze and crackles 10 minutes after nebulization with epinephrine and albutarol [15]. Both studies conclude that computerized lung sound analysis is feasible in young infants with bronchiolitis and that it provides a noninvasive, quantitative measure of wheeze. Considering previous studies that use clinical scores to assess effects of hypertonic saline nebulization there is a cochrane review of 7 randomized, double blind, parallel group, controlled trials including 581 infants with bronchiolitis. Combined results showed an approximate 1 day reduction in length of hospital stay and an improvement in clinical severity score in subjects treated with nebulized hypertonic saline in comparison to normal saline [6]. Two of the included studies were performed in the emergency department and assessed short term effects, as in our study [17,24]. In one study, clinical scores improved, whereas in the other study they did not. In both studies, hypertonic saline was combined with either salbutamol or epinephrine. Considering heterogeneity in effect sizes and combined treatment modalities in all studies, the effect of nebulization with monotherapy hypertonic saline remains unclear. The results from our study indicate, by quantitative assessment, that monotherapy with hypertonic saline nebulization has no short term effects. This is in accordance with a recent study that found no clinical improvement in infants with bronchiolitis and respiratory distress in the emergency department after administration of a single dose of nebulized hypertonic saline [7].

Although clinical severity scores are generally accepted as relatively objective measurements to assess severity of illness, the greatest strength of this study is the computerized acoustic monitor that allows for objective assessment of treatment efficacy. Limitations of this study lie in the observational design and small sample size. Furthermore, the aim of our study was to evaluate short term effects approximately 30 minutes after start of nebulization, but repeat assessments were not performed. Therapeutic effects 2 hours post nebulization can not be ruled out, although previous studies have found no difference in clinical outcome after 120 minutes [24]. Future studies should use digital acoustic monitoring to assess therapeutic effects 2 hours post nebulization or effect from mutliple nebulizations. Studies in ventilated infants could include specific measurements of airway resistance, oxygenation and ventilation index [25]. Caution is also warranted in extrapolation of data to outpatients and those with severe bronchiolitis requiring ventilation, as these patients were excluded from the study.

CONCLUSION

Computerized acoustic monitoring shows that monotherapy with hypertonic saline nebulization has no short term effect on wheezing, respiratory rate, or inspiration/expiration ratio's in children admitted for RSV. The PulmotrackTM is a useful noninvasive tool to quantify bronchus obstruction in young children with RSV bronchiolitis and enables objective evaluation of treatment modalities in both clinical and experimental setting.

TABLE I: Patient characteristics

Clinical and demographic data	n=27	
Age at admission in months (average (SD))	4.9	(4.8)
Male gender (n (%))	18	(67%)
Gestational age < 37 weeks (n (%))	4	(15%)
Temperature >38,5°C at admission (n (%))	13	(48%)
Oxygen saturation <92% at admission (n (%))	11	(41%)
(non-pulmonary) Comorbidity (n (%))	2	(7%)
Antibiotics prescribed pre-admission (n (%))	5	(19%)

TABLE II: Clinical and quantified effect of hypertonic saline nebulization in RSV bronchiolitis

	Pre-nebulization n=27	Post-nebulization n=27	p-value °
Respiratory score			
RDAI total #	5.00 ±1.59	4.74 ±1.61	0.17
RDAI respiratory rate	0.70 ±0.61	0.52 ±0.70	0.23
RDAI accessory muscle	1.67 ±0.88	1.44 ±0.85	**0.01**
RDAI colour	0.81 ±1.00	0.81 ±1.00	0.99
RDAI auscultation	1.85 ±0.36	1.93 ±0.27	0.16
Heart rate	147 ±14.0	145 ±12.9	0.53
Respiratory frequency	50 ±11.0	51 ±12.8	0.61
Digital acoustic monitoring *			
Percentage wheeze	3.4 ±3.84	2.0 ±2.74	0.05
I/E ratio	0.85 ±0.15	0.85 ±0.18	0.93
Number of cough episodes	1.44 ±2.42	2.63 ±3.36	**0.03**

* values are presented as average (SD) during 5 minute interval
# RDAI= mean respiratory distress assessment index score, range 0-12
° Wilcoxon signed rank test

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GENERAL DISCUSSION

GENERAL DISCUSSION

Since its discovery 60 years ago, we have greatly improved our knowledge of RSV disease. Many risk factors for development of serious disease have been identified that include prematurity, pre-existing medical conditions, in utero influences, and old age [1-16]. There is no therapy and treatment remains supportive with oxygen and fluid maintenance [17-20] (Chapter 6 and 7). The use of hypertonic saline nebulization is controversial [17], but by computerized assessment of wheeze, I showed there are no clinically significant effects (Chapter 8). The majority of infants hospitalized with RSV bronchiolitis were previously healthy. Understanding why these children develop severe RSV bronchiolitis, whereas others only have mild RSV infection, is difficult to unravel. The greatest independent risk factor for developing serious disease is young age [1,8,21]. Waning maternal antibodies and the ability of RSV to evade systemic immunity offers some explanation, but there is increasing evidence supporting immune mediated pathology to RSV. Interestingly, children with almost absent immunity, such as those undergoing HCT, do not necessarily show progression to severe disease in case of RSV infection (Chapter 3). Furthermore, acute respiratory failure due to RSV infection is uncommon in healthy adults with mature immune systems (Chapter 5). Approximately 20% of association with severe disease can be accounted to polymorphisms in genes regulating early immune responses 22. Pattern recognition receptors, dendritic cells and NK-cells, all involved in the early phases of RSV infection, are immature in young infants and less able to produce interferons and induce proper antiviral states of cells [23-27].

I showed that development of severe RSV bronchiolitis is associated with a more robust cascade of pro-inflammatory responses displayed by higher levels of nasopharyngeal cytokines such as IL-6 (Chapter 1). This observation suggests a critical role for the epithelium as the first line of defense against RSV and is in accordance with the hypothesis of immune mediated pathology. IL-6 can be secreted by macrophages and stimulates the production of neutrophils, the cells that characterize RSV infection. IL-6 induces inflammatory and auto-immune processes in many diseases such as rheumatoid arthritis and is an important mediator of fever and the acute phase response. [28] The result of my study showing that more severe disease is associated with higher levels of IL-6 is supported by others that have shown association with single nucleotide polymorphisms in the IL-6 promoter region [29,30], and higher lung [31], and plasma concentrations [32,33]. Furthermore, high levels of IL-6 appear to be specific for primary RSV infection in the young infant, as this response is not seen in secondary infections in older children [34]. Therefore, differences in innate immune cytokine responses, such as IL-6 induction, may explain, in part, differences in severity of illness seen with RSV infection.

Most studies focus on RSV disease at its height of severity, and not resolution of disease. I examined cytokines and chemokines during course of disease and found a unique pattern of increased local IL-17 production during the convalescent phase of bronchiolitis (Chapter 1). Th17 cells represent a distinct signaling system with pro-inflammatory responses [35-38]. Previous studies detected IL-17 in plasma and tracheal aspirates in infants with RSV bronchiolitis [39-42], but factors determining beneficial or adverse effects remain to be determined. The result from my study could suggest that local IL-17 plays a protective or regulatory role against the development of severe RSV bronchiolitis and that mechanisms determining disease severity are apparently different than those involved in resolution of disease. This is in accordance with another study in which higher plasma IL-17 concentrations were found in moderately ill patients with RSV bronchiolitis when compared with severe cases [39]. Local IL-17 production in the airways of infants during RSV bronchiolitis is induced by NFκβ and IL-6 signaling pathways [43,44] and facilitates neutrophil recruitment and IL-13 production altering T-cell responses [41,45]. Very recent studies in mice have also shown that IL-17 can have protective propensities. Exogenous IL-17 administered to RSV infected neonatal mice promotes neutrophil migration, decreases IFNγ production, decreases viral load and inflammation and increases survival rate [46,47]. Furthermore, Bordetella vaccine prevents RSV infection in mice through induction of IL-17 [48]. Future research is required in infants to determine whether and how IL-17 effector mechanisms regulate disease severity, in which case it may offer therapeutic potential. It is conceivable that the contribution of IL-17 and/or its effector mechanisms differ during the different phases of disease.

I found an association between IL1RL1 intron SNP rs1921622 and RSV disease severity (Chapter 2). A role of IL1RL1 in RSV pathogenesis was further confirmed by a positive relationship between nasopharyngeal IL1RL1-a levels and disease severity (Chapter 2). The role of IL1RL1-a in RSV disease has not yet been studied in humans, but high IL1RL1-a concentrations are correlated with other clinically severe diseases such as acute cardio-pulmonary disease and asthma [49-57]. High IL1RL1-a production in the airways of children with severe RSV bronchiolitis, as found in my study, suggests this molecule may play a role in modifying the inflammatory response to epithelial damage, as it does in severe asthma. The IL1RL1 gene encodes the receptor for IL-33 that stimulates Th2 responses such as IL-4, IL-5 and IL-13, Th17 responses, and eosinophilic inflammation [58-62]. Studies in RSV infected mice have recently shown that inhibition of IL-33 decreases production of Th2 and Th17 cytokines and reduces lung inflammation and severity of illness [58,63]. Clearly, effects of IL1RL1 in RSV disease remain to be determined, but the newly found genetic link, in light of the well established link between IL1RL1 and asthma, makes it an interesting candidate for future research on association with disease severity and post-bronchiolitis complications.

The cascade of immune responses may exert effects beyond RSV. I have shown that approximately half of young infants clear colonizing pneumococci from the nasopharynx during the course of bronchiolitis. In patients that do not clear pneumococci, bacterial loads decrease significantly (Chapter 4). This is surprising as normal carriage episodes for pneumococci last more than 2 months [64]. It has been suggested that bacterial colonization may influence the inflammatory response during viral infection and thus negatively correlate with outcome [65-68]. However, in accordance with another recent study [66], we found no correlation between the presence of pneumococci and RSV disease severity (data not published). I speculate that RSV induced mucosal immune responses, such as high IL-6 and IL-17, and abundant neutrophil influx, exert influence on colonizing bacteria through phagocytosis, degranulation and generation of neutrophil extracellular traps [69,70], as would occur with invading pathogenic bacteria. It is important to recognize that RSV bronchiolitis appears to impact colonization of pneumococci and perhaps other bacteria of the nasopharyngeal microbiome in the upper airways of previously healthy infants. The recent interest in specific viral-microbial associations has shown that RSV bronchiolitis is clearly associated with distinct nasopharyngeal microbiota including the Streptococcus cluster [65,71]. The presence of this combination is characterized by overexpression of host genes linked to the Toll-like receptor pathway and neutrophil and macrophage activation and signaling [65], yet directionality of pathogenesis warrants further study. It is crucial to study the fluctuations in non-invasive bacterial colonization of the nasopharynx that occur during

childhood in relation to viral infection and to recognize the complex dynamics of microbial communities in the upper respiratory tract, especially in the development and evaluation of candidate viral vaccines.

TOWARDS A MODEL FOR THE PATHOGENESIS OF RSV INFECTION:

There are probably multiple factors that influence the development of serious RSV disease, but combining data from this thesis enables a hypothetical schematic representation of some of the circumstances that impact outcome before, during and after primary RSV infection. The epithelium, microbiome, local innate immune responses and the RSV virus are all significant forces to consider.

The pre-infection phase (figure 1) is characterized by homeostasis of the epithelium and microbiome and a pre-existing vulnerability to develop serious RSV disease. Before infection occurs, the nasopharyngeal microbiome of the asymptomatic neonate is in a stable state with respect to the presence or absence of colonizing bacteria such as pneumococci. The presence of pneumococci may facilitate infection as RSV-pneumococcal complexes have been found to show enhanced adherence to uninfected epithelial cells [72,73]. Furthermore, the nasopharyngeal microbiome and transient colonization with Streptococcus is a determinant for infection spread to the lower airways and poses a risk of future asthma development [74]. Therapeutic approaches that target pathogenic bacteria within the nasopharyngeal microbiome could represent a prophylactic approach to the development of serious RSV disease [74]. The young infant is inherently hindered by habitation of immature innate immune cells and signaling responses. In particular patients, additional susceptibility to develop serious RSV disease originates from a genetic predisposition such as polymorphisms in the IL1RL1 gene that encodes the receptor for IL-33. Identifying genetic factors that affect pathogenesis could help pediatricians forecast course of disease. Furthermore, this could allow for expanding of immunoprophylaxis criteria to apparently healthy term infants that are at risk.

RSV binding and fusion to the epithelium elicits the production of a wide variety of cytokines and chemokines during the acute phase of infection (figure 2). Infants that develop serious disease display an overexpression of pro-inflammatory cytokines such as IL-6, IL-33 and IL1RL1a that modify the immune response resulting in sloughing of the epithelium, mucus production and clinical signs of bronchusobstruction. Mediator inhibitors targeting IL-6, IL-33 and TNFα could dampen this heightened immune response and are interesting therapeutic candidates to study. For example, anti-IL-6 agents such as tocilizumab have already been approved for therapeutic use in other inflammatory diseases in children such as systemic juvenile idiopathic arthritis [75,76]. Regarding the IL1RL1-IL-33 effector mechanisms and the newly found genetic link between asthma and RSV, anti-IL-33 antibody, already considered a therapeutic target in murine models of asthma and allergic airway inflammation, might also be considered for RSV infection. In mice, administration of anti-IL-33 antibody, or vaccination against IL33, reduces levels of IL-33, neutrophil infiltration, expression of pro-inflammatory cytokines and airway inflammation [77,78]. A humanized anti-IL-33 antibody for clinical use is currently undergoing phase 1 trials(https://www.anaptysbio.com/anaptysbio-reports-anb020-top-line-phase-1-clinical-trial-results/). TNFα plays a role in the induction of IL-33 [79-81] and systemic inflammation. As with specific asthmatic phenotypes, subsets of patients with RSV infection may benefit from TNFα inhibitors such as golimumab [82,83]. During the acute phase of infection, the potential benefit of antivirals is debatable. In phase 2 trials, GS-5806, a fusion inhibitor that targets the F protein to prohibit cell entry [84] and ALS-008176, a nucleoside analogue that targets RSV polymerase and interferes with RSV protein synthesis [85], induced lower viral load and decreased symptom scores in adults. However, at time of clinical presentation, the relative contribution of immune-mediated airway damage may be much greater than virus-mediated airway damage.

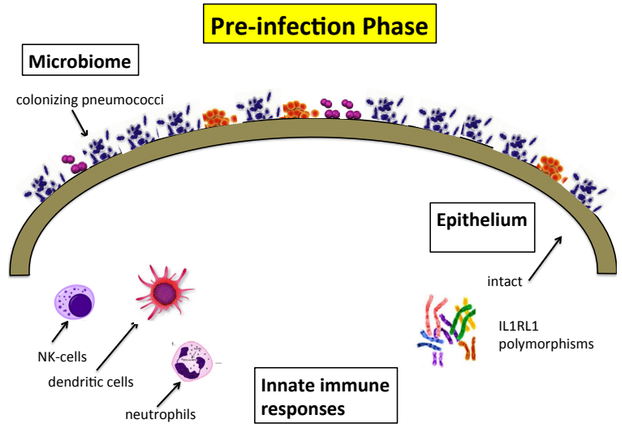
The convalescent phase (figure 3) of disease is characterized by influx of IL-17 that facilitates neutrophil chemotaxis and clearing of the virus. Neutrophils may exert secondary effects on colonizing bacteria through phagocytosis, degranulation and by activation of NET's that can kill bacteria such as pneumococci, hence altering the homeostasis of the microbiome. Dysregulation of innate immune responses, overexpression of IL1RL1a and IL-33, and subsequent epithelial damage contributes not only to acute disease severity, but also the long term increased risk of developing wheeze and asthma. The role of IL1RL1-a in RSV disease has not otherwise been studied in humans, but it may offer a potential therapeutic target. Administration of anti-IL1RL1 antibodies to RSV sensitized mice resulted in attenuated Th2-type cytokine-associated eosinophilic airway inflammation [62]. As suggested before, the influx of IL-17 during this stage of infection might hint towards divergent or regulatory effector mechanisms of TH17 cells depending on phase of disease in which case IL-17 inhibitors would not be useful. It is also important to study the premorbid microbiome in order to understand the role of colonizing bacteria at onset of disease as well as during acute and convalescent phase of disease. Whether a nasopharyngeal microbiomal stable state is an established reference or a continuously changing destination influenced by passing respiratory viruses such as RSV has vital implications for treatments aiming to prevent RSV infection. Furthermore, it has been suggested that targeting pathogenic bacteria within the nasopharyngeal microbiome could prevent post-bronchiolitis complications such as recurrent wheeze and asthma [74].

It is very important, yet unfortunately difficult to study the interactions between the epithelium, microbiome, local innate immune responses and the RSV virus as a whole in a natural setting. Cell lines do not adequately represent the human nasopharyngeal mucosa and modeling an infants' microbiome as a base on which infection occurs would seem nearly impossible. Perhaps novel epithelial culture systems, including air liquid interface culture systems or human lung organoids may provide an opportunity to create the complex model of RSV disease and allow us to better understand innate responses, causes of severe disease, interaction with pneumococci, and identify possible treatments.

CONCLUSION

The results of this thesis support the hypothesis of immune-mediated pathology in RSV and have identified interesting areas for further exploration of factors contributing to development of severe disease in healthy infants. It is of vital importance for future research to unravel the early life immune responses to RSV as they occur within the neonatal epithelial and microbial environment. The unique pathophysiological mechanisms of disease with growing evidence for immune-mediated pathology imply that future treatment strategies should focus on modulating the host immune response to the virus. Defining immune and genetic factors that constitute pathogenicity and the role of the microbiome, will allow us to determine why healthy infants develop serious disease, help pediatricians to forecast course of disease, and identify possible targets for intervention that can potentially benefit young children on a global scale.

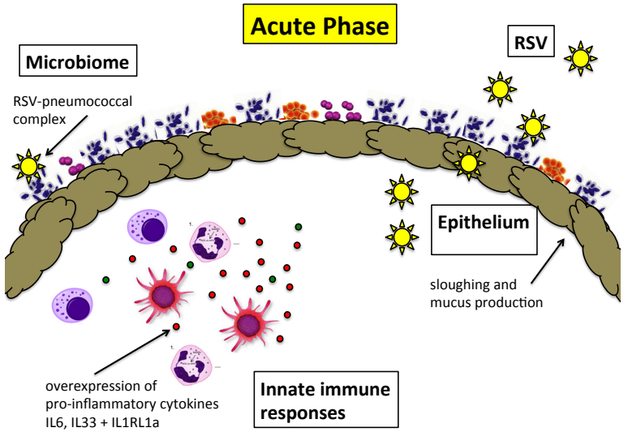
FIGURE I:



CHARACTERIZED BY PRE-EXISTING VULNERABILITY

		Chapter
RSV	• no virus present	
Innate immune responses	• neonates have immature innate immune cells and signaling responses • healthy adults with mature immune systems and children with absent immunity are not particularly sensitive to develop serious RSV disease	**4 + 6**
Epithelium	• intact • genetic predisposition for serious disease through polymorphisms in the IL1RL1 gene	**3**
Microbiome	• homeostasis with transient colonization of pneumococci that may facilitate RSV adherence	**5**
Clinical symptoms	• asymptomatic	
Potential targets for intervention	• identifying high-risk patients for vaccination and immuno-prophylaxis • prophylactic targeting of pathogenic colonizing bacteria may prevent serious disease	

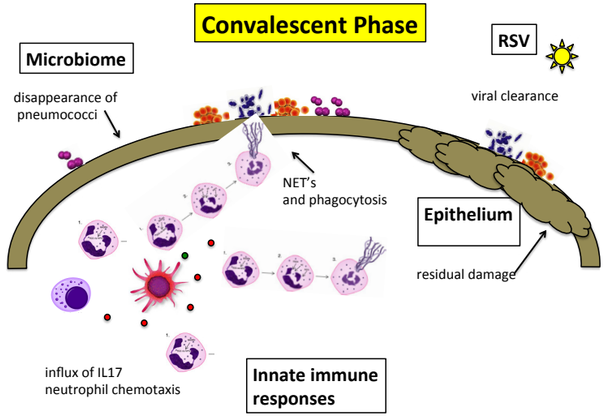
FIGURE II:



CHARACTERIZED BY IMMUNE-MEDIATED PATHOLOGY

		Chapter
RSV	• RSV attachment and fusion to epithelium • RSV can evade host immunity	
Innate immune responses	• over-expression of pro-inflammatory mediators such as IL6, IL33 and IL1RL1a	**2 + 3**
Epithelium	• sloughing of epithelial cells, mucus production and airway plugging	
Microbiome	• disequilibrium of microbiome with RSV-pneumococcal complexes that may facilitate infection spread to the lower airways	
Clinical symptoms	• bronchusobstruction causes wheeze, dyspnea and hypoxia • there is no effective therapy to alleviate clinical symptoms	**7 + 8 + 9**
Potential targets for intervention	• therapies that modify the host immune response, such IL-6, IL-33 and TNFα inhibitors	

FIGURE III:



CHARACTERIZED BY RESOLUTION OF DISEASE

		Chapter
RSV	• viral clearance	
Innate immune responses	• influx of IL17 that facilitates neutrophil chemotaxis • secondary effects on bacteria through phagocytosis and NET's	**2**
Epithelium	• residual damage to epithelium from neutrophils and cytokines such as IL1RL1a and IL-33	
Microbiome	• disappearance of pneumococci and return to stable state	**5**
Clinical symptoms	• symptoms disappear, risk of post-bronchiolitis complications • polymorphism in the IL1RL1 gene cluster may play a role in additive susceptibility for development of post-bronchiolitis wheeze and asthma	**3**
Potential targets for intervention	• anti-IL1RL1a antibodies • prophylactic targeting of pathogenic colonizing bacteria may prevent recurrent wheeze and asthma	

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SUMMARY

SUMMARY

Respiratory syncytial virus (RSV) is the most common cause of respiratory infections in young children. The infection can be asymptomatic, but often causes a bronchiolitis with rhinitis, coughing, feeding problems, dyspnea and apnea. Worldwide, RSV epidemics result in three million hospitalisations each year. In The Netherlands, ± two thousand children are admitted every winter of which ten percent are so severely ill that they can no longer breath independently and require intensive care treatment. This is especially true for newborns, premature babies and those with heart or lung disease. Many healthy babies without risk factors also become seriously ill and the cause is not entirely clear. Virological factors and an immature immune system play a role, but it is increasingly clear that severe symptoms are caused by the natural immune response of the body against the RS virus. After invasion of the airway epithelial cells, a cascade of inflammation is initiated. Immune cells, such as neutrophils, dendritic cells and natural killer cells, migrate to the site of infection and immune particles, such as cytokines and chemokines are released. This causes an inflammatory reaction manifested by fever, mucus production, necrotic cells, edema and thickening of the airways, better known as the clinical picture of bronchiolitis. This immune response of the body against the RS virus is potentially injurious and under normal circumstances moderated by anti-inflammatory reactions and regulatory mechanisms.

The aim of the first part of this thesis is to study immune responses of the body leading to severe RSV illness. The research was designed from a clinical perspective; immune particles in snot from children with mild disease were compared to those with serious illness. In chapter 1 it becomes clear that babies with severe RSV disease have more robust pro-inflammatory responses, displayed by higher IL-6 levels. IL-6 is a cytokine that stimulates the production of neutrophils, the prominent immune cell during the course of RSV infection. During the recovery phase, higher IL-17 concentrations are found which could indicate a regulatory role for this cytokine. In order to better understand the cascade of the immune response, it is important to do “real life” research in patients during acute illness and during the recovery phase.

In chapter 2, an association was found between the IL1RL1 gene and RSV. The IL1RL1 gene is known to play a role in the development of asthma. The symptoms of asthma, including airway hyperreactivity and bronchus obstruction, are similar to those of RSV. Moreover, many babies with RSV subsequently suffer from recurrent wheezing and toddler asthma. The IL1RL1 gene encodes the receptor for IL-33. This cytokine gives a “red flag” signal in response to epithelial damage, after a heart attack for example, and hence plays a modifying role in the immune response. The presence of soluble IL1RL1 inhibits the production of IL-33 and is associated with a poor prognosis in adult patients with acute lung disease. Whether IL1RL1 actually plays a role in the development of post-bronchiolitis complications should be further investigated.

In chapter 3, children with chemotherapy induced immunosuppression undergoing stem cell- or bone marrow transplant are followed. During a twelve year study period, none of the children became severely ill from RSV infection. This supports the hypothesis of immune-mediated pathology, or in other words, that the natural immune response of the body is injurious.

A viral infection is not an isolated event, but occurs in a homeostatic environment of colonizing bacteria, the microbiome. Approximately half of children under two years of age carry potentially pathogenic pneumococci in their nose. Chapter 4 illustrates that pneumococci disappear during the course of RSV infection. The immune response that is triggered by an RSV infection is likely to have a broader effect on the local bacterial environment. This is important information for researchers in the field of therapies and vaccines.

Since its discovery more than sixty years ago, many treatments have been tried that are further explored in the second part of this thesis. Prevention of severe RSV infection is possible for high risk patients such as premature babies. In theory, high risk adult patients could qualify for these monthly injections if they would become seriously ill from RSV. Chapter 5 examines viral pathogens that play a role in adults with respiratory failure in intensive care. RSV is rarely found here. Chapter 6 and 7 provide an overview of treatment for RSV infection. Unfortunately there is no effective therapy and hospitalised children are supported with oxygen en tube feeding until the body clears the virus. There was uncertainty about the efficacy of nebulization with hypertonic saline. In chapter 8, evaluation of digitally measured lung sounds shows that nebulization with hypertonic saline does not improve symptoms. This new method for monitoring wheezing and crackles is a useful and patient-friendly tool to assess the effect of future therapies.

In the general discussion, results from the various studies are combined and a hypothetical model of (severe) RSV infection is presented. The factors that play a role are; the epithelium, the microbiome, the immune response and the RS virus. Before an RSV infection, the pre-infection phase, the epithelium is intact and the microbiome in balance. An immature immune system or genetic predisposition, such as variations in the IL1RL1 gene, makes particular babies vulnerable to become seriously ill from an RSV infection. The ability to identify these high-risk patients would make targeted vaccination and prophylaxis possible. During the acute phase, there is an over-expression of pro-inflammatory mediators such as IL-6 and IL-33. This results in an exaggerated inflammatory reaction causing damage to epithelial cells and microbial imbalance. Present pneumococci facilitate adhesion and spreading of RSV to the lower respiratory tract. Mucus production and bronchus obstruction give rise to a bronchiolitis for which no treatment is available. Therapy directed against this immune-mediated pathology might be effective. The launched antiviral response has secondary effect on colonizing bacteria. During the convalescent- or recovery phase, pneumococci disappear and the microbiome stabilizes. The excessive inflammatory response can cause permanent epithelial damage. Some babies may be more susceptible to this through variations in the IL1RL1 gene. Anti-IL1RL1a antibodies and treatment of pathogenic colonizing bacteria may protect against post-bronchiolitis complications.

The results of this thesis support the hypothesis that the natural immune response of the body against RSV is injurious. Several factors have been identified that can explain why healthy babies become seriously ill and may serve as potential targets for intervention. Treatment strategies that focus on modifying the immune response will be more effective than antivirals, unless these can be administered even before the immune cascade is initiated. To unravel the pathophysiology of RSV infection it is necessary that research takes place in a natural setting, taking into account pre-existing vulnerability of patients and effects on the microbiome.

SAMENVATTING

Respiratoir syncytieel virus (RSV) is de meest voorkomende oorzaak van luchtweginfecties bij jonge kinderen. De infectie kan zonder symptomen verlopen, maar veelal ontstaat het beeld van een bronchiolitis, zich uitend in verkoudheid, hoesten, voedingsproblemen, benauwdheid en een stokkende ademhaling. RSV epidemieën veroorzaken wereldwijd drie miljoen ziekenhuisopnames per jaar. In Nederland worden iedere winter ± tweeduizend kinderen opgenomen waarvan tien procent zo ernstig ziek wordt dat zij niet meer zelfstandig kunnen ademen en intensive care behandeling nodig hebben. Dit geldt vooral voor pasgeborenen, prematuren en baby's met een hart- of longaandoening. Ook gezonde baby's zonder risicofactoren worden ernstig ziek en de oorzaak daarvan is niet geheel duidelijk. Virologische factoren en een onrijp afweersysteem spelen daarbij een rol, maar het wordt steeds duidelijker dat ernstige symptomen veroorzaakt worden door de natuurlijke afweerreactie van het lichaam tegen het RS virus. Nadat het virus is doorgedrongen tot de epitheelcellen van de luchtwegen ontstaat een cascade van inflammatie. Afweercellen, zoals neutrofielen, dendritische cellen en natural killer cellen, migreren naar de plaats van infectie en afweerstofjes, zoals cytokines en chemokines, komen vrij. Hierdoor ontstaan ontstekingsverschijnselen van koorts, slijmproductie, necrotische cellen, oedeem en verdikking van de luchtwegen passend bij het klinisch beeld van een bronchiolitis. Deze afweerreactie van het lichaam tegen het RSV virus is potentieel ziekmakend en normaal gesproken brengen anti-inflammatoire reacties en regulatiemechanismen deze cascade weer tot rust.

Het doel van het eerste deel van dit proefschrift is onderzoek naar de afweerreacties van het lichaam die lijden tot ernstige ziekte. De onderzoeken werden opgezet vanuit een klinisch perspectief: afweerstofjes in snot van kinderen met milde ziekteverschijnselen werden vergeleken met die van kinderen met ernstige ziekte. In hoofdstuk 1 wordt duidelijk dat baby's met ernstige RSV ziekte robuustere pro-inflammatoire responses hebben, zich uitend in hogere IL-6 waarden. IL-6 stimuleert de productie van neutrofielen, één van de afweercellen die een prominente rol speelt tijdens RSV infectie. Tijdens de herstelfase worden hogere IL-17 waarden gevonden wat zou kunnen wijzen op een regulerende rol van dit cytokine. Om een beter inzicht te krijgen in de cascade van de immuunrespons is het belangrijk om "real life" onderzoek te doen bij patiënten tijdens acute ziekte en tijdens de herstelfase.

In hoofdstuk 2 wordt een associatie gevonden tussen het IL1RL1 gen en RSV. Van het IL1RL1 gen is bekend dat deze een rol speelt bij de ontwikkeling van astma. De symptomen van astma, zoals hyperreactiviteit en obstructie van de luchtwegen, zijn vergelijkbaar met die van RSV. Bovendien krijgen veel baby's die RSV hebben doorgemaakt daaropvolgend klachten van recidiverend piepen en peuterastma. Het IL1RL1 gen codeert de receptor voor IL-33. Van dit cytokine is bekend dat het een "rode vlag" signaal geeft bij epitheelschade, bijvoorbeeld bij een hartinfarct, en op die wijze een modificerende rol heeft in de immuunrespons. Aanwezigheid van oplosbaar IL1RL1 remt de productie van IL-33 en is geassocieerd met een slechte prognose bij volwassen patiënten met acute longziekte. Of IL1RL1 daadwerkelijk een rol speelt bij het ontwikkelen van post-bronchiolitis complicaties moet verder onderzocht worden.

In hoofdstuk 3 worden kinderen gevolgd die een stamcel- of beenmergtransplantatie ondergaan en door chemotherapie een volledig onderdrukt immuunsysteem hebben. Tijdens een studieperiode van 12 jaar zijn geen van de kinderen ernstig ziek geworden door infectie met RSV. Dit ondersteunt de hypothese van immuun-gemedieerde pathologie, oftewel dat vooral de natuurlijke afweerreactie van het lichaam ziekmakend is.

Een virale infectie staat niet op zichzelf, maar ontstaat in een homeostatisch milieu van koloniserende bacteriën, het microbioom. Ongeveer de helft van kinderen onder de twee jaar draagt potentieel ziekmakende pneumokokken in zijn neus. In hoofdstuk 4 is te zien dat pneumokokken verdwijnen tijdens het doormaken van een RSV infectie. De afweerreactie die op gang wordt gebracht door een RSV infectie heeft vermoedelijk een breder effect op het lokale bacteriële milieu. Bij onderzoek naar therapieën en vaccinaties zijn dit belangrijke gegevens om mee te nemen.

Sinds het RS virus meer dan zestig jaar geleden ontdekt werd, zijn er vele behandelmethoden geprobeerd die nader worden bekeken in het tweede deel van dit proefschrift. Preventie van ernstige RSV infectie is mogelijk bij hoog risico patiënten zoals te vroeg geboren baby's. In theorie zouden volwassenen met risicofactoren ook in aanmerking kunnen komen voor deze maandelijkse injecties als zij ernstig ziek zouden worden van RSV. In hoofdstuk 5 wordt gekeken naar virale verwekkers die een rol spelen bij volwassenen met respiratoir falen op een intensive care. RSV wordt hier nauwelijks gevonden. In hoofdstuk 6 en 7 wordt een overzicht gegeven van behandeling van RSV infectie. Helaas is er geen effectieve therapie en worden opgenomen kinderen ondersteund met zuurstof en sondevoeding totdat het lichaam het virus klaart. Er bestond onduidelijkheid over de werkzaamheid van verneveling met hypertoon zout. In hoofdstuk 8 is met digitaal gemeten longgeluiden te zien dat verneveling met hypertoon zout geen verbetering geeft van symptomen. Deze nieuwe digitale meting is een bruikbaar en patiëntvriendelijk instrument om het effect van toekomstige therapieën te evalueren.

In de discussie worden de gegevens uit de verschillende onderzoeken gecombineerd en wordt een hypothetisch model van (ernstige) RSV infectie gepresenteerd. De factoren die hierbij een rol spelen zijn; het epitheel, het microbioom, de afweerreactie en het RS virus. Voordat een RSV infectie plaatsvindt, de pre-infectie fase, is het epitheel intact en het microbioom in balans. Een onrijp afweersysteem of een genetische predispositie, zoals variatie in het IL1RL1 gen, maakt sommige baby's kwetsbaar om ernstig ziek te worden van een RSV infectie. Het kunnen identificeren van deze hoog risico patiënten zou gerichte vaccinatie en profylaxe mogelijk kunnen maken. Tijdens de acute fase is er een over-expressie van pro-inflammatoire mediatoren zoals IL-6 en IL-33. Dit resulteert in een ontstekingsreactie met schade aan epitheelcellen en een disbalans in het microbioom. Aanwezige pneumokokken faciliteren hechting en verspreiding van RSV naar de lagere luchtwegen. Mucusproductie en bronchusobstructie geeft bij de patiënt het beeld van een bronchiolitis waarvoor geen behandeling voorhanden is. Therapie gericht op deze immuun gemedieerde pathologie zou effectief kunnen zijn. De op gang gebrachte antivirale reactie heeft een secundair effect op koloniserende bacteriën. Tijdens de convalescentie- of herstelfase verdwijnen pneumokokken en stabiliseert het microbioom. De overmatige ontstekingsreactie kan

blijvende epitheelschade tot gevolg hebben. Sommige baby's kunnen hiervoor extra vatbaar zijn door variaties in het IL1RL1 gen. Anti-IL1RL1a antilichamen en behandeling van pathogene koloniserende bacteriën zouden kunnen beschermen tegen post-bronchiolitis complicaties.

De resultaten uit dit proefschrift ondersteunen de hypothese dat de natuurlijke afweerreactie van het lichaam tegen RSV ziekmakend is. Er zijn verschillende factoren geïdentificeerd die kunnen verklaren waarom gezonde baby's ernstig ziek worden en die potentiële targets kunnen zijn voor interventie. Behandelstrategieën die zich richten op het modificeren van de immuunrespons zullen effectiever zijn dan antivirale middelen, tenzij deze kunnen worden toegediend nog voordat de afweercascade op gang is gebracht. Om de pathofysiologie van RSV infectie te ontrafelen is onderzoek in een natuurlijke setting noodzakelijk, rekening houdend met pre-existente kwetsbaarheid van patiënten en effecten op het microbioom.

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OVER DE AUTEUR

Tina Faber werd geboren in het voormalige Diakonessenziekenhuis in Leeuwarden. In 1980, tijdens het eerste jaar van het Stedelijk Gymnasium, verhuisde zij met haar ouders, twee zusjes en broertje naar Canada. In Burlington, Ontario maakte zij Nelson Highschool af en studeerde daarna Psychologie aan de McGill University in Montreal, Quebec. In 1990 emigreerde Tina terug naar Nederland waar zij Geneeskunde studeerde aan de Universiteit Utrecht. Zij behaalde haar artsenbul in 1995 en is daarna gaan werken in het Wilhelmina Kinderziekenhuis in Utrecht. Zij begon in 1997 met de opleiding tot kinderarts onder begeleiding van prof. dr. J.L.L. Kimpen en deed vervolgens de subspecialisatie tot infectioloog-immunoloog onder begeleiding van dr. T.F.W. Wolfs. Zij liep o.a. stage in Zimbabwe en in Washington op de luchtmachtbasis Patuxent River en in Georgetown Children's Medical Center. Sinds 2006 is zij staflid Kindergeneeskunde in het Medisch Centrum Leeuwarden (MCL), dit ziekenhuis is gebouwd op het terrein waar zij vroeger speelde aan de Badweg. In het MCL heeft zij haar promotieonderzoek gedaan, onder toeziend oog van prof. dr. L.J. Bont en prof. dr. J.L.L. Kimpen. Tina is opleider voor de Kindergeneeskunde, plaatsvervangend opleider voor de Internationale Gezondheidszorg en Tropengeneeskunde, medisch manager van de vakgroep en voorzitter van het FKC (Friese kinderartsen). Zij woont samen met Jan Willem en hun drie zonen, Storm, Wolf en Rover.

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