Respiratory syncytial virus (RSV) bronchiolitis

Respiratory syncytial virus (RSV) bronchiolitis

Clinical and immunological determinants of short-term and long-term airway morbidity

Respiratoir syncytieel virus (RSV) bronchiolitis

Klinische en immunologische determinanten van korte en lange termijn luchtwegmorbiditeit

met een samenvatting in het Nederlands

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht op gezag van de
Rector Magnificus, Prof. dr. W.H. Gispen, ingevolge het besluit
van het College voor Promoties in het
openbaar te verdedigen op dinsdag 12 juni 2001
des middags te 4.15 uur

door

Louis Bont
geboren op 16 maart 1970, te Amsterdam

Promotoren:
Prof. dr. JLL Kimpen, Faculteit der Geneeskunde, Universiteit Utrecht
Prof. dr. CJ Heijnen, Faculteit der Geneeskunde, Universiteit Utrecht

The studies in this thesis were funded by the Dutch Asthma Foundation, The Netherlands, grant number 32.96.08.

Cover: children from the cohort study described in this thesis (design: Mrs. M. Bont-Sons). Published with kind permission by parents.

"I conclude that this disorder (asthma) starts with a common cold,
especially in the rainy season,
and that the patient is forced to gasp for breath day and night"
(Maimonides, 1170 A.D.)

Contents

I General introduction

1.1 Virology of respiratory syncytial virus

RSV is a single stranded enveloped RNA virus. It is a member of the genus pneumoviridae within the family of paramyxoviridae. Other paramyxoviridae include morbillivirus (measles), mumps virus and parainfluenzae viridae. The RSV genome comprises 15,222 nucleotides yielding ten major proteins[1]. The F (fusion) and G (attachment) glycoproteins are the major surface antigenic determinants[2]. Two antigenic subtypes of RSV are distinguished, designated A and B[3;4]. Distinction is mainly based on polymorphisms of the G protein[3;5]. During yearly winter epidemics both strains usually circulate within the community[6]. Although several studies have also shown that RSV type A is associated with more severe disease, others have not been able to confirm this [7-11].

1.2 Epidemiology

More than 50% of all infants are infected with RSV during the first year of life and at age 2 more than 90% of infants have been infected[12;13]. RSV is the most important cause of viral lower respiratory tract infection (LRTI) during infancy worldwide[2]. Hospitalization is required in up to 3% of infants < 1 year of age with RSV infection [14-16]. In hospitalized infants with RSV infection mechanical ventilation is needed in 7-21% of cases[17-19]. In developing countries mortality rates up to 7% have been mentioned, mortality in industrialized countries is low and probably < 1%[20;21].

1.3 Clinical manifestations

RSV bronchiolitis predominantly occurs in children aged less than six months of age. Typically, the child first experiences a mild upper respiratory tract infection with serous nasal discharge. After a few days this is followed by severe coughing, shortness of breath and an audible wheeze on expiration. Impaired oxygen saturation is a key phenomenon in the disease resulting occasionally in cyanosis, tachypnea, inter- and sub-costal retractions and nasal flaring. Gradual recovery may take several weeks, however in case of hospitalization the child can usually be discharged after less than one week.

1.4 Diagnosis

Bronchiolitis is a common disease of the lower respiratory tract of infants resulting from inflammatory obstruction of the small airways [22]. Bronchiolitis is a clinical diagnosis, that may be suspected in infants with clinical symptoms of bronchiolitis during the winter season. To confirm RSV infection in infants with bronchiolitis, direct immunofluorescence for RSV in swabs or washings from the nasopharynx has proven high sensitivity and specificity[23;24]. More recently, RSV can also be detected in nasopharyngeal specimens by polymerase chain reaction PCR[25-28].

1.5 Pathogenesis

Evidence suggests that RSV bronchiolitis has an immune-mediated pathogenesis in which an aberrant immune response to the virus facilitates the development of

disease[29]. In hospitalized infants with natural RSV bronchiolitis elevated IgE and eosinophilic cationic proteins (ECP) in airway secretions have been reported, suggesting that immunological host factors may enhance severity of disease during lower respiratory tract infection with RSV[30;30;31;31-33]. In addition, the relation between RSV and subsequent recurrent wheezing suggests that atopy could underlie both RSV bronchiolitis and the subsequent sequelae. Indeed, it was reported that RSV bronchiolitis is a risk factor for the subsequent development of allergic asthma[34]. However, others did not find an association between RSV and atopy[30;31;35-39]. Moreover, an immune-mediated pathogenesis is further supported by the vaccine-enhanced illness observed during a trial with formalin-inactivated RSV[40]. Finally, data from RSV infection in the mouse model have shown that both CD4+ and CD8+ T cells are required for full development of illness and pulmonary inflammation[41]. In addition, it was shown in the mouse model that RSV infection after immunization with the RSV G protein is characterized by Th2-type immune responses[42], which are associated with the atopy syndrome[43;44].
Immune-mediated enhancement of disease does not easily explain age distribution of infants with RSV bronchiolitis. In particular, the occurrence of RSV infection necessitating mechanical ventilation in neonates and healthy preterm infants suggests that maturation-related mechanisms could play a role[45;46]. The immune response of neonates is characterized by immature cellular immunity which makes these infants more susceptible for severe infections with intracellular pathogens[47-50].

1.6 Treatment and prevention

There is no specific treatment for RSV bronchiolitis[51;52]. Anti-viral medication has not been proven to be effective[53-55]. Conflicting results have been reported on the effect of systemic corticosteroids[52]. A recent meta-analysis showed a borderline significant benefit of the use of systemic corticosteroids in RSV bronchiolitis[56]. No evidence exists that bronchodilators, such as β2-agonists, are indicated for routine use, but may be considered on a trial and error base in individual patients[51;52;57]. Symptomatic treatment consists of oxygen suppletion. Fluid administration may be used to prevent or correct dehydration.
Currently, no vaccine for RSV is available. In the 1960s a formalin-inactivated vaccine was used in infants[40]. No protection against naturally-acquired RSV was observed. In contrast, enhanced disease and increased mortality were observed during RSV infection following vaccination. Several strategies for save and effective vaccination, including immunization with attenuated strains and sub-unit vaccines are under investigation at the moment[58-61].
In the absence of a vaccine, passive immunization strategies were developed. Both donor-derived (RSV-IG) and RSV-specific monoclonal antibodies (palivizumab) have been proven to be safe and effective in reducing hospitalization in case or RSV infection in high-risk infants[62;63]. However, some questions about the use of RSV prophylaxis still have to be addressed. No data are available whether passive immunization can prevent RSV bronchiolitis in non-high-risk infants. In addition, it is not known whether RSV prophylaxis in high-risk infants results in a lower risk for mechanical ventilation and intensive care admission.

1.7 Relevance of prognostic models

The development of short-term prognostic models for RSV bronchiolitis are relevant, because clinicians can use them for practical preventive and treatment strategies. Knowledge on estimated risk factors for adverse outcome of disease in case of RSV bronchiolitis are valuable when developing algorithms for patient management. Prognostic models help to identify children who have the greatest benefit of RSV prophylaxis. In addition, prognostic models may be used to identify infants requiring hospitalization in case of RSV infection. Currently, hospitalization will often be considered in case RSV infection is diagnosed in a preterm neonate with only upper respiratory tract symptoms, because of suspected risk for clinical deterioration. Whether it is actually necessary to advocate hospitalization in this case is not known. Furthermore, it is not known whether RSV infection is associated with sudden infants death syndrome (SIDS) and if so, which group of infants has the highest risk. Some evidence exists that such an association exists but accurate estimation of risk in individual infants is not yet possible[64;65]. In addition to the use for decision making by clinicians, parents are obviously interested in the future course of disease. Parents usually wish information on the expected duration of hospitalization as well as the chance their child will develop "asthma"?

In addition to the direct practical use, predictive models for the clinical outcome of RSV bronchiolitis may lead to a better perception of pathogenetic mechanisms that could play a role. The high risk for adverse outcome in case of RSV infection in healthy neonates has lead to the hypothesis that maturation related mechanisms play a role in severe RSV bronchiolitis in this age group[46;66]. The studies on RSV bronchiolitis in infants with congenital heart disease have indicated that pulmonary hypertension could be a pathogenetic factor in this group of infants[67].

Predictive models can also be used in intervention studies. Inclusion of high-risk infants will lead to an over-all higher incidence of RSV bronchiolitis observed in the study group. This will reduce the number of subject required for the study to draw statistically significant conclusions. Efficacy studies of RSV-specific immunoglubulines and monoclonal antibodies as prophylaxis for severe RSV have been performed in high-risk infants[62;63] would not have been feasible when performed in a general pediatric population. Other subgroups identified to be at high risk for severe disease based on a prognostic model might benefit in the future from prophylactic or therapeutic possibilities. Finally, predictive models can be applied when stratification of patients prior to the intervention is warranted.

1.8 Aims of the thesis

The principal aim of this thesis was to elucidate the potential role of regulatory cytokines produced by lymphocytes and antigen-presenting cells in the pathogenesis of RSV bronchiolitis in relation to short-term (severity) and long-term outcome (recurrent wheezing) of disease. Therefore, cytokine profiles were studied in a cohort of hospitalized infants with RSV bronchiolitis in relation to outcome of acute disease as well as long-term sequelae. Furthermore, we investigated whether simple clinical observation, such as post-conceptional age and the presence or absence of airflow limitation during RSV bronchiolitis can be used as practical predictors for short-term respectively long-term outcome.

In the studies of this thesis the following hypotheses are tested:
The duration of the period that prematurely born children are at risk for severe RSV infection resulting in mechanical ventilation (MV) is determined by the post-conceptional age (**Chapter 5**).
Clinical and immunological parameters during RSV are associated with disease severity (**Chapter 6 to 8**).
Cell-mediated immunity mounted during RSV bronchiolitis provides protection against subsequent reinfection (**Chapter 9**).
Long-term airway morbidity following RSV bronchiolitis can be estimated using clinical and immunological predictors (**Chapter 10, 11**).

1.9 Outline of the studies

1.9.1 Short-term airway morbidity following RSV infection

Clinical and immunological determinants of RSV bronchiolitis resulting in MV are discussed in **Chapter 2**. Risk factors for RSV bronchiolitis resulting in the requirement of MV include neonatal status and prematurity with or without chronic lung disease[19;46;67-70]. Although these risk factors are well established, pathogenetic mechanisms that lead to this most severe form of RSV bronchiolitis are largely unidentified.
Safe and effective RSV prophylaxis is currently available for prematurely born infants. Widespread administration to all preterm infants could be considered. However, the need for such a strategy can be criticized since the majority of preterm infants will never develop severe RSV infection when they do not receive RSV prophylaxis. **Chapter 5** describes until what age prematurely born children have most benefit of RSV prophylaxis to prevent RSV bronchiolitis resulting in MV.
In this thesis an attempt is made to asses a potential role for cellular immunity in the development of RSV bronchiolitis resulting in MV. **Chapter 6** describes differences in T cell responses upon non-specific stimuli in the peripheral blood between mechanically ventilated and non-ventilated hospitalized infants with RSV bronchiolitis. In **Chapter 7** local differences in in vivo T cell cytokine levels differences between ventilated and non-ventilated infants are analyzed.

Antigen-presenting cells, including monocytes and macrophages, have a regulatory role in the development of cellular immunity. In particular, IL-12 has been mentioned to play an important role in the initiation of cellular immunity. In **Chapter 8** the predictive value of monocyte IL-12 production for duration of MV is shown. Subsequently, we present the first predictive model for duration of MV in RSV infection.

1.9.2 Long-term airway morbidity following RSV bronchiolitis

In long-term follow-up studies it has been appreciated that 30-70% of the infants will have recurrent episodes of wheezing during early childhood (reviewed in **Chapter 3**). Little is known about clinical and non-clinical factors that determine long-term airway morbidity following RSV bronchiolitis. Therefore, this prospective follow-up study was undertaken to identify prognostic factors for the occurrence of recurrent episodes of wheezing during early childhood following RSV bronchiolitis.

Previous studies attempted to delineate clinical risk factors for airway morbidity following RSV LRTI[37;71]. However, no clinical risk factors were identified thus far. **Chapter 10** describes the predictive value of signs of airflow limitation during RSV bronchiolitis for subsequent recurrent wheezing.

Studies have indicated that atopic status plays an important role in long-term airway morbidity following RSV bronchiolitis, however other studies could not confirm this finding [34;39;72]. Atopy is considered a condition that is the result of Th2-like cytokine responses[44]. We therefore investigated whether Th2-type immune responses during RSV bronchiolitis in the peripheral blood (**Chapter 11**) are associated with subsequent recurrent wheezing. Furthermore, in line with our study on the predictive value of monocyte IL-12 response for duration of MV[73], we assessed whether monocyte cytokine responses in the peripheral blood during RSV bronchiolitis may be of predictive value for long-term airway morbidity (**Chapter 11**).

Reference List

1 Collins PL, Hill MG, Camargo E, Grosfeld H, Chanock RM, Murphy BR. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc Natl Acad Sci U S A 1995; 92:11563-11567.

2 Collins PL, McIntosh K, Chanock RM. Respiratory syncytial virus. Field Virology. Raven Press, 1996: 1313-1351.

3 Anderson LJ, Hierholzer JC, Hendry RM, Fernie BF, Stone Y, McIntosh K. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis 1985; 151:626-633.

4 Mufson MA, Orvell C, Rafnar B, Norrby E. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol 1985; 66:2111-2124.

5 Johnson PR, Spriggs MK, Olmsted RA, Collins PL. The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: extensive sequence divergence between antigenically related proteins. Proc Natl Acad Sci U S A 1987; 84:5625-5629.

6 Hall CB, Walsh EE, Schnabel KC, Long CE, McConnochie KM, Hildreth SW, Anderson LJ. Occurrence of groups A and B of respiratory syncytial virus over 15 years: associated epidemiologic and clinical characteristics in hospitalized and ambulatory children. J Infect Dis 1990; 162:1283-1290.

7 Walsh EE, McConnochie KM, Long CE, Hall CB. Severity of respiratory syncytial virus infection is related to virus strain. J Infect Dis 1997; 175:814-820.

8 Hornsleth A, Klug B, Nir M, Johanson J, Hansen KS, Christensen LS, Larsen LB, Tech S. Severity of respiratory syncytial virus disease related to type and genotype of virus and to cytokine values in nasopharyngeal secretions. Pediatr Infect Dis J 1998; 17:1117-1121.

9 Kneyber MJC, Brandenburg AH, Rothbarth PhH, De Groot R, Ott A, Van Steensel-Moll HA. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch Dis Child 1996; 75:137-140.

10 McIntosh ED, De Silva LM, Oates RK. Clinical severity of respiratory syncytial virus group A and B infection in Sydney, Australia. Pediatr Infect Dis J 1993; 12:815-819.

11 Tsutsumi H, Onuma M, Nagai K, Yamazaki H, Chiba S. Clinical characteristics of respiratory syncytial virus (RSV) subgroup infections in Japan. Scand J Infect Dis 1991; 23:671-674.

12 Glezen WP, Denny FW. Epidemiology of acute lower respiratory disease in children. N Engl J Med 1973; 288:498-505.

13 Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. Am J Dis Child 1986; 140:543-546.

14 Karron RA, Singleton RJ, Bulkow L, Parkinson A, Kruse D, DeSmet I, Indorf C, Petersen KM, Leombruno D, Hurlburt D, Santosham M, Harrison LH. Severe respiratory syncytial virus disease in Alaska native children. RSV Alaska Study Group. J Infect Dis 1999; 180:41-49.

15 Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 1999; 282:1440-1446.
16 Wang EEL, Law BJ. Respiratory syncytial virus infection in pediatric patients. Seminars in Pediatric Infectious Diseases 1998; 9:146-153.
17 Outwater KM, Crone RK. Management of respiratory failure in infants with acute viral bronchiolitis. Am J Dis Child 1984; 138:1071-1075.
18 Frankel LR, Lewiston NJ, Smith DW, Stevenson DK. Clinical observations on mechanical ventilation for respiratory failure in bronchiolitis. Pediatr Pulmonol 1986; 2:307-311.
19 Tissing WJE, Van Steensel-Moll HA, Offringa M. Risk factors for mechanical ventilation in respiratory syncytial virus infection. Eur J Pediatr 1993; 152:125-127.
20 Simoes EAF. Respiratory syncytial virus infection. Lancet 1999; 354:847-852.
21 Cherian T, Simoes EAF, Steinhoff MC. Bronchiolitis in tropical south India. Am J Dis Child 1990; 144:1026-1030.
22 Behrman RE, Kliegman R, Arvin AM, Nelson WE. Nelson textbook of Pediatrics. 15 ed. 1996.
23 Kumar ML, Super DM, Lembo RM, Thomas FC, Prokay SL. Diagnostic efficacy of two rapid tests for detection of respiratory syncytial virus antigen. J Clin Microbiol 1987; 25:873-875.
24 Olsen MA, shuck KM, Sambol AR. Evaluation of Abbott TestPack RSV for the diagnosis of respiratory syncytial virus infections. Diagn Microbiol Infect Dis 1993; 16:105-109.
25 Paton AW, Paton JC, Lawrence AJ, Goldwater PN, Harris RJ. Rapid detection of respiratory syncytial virus in nasopharyngeal aspirates by reverse transcription and polymerase chain reaction amplification. J Clin Microbiol 1992; 30:901-904.
26 Henkel JH, Aberle SW, Kundi M, Popow-Kraupp T. Improved detection of respiratory syncytial virus in nasal aspirates by seminested RT-PCR. J Med Virol 1997; 53:366-371.
27 Tang YW, Heimgartner PJ, Tollefson SJ, Berg TJ, Rys PN, Li H, Persing DH, Wright PF. A colorimetric microtiter plate PCR system detects respiratory syncytial virus in nasal aspirates and discriminates subtypes A and B. Diagn Microbiol Infect Dis 1999; 34:333-337.
28 Gonzalez IM, Karron RA, Eichelberger M, Walsh EE, Delagarza VW, Bennett R, Chanock RM, Murphy BR, Clements-Mann ML, Falsey AR. Evaluation of the live attenuated cpts 248/404 RSV vaccine in combination with a subunit RSV vaccine (PFP-2) in healthy young and older adults. Vaccine 2000; 18:1763-1772.
29 Graham BS, Johnson TR, Peebles RS. Immune-mediated disease pathogenesis in respiratory syncytial virus infection. Immunopharmacology 2000; 48:237-247.
30 Garofalo RP, Kimpen JL, Welliver RC, Ogra PL. Eosinophil degranulation in the respiratory tract during naturally acquired respiratory syncytial virus infection. J Pediatr 1992; 120:28-32.
31 Garofalo RP, Ahlstedt DA, Welliver RC. Peripheral blood eosinophil counts and eosinophil cationic protein content of respiratory secretions in bronchiolitis: relationship to severity of disease. Pediatr Allergy Immunol 1994; 5:111-117.
32 Welliver RC, Kaul TN, Ogra PL. The appearance of cell-bound IgE in respiratory-tract epithelium after respiratory-syncytial-virus infection. N Engl J Med 1980; 303:1198-1202.
33 Welliver RC, Duff AL. The relationship of RSV-specific immunoglobulin E-antibody responses in infancy, recurrent wheezing and pulmonary function at age 7-8 years. Pediatr Pulmonol 1993; 15:19-27.
34 Sigurs N, Bjarnason R, Sigursbergsson F, Kjellman B, Brorksten B. Asthma and immunoglobulin E Antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. Pediatrics 1995; 95:500-505.
35 Sly PD, Hibbert ME. Childhood asthma following hospitalization with acute viral bronchiolitis in infancy. Pediatr Pulmonol 1989; 7:153-158.
36 Sims DG, Garner PS, Weightman D, Turner MW, Soothill JF. Atopy does not predispose to RSV bronchiolitis or postbronchiolitic wheezing. BMJ 1981; 282:2086-2091.
37 Pullan CR, Hey EN. Wheezing, asthma and pulmonary dysfunction 10 years after infection with respiratory syncytial virus infection in infancy. BMJ 1982; 284:1665-1669.
38 Eisen AH, Bacal HL. The relationship of acute bronchiolitis to bronchial asthma - a 4 tot 14 year follow-up. Pediatrics 1963; 31:859-861.
39 Stein RT, Sherill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13. Lancet 1999; 354:541-545.

40 Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 1969; 89(4):405-421.
41 Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991; 88:1026-1033.
42 Johnson TR, Johnson JE, Roberts SR, Wertz GW, PArker RA, Graham BS. Priming with secreted glycoprotein G of respiratory syncytial virus (RSV) augments interleukin-5 production and tissue eosinophilia after RSV challenge. J Virol 1998; 72(4):2871-2880.
43 Umetsu dt, DeKruyff RH. Th1 and Th2 cd4+ cells in human allergic diseases. J Allergy Clin Immunol 1997; 100:1-6.
44 Allergy and asthma: glossary. Nature 1999; 402:B39.
45 Lebel MH, Gauthier M, Lacroix J, Rousseau E, Buithieu M. Respiratory failure and mechanical ventilation in severe bronchiolitis. Arch Dis Child 1989; 64:1431-1437.
46 Hall CB, Kopelman AE, Douglas RG, Geiman JM, Meagher MP. Neonatal respiratory syncytial virus infection. N Engl J Med 1979; 300:393-396.
47 Holt PG. Postnatal maturation of immune competence during infancy and childhood. Pediatr Allergy Immunol 1995; 6:59-70.
48 Murgita RA, Witzel H. Regulation of immune functions in the fetus and newborn. Prog Allergy 1981; 29:54-133.
49 Wilson CB. Immunological basis for increased susceptibility of the neonate to infection. J Pediatr 1986; 108:1-12.
50 Wilson CB, Lewis DB, English BK. Immunity in the neonate. Semin Pediatr Infect Dis 1999; 10(2):83-90.
51 Kneyber MC, Moll HA, De Groot R. Treatment and prevention of respiratory syncytial virus infection [In Process Citation]. Eur J Pediatr 2000; 159:399-411.
52 van Woensel J, Kimpen J. Therapy for respiratory tract infections caused by respiratory syncytial virus. Eur J Pediatr 2000; 159:391-398.
53 Wheeler JG, Wofford J, Turner RB. Historical cohort evaluation of ribavirin efficacy in respiratory syncytial virus infection. Pediatr Infect Dis J 1993; 12:209-213.
54 Moler FW, Steinhart CM, Ohmit SE, Stidham GL. Effectiveness of ribavirin in otherwise well infants with respiratory syncytial virus-associated respiratory failure. Pediatric Critical Study Group. J Pediatr 1996; 128:422-428.
55 Law BJ, Wang EE, MacDonald N, McDonald J, Dobson S, Boucher F, Langley J, Robinson J, Mitchell I, Stephens D. Does ribavirin impact on the hospital course of children with respiratory syncytial virus (RSV) infection? An analysis using the pediatric investigators collaborative network on infections in Canada (PICNIC) RSV database. Pediatrics 1997; 99:E7.
56 Garrison MM, Christakis DA, Harvey E, Cummings P, Davis RL. Systemic corticosteroids in infant bronchiolitis: A meta-analysis. Pediatrics 2000; 105:E441-E446.
57 Flores G, Horwitz RI. Efficacy of beta2-agonists in bronchiolitis: a reappraisal and meta- analysis. Pediatrics 1997; 100:233-239.
58 Plotnicky-Gilquin H, Huss T, Aubry JP, Haeuw JF, Beck A, Bonnefoy JY, Nguyen TN, Power UF. Absence of lung immunopathology following respiratory syncytial virus (RSV) challenge in mice immunized with a recombinant RSV G protein fragment. Virology 1999; 258:128-140.
59 Groothuis JR, King SJ, Hogerman DA, Paradiso PR, Simoes EAF. Safety and immunogenicity of a purified F protein respiratory syncytial virus (PFP-2) vaccine in seropositive children with bronchopulmonary dysplasia. J Infect Dis 1998; 177:467-469.
60 Piedra PA, Grace S, Jewell A, Spinelli S, Hogerman DA, Malinoski F, Hiatt PW. Sequential annual administration of purified protein vaccine against respiratory syncytial virus in children with cystic fibrosis. Pediatr Infect Dis J 1998; 17:217-224.
61 Waris ME, tsou c, Erdman DD, Day DB, Anderson LJ. Priming with live respiratory syncytial virus (RSV) prevents the enhanced pulmonary inflammatory response seen after RSV challenge in BALB/c mice immunized with formalin-inactivated RSV. J Virol 1997; 71:6935-6939.
62 Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. The PREVENT Study Group. Pediatrics 1997; 99:93-99.

63 The IMpact-RSV study group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 1998; 102(3):531-537.
64 Uren EC, Williams AL, Jack I, Rees JW. Association of respiratory virus infections with sudden infant death syndrome. Med J Aust 1980; 1:417-419.
65 Downham MA, Gardner PS, McQuillin J, Ferris JA. Role of respiratory viruses in childhood mortality. Br Med J 1975; 1(5952):235-239.
66 Bont L, Kimpen JLL. Mechanical ventilation for respiratory syncytial virus (RSV) bronchiolitis: determinants and pathophysiological mechanisms. submitted 2000.
67 McDonald NE, Hall CB, Suffin SC, Alexon C, Harris PJ, Manning JA. Respiratory syncytial virus infection in infants with congenital heart disease. N Engl J Med 1982; 307:397-400.
68 Opavski MA, Stephens D, Wang EEL. Testing models predicting severity of respiratory syncytial virus infections on the PICNIC RSV database. Arch Pediatr Adolesc Med 1995; 149:1217-1220.
69 Stevens TP, Sinkin RA, Hall CB, Maniscalo WM, McConnochie KM. Respiratory syncytial virus and premature infants born at 32 weeks' gestation or earlier. Arch Pediatr Adolesc Med 2000; 154:55-61.
70 Wang EEL, Law BJ, Stephens D. Pediatric Investigators Collaborative Network on infections in Canada (PICNIC) prospective study of risk factors and outcomes in patients hospitalized with respiratory syncytial viral lower respiratory tract infection. J Pediatr 1995; 126:212-219.
71 Sims DG, Downham MA, Gardner PS. Study of 8-year-old children with a history of respiratory syncytial virus bronchiolitis in infancy. BMJ 1978; 1:11-17.
72 Scott R, Pullan CR, Scott M, McQuillin J. Cell-mediated immunity in respiratory syncytial virus disease. J Med Virol 1984; 13(1):105-114.
73 Bont L, Kavelaars A, Heijnen CJ, van Vught AJ, Kimpen JL. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. J Infect Dis 2000; 181:1772-1775.

2 Mechanical Ventilation For Respiratory Syncytial Virus Bronchiolitis: Prognostic Determinants And Pathophysiological Mechanisms

L Bont, JLL Kimpen

Submitted for publication

2.1 Abstract

RSV bronchiolitis resulting in respiratory insufficiency is frequently encountered during the winter season at Paediatric Intensive Care Units. Pre-existent cardiac or pulmonary compromise have been documented as clinical risk factors for severe RSV bronchiolitis. In addition to this group of infants with pre-morbidity, a large proportion of mechanically ventilated RSV bronchiolitis patients are previously healthy full term infants or premature neonates without predisposing risk factors. In general, infants at this early age have maturation-related deficient cellular immunity. Several studies show an association between decreased cellular immunity and severe RSV bronchiolitis, indeed suggesting that a maturation-related defect of the cellular immune system facilitates severe RSV. In addition, low virus-specific antibody titres prior to RSV bronchiolitis have been shown to be a risk factor for severe RSV bronchiolitis. Conflicting data have been reported on the role of the virus strain in severe RSV bronchiolitis, but it is suggested that host factors are of greater importance. Studies in the mouse model have demonstrated that the immune system, in particular T cells, can enhance pulmonary inflammation during RSV infection. It is not yet clear how RSV infection in the animal model bears relevance for RSV bronchiolitis during infancy. In conclusion, severe pre-existent cardiac, pulmonary and immunological morbidity are important determinants of severe RSV bronchiolitis resulting in MV in a subgroup of patients. In healthy preterm infants and neonates immaturity of physiological functions, in particular the immune system, appears to be an important factor in the pathogenesis of RSV bronchiolitis resulting in MV.

2.2 Introduction

Respiratory syncytial virus (RSV) is the most important cause of bronchiolitis during infancy world-wide[1]. Hospitalisation rates for RSV illness are 1-30 cases per 1000 infants < 1 year of age[2-4]. In hospitalised infants with RSV bronchiolitis mechanical ventilation is required in 7-21% of cases[5-7].

The pathogenesis of RSV bronchiolitis necessitating mechanical ventilation (MV) is not well understood. Congenital heart disease, prematurity with or without chronic lung disease, age < 6 weeks and compromised immune function are well established clinical risk factors for severe disease[7-12]. The presence of pre-existent severe cardiac or pulmonary compromise likely explains the development of respiratory insufficiency in case of RSV bronchiolitis in some of these patients. Difficult to explain is the occurrence of RSV bronchiolitis resulting in MV in healthy neonates. It has been suggested that anatomically immature airways play a role[13;14]. Swelling of bronchial epithelium in relatively small airways could lead to severe respiratory distress. However, direct evidence for this hypothesis is still lacking. RSV bronchiolitis has been considered as an, at least partially, immunopathological condition[15;16]. However, it was already questioned by Hall et al. whether an immune-mediated pathogenesis fully explains the high frequency of severe RSV disease in neonates[8].

In the present paper we review determinants and possible pathophysiological mechanisms that have been described in RSV lower respiratory tract infection resulting in mechanical ventilation.

2.3 Clinical characteristics

Infants with congenital heart disease, chronic lung disease and to a lesser extent compromised T-cell function are at risk for severe RSV bronchiolitis[7;9;9-12;17]. It has been well established that many other pathogens can cause severe bronchiolitis at young age in these patient populations. It may well be that the pre-existent morbidity in combination with a vigorous immune response facilitates severe respiratory compromise in these infants during infection due to various pathogens, including RSV. Why neonates and prematurely born infants without chronic lung or heart disease are at risk for severe RSV bronchiolitis resulting in MV is more puzzling. Recurrent apneas and respiratory insufficiency are the main causes of initiating MV. We recently showed that preterm infants are at high risk for MV until a post-conceptional age of 44 weeks[18]. We also investigated the distribution of post-conceptional age in healthy term infants who were ventilated for RSV infection. Again, the vast majority of these healthy term infants had not reached a post-conceptional age of 45 weeks. Thus, a common characteristic of both healthy term and healthy preterm infants seems to put them at increased risk for RSV bronchiolitis resulting in MV until a post-conceptional age of 44 weeks is reached.

Although the mortality of RSV bronchiolitis resulting in MV is low, the clinical course on PICU varies. In particular, duration of mechanical ventilation varies from a few days to more than one month[19-22]. To date, two observational studies have attempted to identify clinical predictors for duration of MV[20;22]. Tasker et al. demonstrated that the combination of a high mean airway pressure (MAP) and a high alveolar-arterial oxygen gradient ($AaDO_2$) during the first 48 hours accurately identified infants with the longest

duration of MV[20]. In addition, we have shown the prognostic value of the ventilation index (VI) during the first 48 hours for duration of MV[22]. Of interest is that both studies were able to identify predictors for duration of MV during the first 48 hours after initiation of MV. Together, these observations suggest that events that occur early in the course of RSV bronchiolitis resulting in MV are more important determinants of disease outcome than events that occur later during MV.

2.4 Histopathological characteristics

Studies on pulmonary pathology would provide more direct insight in the immune response as well as anatomical characteristics of children during RSV bronchiolitis. However, bronchial biopsy studies or bronchoalveolar lavage (BAL) studies in the very young infants can not be performed because of obvious ethical reasons. Sparse post-mortem studies are found in literature[23-25]. Lack of pathological studies is explained by the low mortality rate of RSV bronchiolitis. The estimated mortality in hospitalised patients with RSV bronchiolitis varies from <0.5% in healthy infants to 0.5-2% in high-risk infants[26]. Two reports on post-mortem findings after RSV bronchiolitis were provided by British investigators in the early 70s[23;24]. These studies describe histological changes in infants dying with respiratory illness caused by RSV. Aherne et al. showed the general histological picture of peribronchial lymphocyte infiltration, necrosis of bronchial epithelium and mucus plugs in the small bronchioles[24]. Downham et al. assessed viral load in 5 infants by quantifying fluorescent viral antigen. Interestingly, in 3 of 5 infants scanty amount of RSV was found, suggesting that large viral load is not required for most severe RSV bronchiolitis [23]. However, some limitation of these studies should be noted. Some of the children had severe congenital cardiac abnormalities, a history of asthma or strong microbiological signs of secondary bacterial infection. In addition, a period of ≥ 48 hours between death and post-mortem examination might have diminished the quantity of epithelial cells showing RSV-specific immunofluorescence. As a result of the non-uniform inclusion criteria and the delay between death and autopsy, careful interpretation of the histological findings in the studies described is warranted.

2.5 Virological characteristics

Virological studies in infants with RSV bronchiolitis resulting in MV have focused on quantification of RSV in the airways. In general, higher RSV titres are found in nasopharyngeal samples from mechanically ventilated infants than in hospitalised non-ventilated infants[27;28]. These findings appear to be in contradiction with the autopsy studies mentioned earlier. However, the differences may be explained by different methods of quantification of viral load (fluorescence versus titration) and the high prevalence of pre-existent severe morbidity in the autopsy study.

In addition to viral load, it was attempted to relate virus type to the risk for MV among hospitalised infants with RSV bronchiolitis. Two antigenic variants of RSV circulate concomitantly during epidemics, type A and B[29]. Walsh et al. investigated the infecting type (A or B) among 265 hospitalised infants with a respiratory tract infection caused by RSV[30]. They showed that RSV types were equally prevalent in this group of patients,

but that MV was required more often in infants infected with type A than type B. It was suggested that among infants requiring MV, type A was seen most often in infants without underlying medical conditions (congenital heart disease, chronic lung disease), whereas type B was found mainly in infants with underlying medical conditions. Some other studies have also shown that RSV type A is associated with more severe disease, but the subject remains controversial[31-34]. One study indicated that disease severity in a group of hospitalised non-ventilated infants was associated with viral genotype[31]. In this study, a specific genotypic variant of antigenic type B RSV was strongly associated to disease severity .

2.6 Immunological characteristics

The role of cellular immunity has been mentioned in the pathogenesis of RSV bronchiolitis [16]. It has been speculated that cell-mediated immunity against RSV contributes importantly to disease manifestations. Two clinical observations lead to the view that disease manifestations during RSV infection are at least partially caused by the immune response. The first observation that specific immunity against RSV may be harmful came from the trials with a formalin-inactivated experimental vaccine in the 1960s[35]. Vaccination resulted in augmented disease upon subsequent natural RSV infection in vaccinated infants as compared to controls. An immune-mediated pathogenesis of RSV bronchiolitis is further supported by the clinical and epidemiological similarity between RSV bronchiolitis and childhood asthma. The clinical picture of both RSV bronchiolitis and childhood asthma is characterised by upper respiratory tract symptoms, such as rhinitis, followed by wheezing respiration as a sign of airflow limitation. In addition, RSV bronchiolitis is followed in 30-70% of the cases by recurrent episodes during early childhood[13;36-38].

In apparent contrast with the assumption that pathology in RSV is primarily mediated through the immune system, are the following clinical observations. Infants, but also older children and adults with a primary immunodeficiency disorder or impaired cell-mediated immunity due to chemotherapy have increased risk for severe course of disease [39;40;40-44]. In these patients severe viral pneumonia is seen and wheezing respiration is unusual. This clearly indicates that cellular immunity can protect against severe RSV bronchiolitis. However, considering the differences in age at onset of RSV and clinical presentation of these patients it is questionable whether RSV lower respiratory tract illness in patients with an immunodeficiency is the same disease entity as RSV bronchiolitis during infancy.

The role of humoral immunity against RSV has been questioned over time. The commonly held view in the early seventies was that serum antibody could be responsible in part for enhanced disease[45]. Chanock et al. observed that disease occurred during early infancy when maternal antibodies are universally present[45]. In addition, it was shown by Welliver et al. in the 80s that virus-specific IgE in the respiratory tract during RSV bronchiolitis is associated with disease severity[46;47]. However, it is conceivable that high virus-specific antibody levels mounted during RSV do not cause severe disease, but reflect a protective immune response. More recent data have indeed suggested a protective role for naturally occurring virus-specific antibodies. Low cord blood neutralising antibodies have been shown to be associated with severe disease[2]. However, to our

knowledge, it has not been shown that low neutralising antibodies result in increased risk for RSV bronchiolitis resulting in MV. In addition to the observation that transplacental transfer of RSV-specific antibodies may prevent RSV bronchiolitis, it was shown that prophylactic administration of RSV-specific immunoglobulines or monoclonal antibodies to high-risk infants can prevent hospitalisation in this specific group of infants[48-50]. Whether antibody prophylaxis prevents RSV bronchiolitis resulting in MV is not known.

Immunological studies have shown that lower peripheral blood T cell counts are seen in infants during RSV bronchiolitis resulting in MV than in hospitalised non-ventilated infants with RSV bronchiolitis[51]. In addition, we and others showed that the immune response during RSV bronchiolitis resulting in MV is characterised by low T cell proliferative responses and IFN-γ production upon non-specific stimuli[52;53]. Recently, we performed the first study on cytokine levels in nasopharyngeal aspirates in a large group of mechanically ventilated infants. Our findings showed that also *in vivo interferon (IFN)*-γ levels in nasopharyngeal aspirates are severely decreased in mechanically ventilated infants as compared to hospitalised non-ventilated infants (unpublished observations). In a different study in mechanically ventilated RSV bronchiolitis infants, we investigated the role of monocyte interleukin (IL)-12, a cytokine produced by antigen-presenting cells that is required for the initiation of cellular immunity[22;54]. Duration of mechanical ventilation of infants with RSV bronchiolitis was strongly inversely related to monocyte IL-12 production at initiation of MV, suggesting a crucial role for cellular immunity during recovery from RSV bronchiolitis requiring MV [22].

Animal studies have indicated a possible role of Th2-like cytokine profiles in the pathogenesis of RSV bronchiolitis[55]. Controversial results have been found in human RSV[53;56-58]. In most human studies Th1/Th2 cytokine profiles are expressed as IFN-γ/IL-4 ratios. In hospitalised RSV infected infants decreased IFN-γ/IL-4 ratios were found after polyclonal stimulation as compared to healthy controls[57]. In a similar study, decreased IFN-γ/IL-4 ratios after in vitro allergen stimulation were found five months after hospitalisation for bronchiolitis[56]. In contrast, we recently reported normal IFN-γ/IL-4 ratios after polyclonal stimulation on admission, and again 3-4 weeks later during the convalescent phase in RSV infected non-ventilated infants [53;58]. In mechanically ventilated, however, no IFN-γ/IL-4 ratio could be calculated of on admission because both IFN-γ and IL-4 production were below detection limits[53]. To date no data are available on Th1/Th2 cytokine profiles during RSV bronchiolitis resulting in MV.

2.7 Animal models

Murine models have provided insight into the possible mechanisms by which RSV could lead to disease[59-64]. It was shown by Graham et al. that T cell depleted BALB/c mice showed prolonged viral excretion after intranasal infection with human RSV but became less ill than immunocompetent BALB/c mice[59]. In T cell transfer experiments in murine models the differential role of CD4+ and CD8+ cells was studied[62]. After injection of RSV specific CD4+ T cells into BALB/c mice, the recipient mice developed more respiratory distress and weight loss upon RSV infection than non-transferred normal mice. The effect of injection of RSV-specific CD8+ cells prior to RSV infection were simi-

lar but moderate. Altogether, these animal experiments show that T cells are capable of enhancing disease during RSV infection in mice.

In murine models, most previous studies have shown a predominance of Th2 over Th1 cytokines during RSV infection[55]. However, it has not been established how these cytokine profiles relate to disease in mice. Administration of exogenous IL-12 lead to a complete alteration of cytokine profiles in CD4+ T cells in the lungs of mice subsequently challenged with RSV[65]. IL-12 treatment caused CD4+ T cells to make higher levels of IFN-γ and lower levels of IL-4 and IL-5 and reduced the pulmonary eosinophilia found in the lungs of the challenged mice, but IL-12 treatment did not decrease disease severity. These experiments illustrate the complex relation between immune pathology during RSV infection and clinical disease.

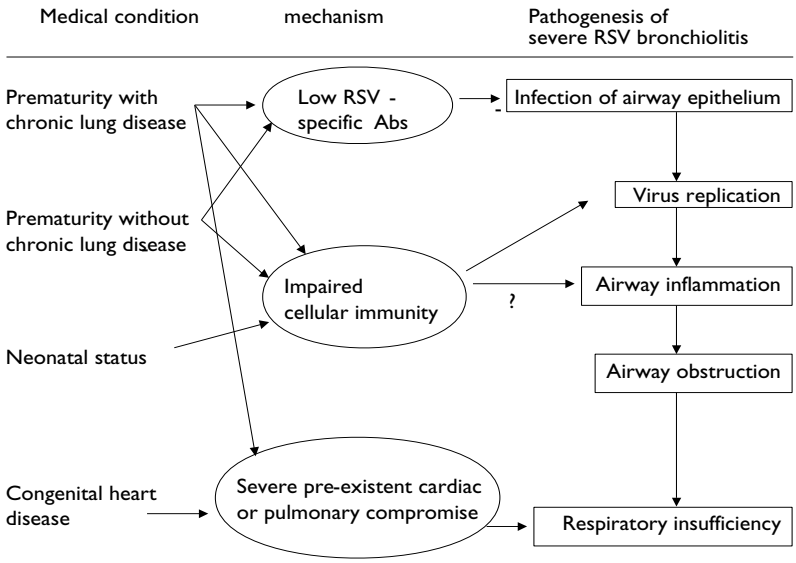
To what degree the animal model bears relevance to human RSV remains to be established. Most animal model studies were performed in BALB/c mice. In various infectious and non-infectious models BALB/c have shown to have a strong preferential activation of Th2 immune responses, including allergic responses[66-69]. To our knowledge, no animal model exists for respiratory insufficiency caused by RSV. RSV in animal models, including BALB/c mice, does not lead to animal death. Moreover, it was established that young mice develop less pulmonary inflammation upon natural primary infection than older mice[70]. Consequently, the BALB/c mouse appears not a very suitable model for either neonatal RSV bronchiolitis or RSV bronchiolitis resulting in MV.

2.8 Summary

Different patient groups need to be distinguished in order to understand the pathophysiology of RSV bronchiolitis resulting in MV (Figure 2.1). In infants with severe pre-existing pulmonary or cardiac morbidity pathophysiological mechanisms underlying the occurrence of RSV bronchiolitis resulting in MV are likely comparable to those in lower respiratory tract infections caused by other pathogens. Pathogenesis of disease can be well explained without an immune-mediated pathogenesis. The most intriguing question is why healthy neonates without chronic lung or heart disease are at risk for RSV bronchiolitis resulting in MV.

Airway inflammation is the hallmark of RSV bronchiolitis resulting in MV. Two different mechanisms appear to contribute to the development of airway inflammation. On the one hand, airway inflammation results from necrosis of airway epithelial cells which is directly caused by the cytopathological effect of RSV[71-73]. Decreased cellular immunity in young infants would hereby allow for more widespread viral replication resulting in more extensive damage to the airways. On the other hand, the immune response to RSV may directly damage the airways resulting in inflammation and more airway destruction. Indeed, animal studies support the concept that T cells can directly cause airway inflammation. Immunopathogenetic factors contributing to respiratory insufficiency during human RSV, however, are largely unidentified.

Figure 2.1 *Schematic presentation of pathogenetic pathways in the development of RSV bronchiolitis resulting in respiratory insufficiency*



Immaturity of the immune system in preterm infants and neonates could play a role in RSV bronchiolitis resulting in MV. Fetal and early post-natal life is associated with a physiological immune deficiency[74-76]. A consequence of this immune deficiency is susceptibility to infections with intracellular pathogens, including cytomegalovirus (CMV), Toxoplasma gondii, herpes simplex virus (HSV), enteroviruses, human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* during the first 4 to 8 weeks after birth[77-82]. Deficient immune function in neonates is characterised by relative deficient functioning of innate and antigen-specific immunity[76;83]. Defective functions of cells of the innate immune system consist of delayed recruitment of neutrophils and monocytes to infected tissue and diminished NK cell cytotoxicity[84]. During the neonatal period antigen-presentation by macrophages and dendritic cells is less efficient, possibly due to deficient IL-12 production, resulting in slow development of antigen-specific immunity[75;76;85]. In addition, T cell-mediated responses are delayed during the first 4 to 8 weeks of postnatal age, accompanied by impaired capacity to produce IFN-g[74;76;83;86;87]. Some evidence described in the current paper has shown that, indeed, immature cellular immunity could play a role in RSV bronchiolitis resulting in MV. Whether all immune functions mentioned, however, are involved in RSV bronchiolitis resulting in MV is uncertain.

In addition to maturation of immune responses neonatal maturation includes physiological changes in airway anatomy and functioning. Little is known about normal ontogeny of airway anatomy and physiology in young infants[88-90]. It was shown in a large birth cohort study that impaired airway function at birth is associated with increased risk for wheezing illness[91]. In addition, it has been suggested that the same mechanism could

underlie the occurrence of RSV bronchiolitis [13]. Whether this is true, in particular for RSV bronchiolitis resulting in MV, needs further study.

RSV research in infants has traditionally put most emphasis on hospitalised non-ventilated infants. A minority of immunological and virological studies on RSV bronchiolitis have included mechanically ventilated infants. Due to severe pre-existent medical conditions a number of patients with RSV bronchiolitis requiring MV would be less eligible for such studies. However, the majority of infants with RSV bronchiolitis requiring MV are term or preterm infants without any pre-existent morbidity. The possibility to take tracheobronchial aspirates allows for more detailed study of pathological conditions in the lower airways in this sub-group of infants with RSV bronchiolitis.

We conclude that differences exist in the pathogenesis of RSV bronchiolitis resulting in MV between infants with and without severe pre-existent morbidity. Cardiac, pulmonary or immunological compromise prior to RSV infection is probably the most important pathogenetic factor in the latter. In healthy preterm and term infants maturational factors are most important in the development of RSV bronchiolitis resulting in MV. Maturational factors mechanisms in RSV resulting in MV include small airways, and perhaps more important, immaturity of the immune system.

References

1 Collins PL, McIntosh K, Chanock RM. Respiratory syncytial virus. Field Virology. Raven Press, 1996: 1313-1351.

2 Karron RA, Singleton RJ, Bulkow L, Parkinson A, Kruse D, DeSmet I, Indorf C, Petersen KM, Leombruno D, Hurlburt D, Santosham M, Harrison LH. Severe respiratory syncytial virus disease in Alaska native children. RSV Alaska Study Group. J Infect Dis 1999; 180:41-49.

3 Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 1999; 282:1440-1446.

4 Wang EEL, Law BJ. Respiratory syncytial virus infection in pediatric patients. Seminars in Pediatric Infectious Diseases 1998; 9:146-153.

5 Outwater KM, Crone RK. Management of respiratory failure in infants with acute viral bronchiolitis. Am J Dis Child 1984; 138:1071-1075.

6 Frankel LR, Lewiston NJ, Smith DW, Stevenson DK. Clinical observations on mechanical ventilation for respiratory failure in bronchiolitis. Pediatr Pulmonol 1986; 2:307-311.

7 Tissing WJE, Van Steensel-Moll HA, Offringa M. Risk factors for mechanical ventilation in respiratory syncytial virus infection. Eur J Pediatr 1993; 152:125-127.

8 Hall CB, Kopelman AE, Douglas RG, Geiman JM, Meagher MP. Neonatal respiratory syncytial virus infection. N Engl J Med 1979; 300:393-396.

9 McDonald NE, Hall CB, Suffin SC, Alexon C, Harris PJ, Manning JA. Respiratory syncytial virus infection in infants with congenital heart disease. N Engl J Med 1982; 307:397-400.

10 Opavski MA, Stephens D, Wang EEL. Testing models predicting severity of respiratory syncytial virus infections on the PICNIC RSV database. Arch Pediatr Adolesc Med 1995; 149:1217-1220.

11 Stevens TP, Sinkin RA, Hall CB, Maniscalo WM, McConnochie KM. Respiratory syncytial virus and premature infants born at 32 weeks' gestation or earlier. Arch Pediatr Adolesc Med 2000; 154:55-61.

12 Wang EEL, Law BJ, Stephens D. Pediatric Investigators Collaborative Network on infections in Canada (PICNIC) prospective study of risk factors and outcomes in patients hospitalized with respiratory syncytial viral lower respiratory tract infection. J Pediatr 1995; 126:212-219.

13 Stein RT, Sherill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13. Lancet 1999; 354:541-545.

14 McBride JT. Pulmonary function changes in children after respiratory syncytial virus infection in infancy. J Pediatr 1999; 135:28-32.

15 Kimpen JLL. Respiratory syncytial virus immunology. Pediatr Allergy Immunol 1996; 7:S86-90.

16 Openshaw PJM. Immunopathological mechanisms in respiratory syncytial virus disease. Springer Semin Immunopathol 1995; 17:187-201.
17 Lebel MH, Gauthier M, Lacroix J, Rousseau E, Buithieu M. Respiratory failure and mechanical ventilation in severe bronchiolitis. Arch Dis Child 1989; 64:1431-1437.
18 Bont L, Van Vught HJ, Kimpen JLL. Prophylaxis against respiratory syncytial virus (RSV) in premature infants. Lancet 1999; 354(9183):1003-1004.
19 Smith DW, Frankel LR, Mathers LH, Tang AT, Ariagno RL, Prober CG. A controlled trial of aerosolized ribavirin in infants receiving mechanical ventilation for severe respiratory syncytial virus infection. N Engl J Med 1991; 325(1):24-29.
20 Tasker RC, Gordon I, Kiff K. Time course of severe respiratory syncytial virus infection in mechanically ventilated infants. Acta Paediatr 2000; 89(8):938-941.
21 Meert KL, Sarnaik AP, Gelmini MJ, Lieh-Lai MW. Aerosolized ribavirin in mechanically ventilated children with respiratory syncytial virus lower respiratory tract disease: a prospective, double-blind, randomized trial. Crit Care Med 1994; 22(4):566-572.
22 Bont L, Kavelaars A, Heijnen CJ, van Vught AJ, Kimpen JL. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. J Infect Dis 2000; 181:1772-1775.
23 Downham MA, Gardner PS, McQuillin J, Ferris JA. Role of respiratory viruses in childhood mortality. Br Med J 1975; 1(5952):235-239.
24 Aherne W, Bird T, Court SD, Gardner PS, McQuillin J. Pathological changes in virus infections of the lower respiratory tract in children. J Clin Pathol 1970; 23:7-18.
25 Neilson KA, Yunis EJ. Demonstration of respiratory syncytial virus in an autopsy series. Pediatr Pathol 1990; 10:491-502.
26 Simoes EAF. Respiratory syncytial virus infection. Lancet 1999; 354:847-852.
27 Buckingham SC, Bush AJ, Devincenzo JP. Nasal quantity of respiratory syncytial virus correlates with disease severity in hospitalized infants. Pediatr Infect Dis J 2000; 19:113-117.
28 Hall CB, Douglas-RG J, Geiman JM. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. J Pediatr 1976; 89:11-15.
29 Anderson LJ, Hierholzer JC, Hendry RM, Fernie BF, Stone Y, McIntosh K. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis 1985; 151:626-633.
30 Walsh EE, McConnochie KM, Long CE, Hall CB. Severity of respiratory syncytial virus infection is related to virus strain. J Infect Dis 1997; 175:814-820.
31 Hornsleth A, Klug B, Nir M, Johanson J, Hansen KS, Christensen LS, Larsen LB, Tech S. Severity of respiratory syncytial virus disease related to type and genotype of virus and to cytokine values in nasopharyngeal secretions. Pediatr Infect Dis J 1998; 17:1117-1121.
32 Kneyber MJC, Brandenburg AH, Rothbarth PhH, De Groot R, Ott A, Van Steensel-Moll HA. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch Dis Child 1996; 75:137-140.
33 McIntosh ED, De Silva LM, Oates RK. Clinical severity of respiratory syncytial virus group A and B infection in Sydney, Australia. Pediatr Infect Dis J 1993; 12:815-819.
34 Tsutsumi H, Onuma M, Nagai K, Yamazaki H, Chiba S. Clinical characteristics of respiratory syncytial virus (RSV) subgroup infections in Japan. Scand J Infect Dis 1991; 23:671-674.
35 Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 1969; 89(4):405-421.
36 Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Joseph LK, Symington P, O'Toole S, Myint SH, Tyrell DAJ, Holgate ST. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. BMJ 1995; 310:1225-1228.
37 Johnston SL, Pattemore PK, Sanderson G, Smith S, Campbell MJ, Joseph LK, Cunningham A, Robinson S, Myint SH, Ward ME, Tyrell DAJ, Holgate ST. The relationship between upper respiratory infections and hospital admissions for ashma: a time-trend analysis. Am J Respir Crit Care Med 1996; 154:654-660.
38 Eisen AH, Bacal HL. The relationship of acute bronchiolitis to bronchial asthma - a 4 tot 14 year follow-up. Pediatrics 1963; 31:859-861.
39 Chandwani S, Borkowski W, Krasinski K, Lawrence R, Welliver RC. Respiratory syncytial virus infection in human immunodeficiency virus-infected children. J Pediatr 1990; 117:251-254.

40 Ogra PL, Patel J. Respiratory syncytial virus infection and the immunocompromised host. Pediatr Infect Dis J 1995; 7:246-249.

41 Couch RB, Englund JA, Whimbey E. Respiratory viral infections in immunocompetent and immunocompromised persons. Am J Med 1997; 102:2-9.

42 Hall CB, Powell KR, McDonald NE, Gala CL, Menegus ME, Suffin SC, Cohen HJ. Respiratory syncytial virus infection in children with compromised immune function. N Engl J Med 1986; 315:77-81.

43 McCarthy AJ, Kingman HMKC, Taylor GS, Caul EO, Grier D, Moppett J, Foot ABM, Cornish JM, Oakhill A, Steward CG, Pamphilon DH, Marks DI. The outcome of 26 patients with respiratory syncytial virus infection following allogeneic stem cell transplantation. Bone Marrow Transplant 1999; 24:1315-1322.

44 Milner EM, De la Monte SM, Hutchins GM. Fatal respiratory syncytial virus infection in severe combined immunodeficiency syndrome. Am J Dis Child 1985; 139:1111-1114.

45 Chanock RM, Kapikian AZ, Mills J, Kim HW, Parrot RH. Influence of immunological factors in respiratory syncytial virus disease. Arch Environ Health 1970; 21:347-355.

46 Welliver RC, Kaul TN, Ogra PL. The appearance of cell-bound IgE in respiratory-tract epithelium after respiratory-syncytial-virus infection. N Engl J Med 1980; 303:1198-1202.

47 Welliver RC, Wong DT, Sun M, Middleton EJR, Vaughan RS, Ogra PL. The development of respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection. N Engl J Med 1981; 305:841-846.

48 Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. The PREVENT Study Group. Pediatrics 1997; 99:93-99.

49 The IMpact-RSV study group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 1998; 102(3):531-537.

50 Groothuis JR, Simoes EAF, Levin MJ, Hall CB, Long CE, Rodriguez WJ, Arrobio J, Meissner HD, Fulton DR, Welliver RC, Tristam DA, Siber GR, Prince GA, Raden vM, Hemming VG. prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. N Engl J Med 1993; 329:1524-1530.

51 De Weerd W, Twilhaar WN, Kimpen JL. T cell subset analysis in peripheral blood of children with RSV bronchiolitis. Scand J Infect Dis 1998; 30(1):77-80.

52 Aberle JH, Aberle SW, Dworzak MN, Mandl CW, Rebhandl W, Vollnhofer G, Kundi M, Popow-Kraupp T. Reduced Interferon-gamma expression in peripheral blood mononuclear cells of infants with severe respiratory syncytial virus disease. Am J Respir Crit Care Med 1999; 160:1263-1268.

53 Bont L, Heijnen CJ, Kavelaars A, Aalderen vWMC, Brus F, Draaisma JMTh, Geelen SM, Vught vHJ, Kimpen JLL. Association of peripheral blood cytokine responses with disease severity in respiratory syncytial virus bronchiolitis. Eur Respir J 1999; 14:144-149.

54 Scott P, Trinchieri G. IL-12 as an adjuvant for cell mediated immunity. Semin Immunol 1997; 9(5):285-291.

55 Openshaw PJM. Immunity and immunopathology to respiratory syncytial virus. Am J Respir Crit Care Med 1995; 152:S59-S62.

56 Renzi PM, Turgeon JP, Yang JP, Drblik SP, Marcotte JE, Pedneault L, Spier S. Cellular immunity is activated and a Th2-response is associated with early wheezing in infants after bronchiolitis. J Pediatr 1997; 130:584-593.

57 Roman M, Calhoun WJ, Himton KL, Avendano LF, Simon V, Escobar AM, Gaggero A, Diaz PV. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am J Respir Crit Care Med 1997; 156:190-195.

58 Bont L, Heijnen CJ, Kavelaars A, van Aalderen WM, Brus F, Draaisma JT, Geelen SM, Kimpen JL. Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one-year follow-up study. Am J Respir Crit Care Med 2000; 161(5):1518-1523.

59 Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991; 88:1026-1033.

60 Tang YW, Graham BS. T cell source of type 1 cytokines determines illness patterns in respiratory syncytial virus-infected mice. J Clin Invest 1997; 99:2183-2191.

61 Openshaw PJ, Clarke SL, Record FM. Pulmonary eosinophilic response to respiratory syncytial virus infection in mice sensitized to the major surface glycoprotein G. Int Immunol 1992; 4:493-500.

62 Schwarze J, Makela MJ, Cieslewicz G, Dakhama A, Lahn M, Ikemura T, Joetham A, Gelfand EW. Transfer of the enchancing effect of respiratory syncytial virus infection on subsequent allergic airway sensitization by T lymphocytes. J Immunol 1999; 163:5729-5734.
63 Schwarze J, Cieslewicz G, Hamelmann E, Joetham A, Shultz LD, Lamers MC, Gelfand EW. CD8 T cells are essential in the development of respiratory syncytial virus-induced lung eosinophila and airway hyperresponsiveness. J Immunol 1999; 162:4207-4211.
64 Hussell T, Georgiou A, Sparer TE, Matthews S, Pala P, Openshaw PJ. Host genetic determinants of vaccine-induced eosinophilia during respiratory syncytial virus infection. J Immunol 1998; 161:6215-6222.
65 Hussell T, Khan U, Openshaw PJM. IL-12 treatment attenuates T Helper Cell Type 2 and B Cell responses but does not improve vaccine-enhanced lung illness. J Immunol 1997; 159:328-334.
66 Miralles GD, Stoeckle MY, McDermott DF, Finkelman FD, Murray HW. Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis. Infect Immun 1994; 62:1058-1063.
67 Pope M, Chung SW, Mosmann T, Leibowitz JL, Gorczynski RM, Levy GA. Resistance of naive mice to murine hepatitis virus strain 3 requires development of a Th1, but not a Th2, response, whereas pre-existing antibody partially protects against primary infection. J Immunol 1996; 156:3342-3349.
68 Hoft DF, Lynch RG, Kirchhoff LV. Kinetic analysis of antigen-specific immune responses in resistant and susceptible mice during infection with Trypanosoma cruzi. J Immunol 1993; 151:7038-7047.
69 Liblau RS, Singer SM, McDevitt HO. Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today 1995; 16:34-38.
70 Graham BS, Perkins MD, Wright PF, Karzon DT. Primary respiratory syncytial virus infection in mice. J Med Virol 1988; 26:153-162.
71 Noah TL, Becker S. Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line. Am J Physiol 1993; 265:L472-L478.
72 Tristram DA, Hicks W, Jr., Hard R. Respiratory syncytial virus and human bronchial epithelium. Arch Otolaryngol Head Neck Surg 1998; 124:777-783.
73 Patel JA, Kunimoto M, Sim TC, Garofalo R, Eliott T, Baron S, Ruuskanen O, Chonmaitree T, Ogra PL, Schmalstieg F. Interleukin-1 alpha mediates the enhanced expression of intercellular adhesion molecule-1 in pulmonary epithelial cells infected with respiratory syncytial virus. Am J Respir Cell Mol Biol 1995; 13:602-609.
74 Adkins B. T-cell function in newborn mice and humans. Immunol Today 1999; 20:330-335.
75 Murgita RA, Witzel H. Regulation of immune functions in the fetus and newborn. Prog Allergy 1981; 29:54-133.
76 Wilson CB, Lewis DB, English BK. Immunity in the neonate. Semin Pediatr Infect Dis 1999; 10(2):83-90.
77 Wilson CB. Immunological basis for increased susceptibility of the neonate to infection. J Pediatr 1986; 108:1-12.
78 Starr SE, Toplin MD, Friedman HM. Impaired cellular immunity to cytomegalovirus in congenitally infected children and their mothers. J Infect Dis 1979; 140:500-505.
79 Smith S, Jacobs RF, Wilson CB. Immunobiology of childhood tuberculosis: a window on the ontogeny of cellular immunity. J Pediatr 1997; 131:16-26.
80 Burchett SK, Corey L, Mohan KM, Westall J, Ashley R, Wilson CB. Diminished interferon-gamma and lymphocyte proliferation in neonatal and postpartum primary herpes simplex virus infection. J Infect Dis 1992; 165:813-818.
81 Hayward AR, Herberger M, Saunders D. Herpes simplex virus-stimulated gamma-interferon production by newborn mononuclear cells. Pediatr Res 1986; 20:398-400.
82 Alexander J, Scharton-Kersten TM, Yap G, Roberts CW, Liew FY, Sher A. Mechanisms of innate resistance to Toxoplasma gondii infection. Philos Trans R Soc Lond B Biol Sci 1997; 352:1355-1359.
83 Watson W, Oen K, Ramhdahin R, Harman C. Immunoglobulin and cytokine production by neonatal lymphocytes. Clin Exp Immunol 1991; 83:169-174.
84 Dominguez E, Madrigal JA, Layrisse Z, Cohen SB. Fetal natural killer cell function is suppressed. Immunol 1998; 94:109-114.
85 Holt PG. Postnatal maturation of immune competence during infancy and childhood. Pediatr Allergy Immunol 1995; 6:59-70.
86 Adinolfi M. Ontogeny of human natural and acquired immunity. onbekend 1998.
87 Coutinho GC, Delassus S, Kourilsky P, Bandeira A, Coutinho A. Developmental shift in the patterns of interleukin production in early post-natal life. Eur J Immunol 1994; 24:1858-1862.

88 Zeltner TB, Caduff JH, Gehr P, Pfenninger J, Burri PH. The postnatal development and growth of the human lung. I. Morphometry. Respir Physiol 1987; 67:247-267.
89 Cooney TP, Thurlbeck WM. The radial alveolar count method of Emery and Mithal: a reappraisal 1- postnatal lung growth. Thorax 1982; 37:572-579.
90 Langston C, Kida K, Reed M, Thurlbeck WM. Human lung growth in late gestation and in the neonate. Am Rev Respir Dis 1984; 129:607-613.
91 Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. N Engl J Med 1995; 332:133-138.

3 Long-term consequences of respiratory syncytial virus (RSV) bronchiolitis

L Bont, WMC van Aalderen, JLL Kimpen

Pediatr Respir Rev 2000;1:221-227

3.1 Abstract

Despite differences in study design, follow-up studies consistently show that approximately half of the patients with respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) during infancy go on to have recurrent wheezing episodes during childhood. Respiratory symptoms are associated with abnormal lung function, including bronchial hyperresponsiveness. Wheezing symptoms following RSV LRTI gradually decrease and it appears that during school age airway morbidity is no longer related to RSV LRTI during infancy. Mechanisms underlying the association between RSV LRTI and long-term airway morbidity are poorly understood. On the one hand, abnormal airway function that is congenitally present or acquired before RSV LRTI occurs could be the cause of both RSV LRTI and subsequent recurrent wheezing. On the other hand, it is possible that RSV LRTI causes changes in the lower airways or the immune system that result in long-term airway morbidity. Animal models suggest RSV infection can promote the development of allergic sensitisation, but most studies in humans do not indicate a role for atopy in the development of recurrent wheezing following RSV LRTI.

3.2 Introduction

Maimonides wrote in 1170 A.D.: "I conclude that this disorder (asthma) starts with a common cold, especially in the rainy season, and that the patient is forced to gasp for breath day and night"[1]. This observation might well be the first description of the relation between bronchiolitis and recurrent wheezing. Infants who recover from respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) have an increased risk for the subsequent development of recurrent wheezing during early childhood[2;3]. Recurrent wheezing is accompanied by functional abnormalities of the airways, such as bronchial hyperresponsiveness[4;5]. Although knowledge on clinical, infectious and immunological factors in the pathogenesis of RSV LRTI increases rapidly, mechanisms by which RSV LRTI results in long-term airway morbidity are still poorly understood. Insight in these mechansims is required in order to develop innovative intervention strategies to prevent or treat long-term airway morbidity following RSV LRTI. The aim of this article is to provide an overview of clinical and experimental studies on the relation between RSV LRTI and long-term consequences.

3.3 Design of follow-up studies

During the last 40 years a number of prospective follow-up studies have focused on the relation between RSV LRTI and subsequent airway morbidity[2-16]. Although these studies have generally confirmed that such a relation exists, differences in the incidence of long-term airway morbidity following RSV LRTI and differences in the magnitude of involved risk factors were found. These differences may be explained, at least partially, by the study design used to investigate the relation between RSV LRTI and subsequent airway morbidity. Moreover, the definitions used for the different disease entities that have been used in the studies may be confusing. Definitions used in this article are shown in table 3.1.

A number of large-scale follow-up studies were performed before immunofluorescence for RSV became generally available for RSV diagnosis (table 3.2). In these studies, which are still cited in current literature, bronchiolitis patients were included regardless of the infectious agent. It can not be excluded that even patients with bacterial infections have been included.

Historical (retrospective) follow-up studies have shown the possible epidemiological relation between RSV LRTI and childhood asthma. Since childhood asthma is a heterogeneous disease, longitudinal follow-up studies were required to obtain more valid information. In addition, well defined (matched) control children were needed to estimate the relative risk for airway morbidity following RSV LRTI.

Another explanation for the observed differences in outcome variables in the studies on long-term airway morbidity following RSV bronchiolitis is that severity of the initial RSV LRTI differs between the studies. Most studies focus on hospitalised infants. Only recently, new data of long term consequences of RSV LRTI in non-hospitalised infants have been published[16]. The presence of respiratory wheeze is required in studies focusing on classical RSV bronchiolitis, in other studies any sign of LRTI is sufficient for inclusion. For example, "chest cough" as the sole symptom of LRTI caused by RSV in a 3-year-old child, was sufficient for inclusion in a recently published large longitudinal study on

Table 3.1 *Terms and definitions used throughout the article*

Term	Definition
Asthma[64]	an inflammatory disease of the airway of the lung, characterised by intermittent airway narrowing and variable symptoms of chest tightness, wheeze and shortness of breath
Airway hyperresponsiveness[64]	Characteristic physiological abnormality in asthma with exaggerated airway narrowing in response to bronchoconstrictor stimuli
Allergy[64]	Th2-associated immune reactions to allergens
Atopy[64]	Familial syndrome of asthma, eczema and hay fever. Can be recognised by elevated serum IgE levels, and positive skin prick tests or ELISA tests which detect IgE directed against allergens. Virtually synonymous with allergy.
RSV bronchiolitis[65]	Lower respiratory tract infection with characteristic expiratory wheeze caused by RSV in young infants without history of wheezing respiration.
Lower respiratory tract infection[16]	Infectious disease of the airway of the lung, characterised by shortness of breath, wheezing or severe cough

airway morbidity following RSV LRTI[16]. For practical reasons mechanically ventilated infants are usually excluded. Therefore, little data are available on long term consequences of this group of patients. It may well be that the severity of the initial disease influences the outcome in the long term.

Two types of outcome parameters for airway morbidity can be distinguished: airway symptoms and lung function. To date, most follow-up studies have used standardised questionnaires focusing on airway symptoms. However, bias can occur when questionnaires are filled out by parents. Most importantly, parents might in retrospect forget the occurrence of symptoms that are related to the respiratory state of their chilld (recall bias) or over-report in a certain time-frame. Parents might not understand the questions when they are not put very simple or they might not report the occurrence of events in the past because it is socially unaccepted (for example exposure to cigarette smoke).

Airway symptoms as experienced by the infants or parents are probably best assessed using daily diaries for airway symptoms, because it is a chronological report of symptoms as they are experienced. It is time-consuming to note airway symptoms in a diary for a long period of time, which allows for more non-compliance and dropouts during follow-up. Our experience is that regular contact with parents is time consuming for investigators but can provide parental motivation to note the presence or absence of simple airway symptoms (runny nose, cough, wheeze) in a diary for at least up to two years (Bont and colleagues, submitted for publication).

In addition to symptoms, lung function can be assessed as an objective outcome parameter. Since RSV LRTI is related to airway morbidity up to the age of 6 years, lung function abnormalities are predominantly expected during these years. However, at the age of 0-4

Table 3.2 *Study design and inclusion criteria used in 13 RSV follow-up studies*

Year	Reference	Prospective inclusion	Controls	Hospitalisation	RSV diagnosis	Bronchial obstruction	Age (months)	*n*
1963	Eisen[2]	no	no	yes	no	yes	<24	248
1978	Sims[3;24]	no	yes	yes	yes	yes	<12	35
1981	Gurwitz[4]	no	no	yes	no	yes	<24	48
1982	Pullan[5]	no	yes	yes	yes	no	<12	130
1984	McConnochie[7]	no	yes	no	no	yes	<24	177
1984	Hall[6]	yes	yes	yes	yes	no	<24	29
1986	Welliver[11;23]	yes	no	yes	yes	yes	<6	38
1989	Sly[10]	yes	no	yes	yes	yes	<12	48
1992	Murray[22]	yes	yes	yes	no	no	<12	73
1995	Sigurs[12]	yes	yes	yes	yes	yes	<12	52
1997	Dezateuz[66]	yes	yes	yes	yes	yes	<12	29
1997	Renzi[14]	yes	no	yes	no	yes	<12	26
1999	Stein[16]	yes	yes	no	yes	no	<36	207
2000	Bont[40]	yes	no	yes	yes	no	<12	50

n: number of RSV patients

years only non-conventional techniques for lung function measurement are available. Tidal breathing analysis can be used to assess airway resistance [17;18]. Bronchial hyperresponsiveness can be assessed using trachea auscultation to determine the methacholine concentration to induce wheezing[19]. However, the validity and reliability of these techniques have not yet been fully established. To study airway resistance and compliance, most techniques analyse tidal breathing. In addition, these techniques can measure the effect of bronchodilators. Improvement of lung function analysis techniques during early childhood will enable investigators to focus on the evolution of airway function abnormalities following RSV LRTI on the long term.

3.4 Relation RSV bronchiolitis and airway morbidity

An overview of relevant epidemiological studies analysing the relation between RSV LRTI and airway morbidity is shown in table 3.3. Wheezing episodes following RSV LRTI are found in 42-71% of patients. It is generally assumed that wheezing episodes following RSV LRTI are associated with viral upper respiratory tract infections[5], and not allergen exposure in contrast to children who suffer allergic asthma. This is in line with data from a community based longitudinal study (not focused on RSV) that showed that viral URTI also have been associated with asthma episodes in asthmatic school children[20;21]. Infants who will go on to have wheezing episodes will do so within 12-24 months after the first episode of RSV LRTI[5]. Less than 10% of children with recurrent wheezing following RSV LRTI began to have wheezing episodes after they were 2 years old.
A large number of studies attempted to identify clinical risk factors that determine the development of airway morbidity following RSV bronchiolitis. To date, these risk factors are not well understood. Disease severity and age at the moment of RSV LRTI could not

Table 3.3 *Relation between RSV LRTI during infancy and subsequent development of airway morbidity and atopy*

First author	Main outcome parameter	Duration follow-up (years)	Main finding	Percentage wheeze	Lung function peformed	Relation with atopy
Eisen[2]	Questionnaire	14	Bronchiolitis related to recurrent wheeze	49	no	no
Sims[3]	Questionnaire	8	Atopy not related to wheeze following RSV	56	yes	no
Gurwitz[4]	Bronchial reactivity	8	Hyperresponsiveness after bronchiolitis	52	yes	–
Pullan[5]	Lung function	10	RSV related to subsequent wheeze and low lung function	42	yes	no
McConnochie[7]	Questionnaire	8	9.4% of wheeze in children related to RSV	44	no	no
Hall[6]	Physician diagnosed wheeze	8	wheeze after RSV related to low lung function, but not atopy	45	yes	no
Welliver[11;67]	Physician diagnosed wheeze	2.0	Nasal RSV-specific IgE predicts wheeze	53	yes	–
Sly[10]	Questionnaire	5	Family atopy not related to wheeze after RSV	71	yes	no
Murray[22]	Wheeze	5.5	Personal atopy not related to wheeze after RSV	43	yes	no
Sigurs[12]	Skin prick test, allergen-specific IgE	3	RSV risk factor for allergy	60	no	yes
Renzi[14]	Diary	0.25	Th2 cytokine profile predict wheeze	65	no	–
Dezateux[66]	Lung function	0.7	Diminished t_{tpef}:t_e ratio after RSV	55	yes	–
Stein[16]	Questionnaire	13	Wheezing after RSV up to age 11, not at age 13 no relation with atopy	1	yes	no
Bont[40]	Wheeze (diary)	1.0	Monocyte IL-10 related to recurrent wheeze after RSV	58	no	no

(1) Odd's ratio at age 6 for frequent and infrequent wheeze: 4.3 and 3.2, respectively

be related to subsequent airway morbidity[3;5;6;22]. Male sex appeared to be a risk factor for recurrent wheezing following RSV LRTI in some, but not all studies[7;23;24]. It has been shown by Martinez and colleagues that pre-existent lower levels of lung function in newborns are associated with subsequent incidence of lower respiratory tract illness with or without wheezing in the first year of life[17]. In line with this finding, it has been suggested that infants with recurrent wheezing following RSV LRTI have lower lung function early in infancy, before any respiratory tract infection has occurred[16]. However, this hypothesis needs to be confirmed. In addition to premorbid lung function, atopy is a possible risk factor, that was studied extensively, but results were inconclusive (table 2). Another risk factor for recurrent wheezing is exposition to cigarette smoke

before or after RSV LRTI[3;6;7]. Cigarette smoke has also been shown to be a risk factor for airway morbidity in childhood in the general population[25;26]. Because maternal smoking during pregnancy is a determinant of lung function during early infancy, smoke exposure could contribute to the risk for both hospitalisation for RSV LRTI and the subsequent development of airway morbidity[25]. Parental history of asthma, in particular maternal asthma, has been mentioned as a risk factor for childhood asthma[27;28]. Although parental history of asthma has been associated with RSV bronchiolitis[22], to our knowledge the predictive value of parental history of asthma for recurrent wheezing following RSV LRTI has not yet been investigated.

A few studies have followed infants for more than 10 years to study the evolution of symptoms or lung function abnormalities following RSV LRTI[2;5;16]. These studies generally show that the risk for wheezing following RSV LRTI decreases with age. Stein and colleagues recently published the first prospective longitudinal study of a large cohort that was drawn from the community. Infants were followed for 13 years[16]. They compared infants with RSV LRTI with infants without any LRTI during the first three years of life. The presence of recurrent wheezing was assessed using standardised questionnaires when children in the study were 6, 8, 11 and 13 years old. In addition, lung function tests were performed when children were 11 years old. These lung function tests included assessment of the response to inhaled salbutamol. Frequent wheeze (≥4 wheezing episodes during the last year) and infrequent wheeze (<4 wheezing episodes during the last year) were found more frequently at age 6 in infants with RSV LRTI than in infants without LRTI (relative risk 4.3). Subsequently, the risk decreased markedly and was not significant when infants were 13 years old. Forced expiratory volume in 1 second (FEV1) at age 11 was significantly lower in infants who suffered from RSV LRTI in the past than in infants without LRTI, but this difference was not observed after salbutamol inhalation, suggesting reversible airflow limitation.

Several studies investigated whether treatment of initial RSV bronchiolitis can influence the long-term outcome. The use of ribavirin during initial RSV have been found to reduce the occurrence of wheezing following RSV bronchiolitis by some investigators[29]. However, these data could not be confirmed by others[30]. Similar to the use of ribavirin during RSV bronchiolitis to prevent recurrent wheezing, conflicting data have been reported in the use of inhaled corticosteroids[31;32].

Palivizumb is a humanized monoclonal antibody that reduces hospitalisation rates from 10.6% to 4.8% in preterm infants with and without chronic lung disease[33]. Of interest would be follow-up data of airway symptoms of infants from the trial with palivizumab. These follow-up data may provide evidence whether prevention of RSV also results in the prevention of long-term airway morbidity. This would support the idea that RSV LRTI results from pre-morbid pathology, at least in this specific high-risk group.

3.5 Pathophysiology of airway morbidity following RSV LRTI

3.5.1 Human studies

It has been suggested that children with RSV LRTI in early childhood have pre-existing abnormalities of lung function that manifest during the initial illness and again during recurrences of wheezing episodes[16]. An alternative explanation for the association between RSV LRTI and the subsequent development of recurrent wheezing is that RSV

itself causes changes in the lower airways or caused changes in the immune system that result in long-term airway morbidity. In line with this latter hypothesis, it has been suggested that RSV results in allergy, which subsequently leads to allergic asthmatic symptoms.

Airway damage during RSV LRTI might well be a factor in the pathogenesis of long-term airway morbidity. It is likely that severity of disease during RSV LRTI is related to the magnitude of destruction of airway epithelium. Therefore, studies have attempted to find an association between severity of disease during RSV LRTI and subsequent long-term airway morbidity, but this was not found[3;5]. In a prospective cohort study, we found that mechanically ventilated infants with RSV LRTI without pre-existing airway disease have no larger risk for recurrent wheeze or physician diagnosed asthma than other hospitalised infants with RSV LRTI (unpublished data). This suggests that other mechanisms than destruction of airway epithelium during RSV LRTI are important in the pathogenesis of long-term airway morbidity following.

Allergy is a Th2-driven syndrome of which IgE-mediated disease manifestations are a hallmark (table 1). Considering the clinical similarities between recurrent wheezing following RSV and allergic asthma, the relationship between allergy-related immune responses and recurrent wheezing following RSV LRTI has been studied. For long, it has been believed that RSV bronchiolitis and subsequent airway morbidity are associated with the same or similar pathogenetic mechanisms that cause atopy and allergy. In a prospective cohort study with 47 infants with RSV bronchiolitis in the first year of life and 94 matched controls, Sigurs and colleagues analysed the risk to develop a positive skin prick tests and increased serum IgE against common inhalant and food allergens at age 3 years as signs of atopy[12]. Signs of atopy were found in 32% of infants with a history of RSV bronchiolitis and in 9% of controls. This apparent risk for allergic sensitisation following RSV bronchiolitis was independent of family history of atopy. Contrasting data were found in the study by Stein and colleagues, in which it was shown that RSV LRTI before the fourth birthday is no risk factor for positive skin prick test for common food or inhalation allergens or increased serum IgE after age 6[16]352.

Eosinophils play a clear role in allergic asthma. Therefore, eosinophils and eosinophil activity during RSV LRTI have been studied in relation to the development of recurrent wheezing. In two different studies, Reijonen and Koller found that eosinophilic cationic protein (ECP) levels during bronchiolitis in nasopharyngeal secretions (NPS) and serum, respectively, were associated with recurrent wheezing in the first year of life[13;34-36]. However, in these studies RSV infection was not required for inclusion. A prospective follow-up study by Ehlenfield and colleagues showed that eosinophilia during RSV bronchiolitis is associated with persistent wheezing after 6 years of age, but not with transient wheezing in early childhood[37].

Similar to allergic asthma, Th2-like cytokine responses have been put forward as a potential mechanism by which RSV results in long-term airway morbidity. Renzi showed that peripheral blood lymphocytes of 26 bronchiolitis infants, produced less IFNγ in response to IL-2 and more IL-5 in response to *Dermatophagoides farinae* five months after the acute bronchiolitis in children who developed recurrent wheezing[14], indicating a blunted Th1 response in confirmation with Th2 predominance. However, RSV was diagnosed in only half of the infants. In a different study decreased *ex vivo* IFNg/IL-4 ratios in response to non-specific stimuli were found during RSV LRTI[15]. However, from this

study no follow-up data are available. In contrast with the previous 2 studies, we have recently shown that *ex vivo* IFNg/IL-4 ratios in a whole blood culture system stimulated with phytohemaglutinin (PHA) during RSV LRTI was comparable with normal controls. In addition, IFNγ/IL-4 ratios were not associated with recurrent wheezing during one year follow-up. These apparent differences found in our study may be explained by different inclusion criteria and different culture assays.

During RSV LRTI RSV-specific IgE is secreted in the respiratory tract and correlates with disease severity[38;39]. In addition, it was shown in a cohort of 38 infants that peak titres of RSV-specific IgE in nasopharyngeal secretion (NPS) during RSV LRTI predicted wheezing during a 48 months follow-up period[23]. RSV-specific IgE in NPS are not related to allergic sensitisation during the follow-up period. Thus, the production and release of RSV-specific IgE in the respiratory tract appears to be regulated by mechanisms that are unrelated to atopy. More research is required to investigate the potential role of RSV-specific IgE in airway morbidity following RSV LRTI.

Recently, we have shown that monocyte cytokine profiles in the blood during RSV LRTI are related to disease severity, and also to subsequent airway morbidity[40;41]. In a group of 30 RSV infected infants requiring mechanical ventilation, IL-12 production in whole blood cultures at initiation of mechanical ventilation had predictive value for duration of respiratory insufficiency[41]. In a different study, we investigated whether monocyte cytokine responses during RSV LRTI are also associated with subsequent long-term airway morbidity[40]. Results showed that monocyte IL-10 production 3-4 weeks after hospitalisation was associated with recurrent wheezing during a one year follow-up period and with physician diagnosed asthma. Sherran and colleagues have shown that IL-10 concentrations in NPS are increased during LRTI RSV but no attempt was made to relate IL-10 levels in NPS to subsequent airway morbidity[42]. Further study is required to elucidate by what mechanism monocyte IL-10 responses during RSV may contribute to recurrent wheezing.

3.5.2 Animal studies

In studies of experimental infection with RSV, replication of the virus within the lung has been demonstrated in several animals, including monkey species, mice, rats and guinea pigs[43-46]. None of these animals develop signs of respiratory illness resembling human RSV bronchiolitis. For this reason, discussion persists as to whether animal models are relevant for human RSV infection. Murine RSV infection is the animal model that has been studies most extensively. Signs of illness in mice include weight loss and decreased activity[47]. Objective evidence of respiratory illness was observed in RSV infected BALB/c mice using whole body plethysmography[48]. With this method increased respiration rate and abnormal breathing patterns were observed. Increased responsiveness to methacholine following RSV infection was seen in mice, rats and guinea pigs[48-50]. Consequently, these animals could be suitable models, since increased hyperresponsiveness is also seen in infants with airway morbidity following RSV infection[4]. The major limitation of animal models to study long-term airway morbidity following RSV infection is that persistence of abnormal lung function has not yet been established. Therefore, the major challenge for animal studies in the future is to establish the relevance for the human situation.

From experiments in the mouse model, it has been suggested that RSV infection may interact with immunological mechanisms involved in the development of Th2-like immune responses, including allergic sensitisation[51-57]. In mice who were sensitised to the G protein, RSV infection enhances allergic airway sensitization, resulting in lung eosinophilia and in airway hyperresponsiveness[54;58]. It was shown that transfer of T cells, in particular CD8+ cells, of RSV infected mice into naive mice result in increased eosinophil influx and production of Th2 cytokines following challenge with ovalbumine[59]. In order to reduce morbidity caused by RSV in the animal model, experiments have been designed to induce Th1-like immune responses during RSV infection[60]. Treatment with IL-12 of BALB/c mice, primed with RSV G protein, reduced lung eosinophilia and the production of Th2 cytokines following RSV infection, but did not reduce acute illness, as assessed by weight loss. The latter experiment nicely shows that immune responses can be modified, but indicates that morbidity caused by RSV is much more complex. In addition, we note that most studies were performed in BALB/c mice, which have a genetic predisposition to develop Th2-like immune responses. Further study is required to investigate the interaction between RSV infection and the development of allergy in animal models without a predisposition for Th2-like immune responses.

Animal models have indicated that also non-immune mechanisms could be involved in the pathogenesis of airway hyperresponsiveness following RSV infection. The increased responsiveness to bronchodilators following RSV LRTI observed by Stein and colleagues can not easily be explained by aforementioned immune-mediated mechanisms that might be involved in airway morbidity following RSV LRTI. In different animal models persistent alterations in the development of non-adrenergic non-cholinergic inhibitory neural pathways were seen following RSV infection[61-63]. This change was associated with increased contractility of airway smooth muscle. To test the relevance of these models for humans, future studies on neural regulation of airway tone in humans following RSV are required.

3.6 Conclusions

In the present article we reviewed clinical and experimental data on the relation between RSV LRTI and long-term airway morbidity. Remarkable differences in design, inclusion criteria and measures of clinical outcome exist between follow-up studies. However, most studies show that approximately half of infants with RSV LRTI will have recurrent episodes of wheezing during early childhood. Both early and more recently published data indicate that airway morbidity following RSV LRTI is transient and subsides during school age.

The pathogenesis of recurrent wheezing following RSV bronchiolitis is still poorly understood. Present studies focus on pre-existing lung function defects as the major cause of both RSV bronchiolitis and long-term airway morbidity. Although it appears that atopy does not play a large role in the development of airway morbidity following RSV bronchiolitis, immunological studies on the role of Th2 cytokine responses are still subject of conflict. Immunological research needs to focus on alternative mechanisms in the pathogenesis of airway morbidity following RSV bronchiolitis. Potentially, antigen presenting cells as well as epithelial cells play an important role in the development of airway

morbidity following RSV bronchiolitis. Animal studies provide the opportunity to obtain detailed observations of pathogenetic changes in immune responses and pulmonary function following RSV infection. However, it is urgently required that the relevance of different animal models for human RSV bronchiolitis are established. Better knowledge of the pathogenesis of long-term consequences of RSV bronchiolitis will facilitate the development of innovative intervention strategies to prevent or treat long-term airway morbidity following RSV bronchiolitis.

References

1 Rosner F. Moses Maimonides' Treatise on asthma. Thorax 1981; 36:245-251.
2 Eisen AH, Bacal HL. The relationship of acute bronchiolitis to bronchial asthma - a 4 tot 14 year follow-up. Pediatrics 1963; 31:859-861.
3 Sims DG, Downham MA, Gardner PS. Study of 8-year-old children with a history of respiratory syncytial virus bronchiolitis in infancy. BMJ 1978; 1:11-17.
4 Gurwitz D, Mindorff C, Levinson H. Increased evidence of bronchial reactivity in children with a history of acute bronchiolitis. J Pediatr 1981; 98:551-555.
5 Pullan CR, Hey EN. Wheezing, asthma and pulmonary dysfunction 10 years after infection with respiratory syncytial virus infection in infancy. BMJ 1982; 284:1665-1669.
6 Hall CB, Hall WJ, Gala CL, McGill FB, Leddy JP. Long-term prospective study in children after respiratory syncytial virus infection. J Pediatr 1984; 105:358-364.
7 McConnochie KM, Roghmann KJ. Bronchiolitis as a possible cause of wheezing in childhood: new evidence. Pediatrics 1984; 74:1-10.
8 Mok JYQ, Simpson H. Symptoms, atopy, and bronchial reactivity after lower respiratory infection in infancy. Arch Dis Child 1984; 59:299-305.
9 McConnochie KM, Mark JD, McBride JT, Hall WJ, Brooks JG, Klein SJ, Miller RL, McInerny TK, Nazarian LF, McWhinney JB. Normal pulmonary function measurements and airway reactivity in childhood after mild bronchiolitis. J Pediatr 1985; 107:54-58.
10 Sly PD, Hibbert ME. Childhood asthma following hospitalization with acute viral bronchiolitis in infancy. Pediatr Pulmonol 1989; 7:153-158.
11 Welliver RC, Duff AL. The relationship of RSV-specific immunoglobulin E-antibody responses in infancy, recurrent wheezing and pulmonary function at age 7-8 years. Pediatr Pulmonol 1993; 15:19-27.
12 Sigurs N, Bjarnason R, Sigursbergsson F, Kjellman B, Brorksten B. Asthma and immunoglobulin E Antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. Pediatrics 1995; 95:500-505.
13 Reijonen TM, Korppi M, Kleemola M, Savolainen K, Kuikka l, Mononen I, Remes K. Nasopharyngeal eosinophil cationic protein in bronchiolitis: relation to viral findings and subsequent wheezing. Pediatr Pulmonol 1997; 24:35-41.
14 Renzi PM, Turgeon JP, Yang JP, Drblik SP, Marcotte JE, Pedneault L, Spier S. Cellular immunity is activated and a Th2-response is associated with early wheezing in infants after bronchiolitis. J Pediatr 1997; 130:584-593.
15 Roman M, Calhoun WJ, Himton KL, Avendano LF, Simon V, Escobar AM, Gaggero A, Diaz PV. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am J Respir Crit Care Med 1997; 156:190-195.
16 Stein RT, Sherill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13. Lancet 1999; 354:541-545.
17 Martinez FD, Morgan WJ, Wright AL, Holberg CJ, Taussig LM. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. N Engl J Med 1988; 319:1112-1117.
18 Van der Ent CK, Brackel HJ, van der LJ, Bogaard JM . Tidal breathing analysis as a measure of airway obstruction in children three years of age and older. Am J Respir Crit Care Med 1996; 153:1253-1258.
19 Avital A, Bar YE, Springer C, Godfrey S. Bronchial provocation tests in young children using tracheal auscultation. J Pediatr 1988; 112:591-594.

20 Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Joseph LK, Symington P, O'Toole S, Myint SH, Tyrell DAJ, Holgate ST. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. BMJ 1995; 310:1225-1228.
21 Johnston SL, Pattemore PK, Sanderson G, Smith S, Campbell MJ, Joseph LK, Cunningham A, Robinson S, Myint SH, Ward ME, Tyrell DAJ, Holgate ST. The relationship between upper respiratory infections and hospital admissions for ashma: a time-trend analysis. Am J Respir Crit Care Med 1996; 154:654-660.
22 Murray M, Webb MSC, O'Callaghan C, Swarbrick AS, Milner AD. Respiratory status and allergy after bronchiolitis. Arch Dis Child 1992; 67:482-487.
23 Welliver RC, Sun M, Rinaldo D, Ogra PL. Predictive value of respiratory syncytial virus- specific IgE responses for recurrent wheezing following bronchiolitis. J Pediatr 1986; 109:776-780.
24 Sims DG, Garner PS, Weightman D, Turner MW, Soothill JF. Atopy does not predispose to RSV bronchiolitis or postbronchiolitic wheezing. BMJ 1981; 282:2086-2091.
25 Tager I.B., Hanrahan JP, Tosteson TD, Castile RG, Brown RW, Weiss.S.T., Speizer FE. Lung function, pre- and post-natal smoke exposure, and wheezing in the first year of life. Am Rev Respir Dis 1993; 147:811-817.
26 Meijer GG, Postma DS, van der HS, de Reus DM, Roorda RJ, Koeter GH, van Aalderen WM. Exogenous stimuli and circadian peak expiratory flow variation in allergic asthmatic children. Am J Respir Crit Care Med 1996; 153(1):237-242.
27 Litonjua AA, Carey V.J., Burge H.A., old D.R. Parental history and the risk for childhood asthma. Does mother confer more risk than father? Am J Respir Crit Care Med 1998; 158:176-181.
28 Halonen M, Stern DA, Lohman C, Wright AL, Brown MA, Martinez FD. Two subphenotypes of childhood asthma that differ in maternal and paternal influences on asthma risk. Am J Respir Crit Care Med 1999; 160:564-570.
29 Edell D, Bruce E, Hale K, Khoshoo V. Reduced long-term respiratory morbidity after treatment of respiratory syncytial virus bronchiolitis with ribavirin in previously healthy infants: a preliminary report. Pediatr Pulmonol 1998; 25:154-158.
30 Rodriguez WJ, Arrobio J, Fink R, Kim HW, Milburn C. Prospective follow-up and pulmonary functions from a placebo-controlled randomized trial of ribavirin therapy in respiratory syncytial virus bronchiolitis. Ribavirin Study Group. Arch Pediatr Adolesc Med 1999; 153:469-474.
31 Reijonen T, Korppi M, Kuikka l, Remes K. Anti-inflammatory therapy reduces wheezing after bronchiolitis. Arch Pediatr Adolesc Med 1996; 150:512-517.
32 Berger I, Argaman Z, Schwartz SB, Segal E, Kiderman A, Branski D, Kerem E. Efficacy of corticosteroids in acute bronchiolitis: short-term and long-term follow-up. Pediatr Pulmonol 1998; 26:162-166.
33 The IMpact-RSV study group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 1998; 102(3):531-537.
34 Garofalo RP, Kimpen JL, Welliver RC, Ogra PL. Eosinophil degranulation in the respiratory tract during naturally acquired respiratory syncytial virus infection. J Pediatr 1992; 120:28-32.
35 Kimpen JL, Garofalo R, Welliver RC, Ogra PL. Activation of human eosinophils in vitro by respiratory syncytial virus. Pediatr Res 1992; 32:160-164.
36 Koller DY, Wojnarowski C, Herkner KR, Weinlander G , Raderer M, Eichler I, Frischer T. High levels of eosinophil cationic protein in wheezing infants predict the development of asthma. J Allergy Clin Immunol 1997; 99:752-756.
37 Ehlenfield DR, Cameron K, Welliver RC. Eosinophilia at the time of respiratory syncytial virus bronchiolitis predicts childhood reactive airway disease. Pediatrics 2000; 105:79-83.
38 Welliver RC, Kaul TN, Ogra PL. The appearance of cell-bound IgE in respiratory-tract epithelium after respiratory-syncytial-virus infection. N Engl J Med 1980; 303:1198-1202.
39 Welliver RC, Wong DT, Sun M, Middleton EJR, Vaughan RS, Ogra PL. The development of respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection. N Engl J Med 1981; 305:841-846.
40 Bont L, Heijnen CJ, Kavelaars A, van Aalderen WM, Brus F, Draaisma JT, Geelen SM, Kimpen JL. Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one-year follow-up study. Am J Respir Crit Care Med 2000; 161(5):1518-1523.
41 Bont L, Kavelaars A, Heijnen CJ, van Vught AJ, Kimpen JL. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. J Infect Dis 2000; 181:1772-1775.

42 Sherran P, Jafri H, Carubelli C, Saavedra J, Johnson C, Krisher K, Sanchez PJ, Ramilo O. Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease. Pediatr Infect Dis J 1999; 18:115-122.

43 Hegele RG, Robinson PJ, Gonzalez S, Hogg JC. Production of acute bronchiolitis in guinea-pigs by human respiratory syncytial virus. Eur Respir J 1993; 6:1324-1331.

44 Prince GA, Horswood RL, Berndt J, Suffin SC, Chanock RM. Respiratory syncytial virus infection in inbred mice. Infect Immun 1979; 26:764-766.

45 Prince GA, Jenson AB, Horswood RL, Camargo E, Chanock RM. The pathogenesis of respiratory syncytial virus infection in cotton rats. Am J Pathol 1978; 93:771-791.

46 Belshe RB, Richardson LS, London WT, Sly DL, Lorfeld JH, Camargo E, Prevar DA, Chanock RM. Experimental respiratory syncytial virus infection of four species of primates. J Med Virol 1977; 1:157-162.

47 Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991; 88:1026-1033.

48 Van Schaik SM, Enhorning G, Vargas I, Welliver RC. Respiratory syncytial virus affects pulmonary function in BALB/c mice. J Infect Dis 1998; 177 (2):269-276.

49 Sorkness R, Clough JJ, Castleman WL, Lemanske-RF J. Virus-induced airway obstruction and parasympathetic hyperresponsiveness in adult rats. Am J Respir Crit Care Med 1994; 150:28-34.

50 Buckner CK, Songsiridej V, Dick EC, Busse WW. In vivo and in vitro studies on the use of the guinea pig as a model for virus-provoked airway hyperreactivity. Am Rev Respir Dis 1985; 132:305-310.

51 Openshaw PJ, Clarke SL, Record FM. Pulmonary eosinophilic response to respiratory syncytial virus infection in mice sensitized to the major surface glycoprotein G. Int Immunol 1992; 4:493-500.

52 Hussell T, Spender IC, Georgiou A, O'Garra A, Openshaw PJM. Th1 and Th2 cytokine induction in pulmonary T cells during infection with respiratory syncytial virus. J General Virol 1996; 77:2447-2455.

53 Openshaw PJM. Immunopathological mechanisms in respiratory syncytial virus disease. Springer Semin Immunopathol 1995; 17:187-201.

54 Schwarze J, Cieslewicz G, Hamelmann E, Joetham A, Shultz LD, Lamers MC, Gelfand EW. IL-5 and eosinophils are essential for the development of airway hyperresponsiveness following acute respiratory syncytial virus infection. J Immunol 1999; 162:2997-3005.

55 Alwan WH, Record FM, Openshaw PJM. Phenotypic and functional characterization of T cell lines specific for individual respiratory syncyctial virus proteins. J Immunol 1993; 150:5211-5218.

56 Jackson M, Scott R. Different patterns of cytokine induction in cultures of respiratory syncytial virus (RS) virus-specific human Th cell lines following stimulation with RS virus and RS virus proteins. J Med Virol 1996; 49:161-169.

57 Hussell T, Baldwin CJ, O'Garra A, Openshaw PJM. CD8+ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. Eur J Immunol 1997; 27:3341-3349.

58 Johnson TR, Johnson JE, Roberts SR, Wertz GW, PArker RA, Graham BS. Priming with secreted glycoprotein G of respiratory syncytial virus (RSV) augments interleukin-5 production and tissue eosinophilia after RSV challenge. J Virol 1998; 72(4):2871-2880.

59 Schwarze J, Makela MJ, Cieslewicz G, Dakhama A, Lahn M, Ikemura T, Joetham A, Gelfand EW. Transfer of the enchancing effect of respiratory syncytial virus infection on subsequent allergic airway sensitization by T lymphocytes. J Immunol 1999; 163:5729-5734.

60 Hussell T, Khan U, Openshaw PJM. IL-12 treatment attenuates T Helper Cell Type 2 and B Cell responses but does not improve vaccine-enhanced lung illness. J Immunol 1997; 159:328-334.

61 Colasurdo GN, Hemming VG, Prince GA, Loader JE, Graves JP, Larsen GL. Human respiratory syncytial virus affects nonadrenergic noncholinergic inhibition in cotton rat airways. Am J Physiol 1995; 268:L1006-L1011.

62 Colasurdo GN, Hemming VG, Prince GA, Gelfand AS, Loader JE, Larsen GL. Human respiratory syncytial virus produces prolonged alterations of neural control in airways of developing ferrets. Am J Respir Crit Care Med 1998; 157:1506-1511.

63 Larsen GL, Colasurdo GN. Neural control mechanisms within airways: disruption by respiratory syncytial virus. J Pediatr 1999; 135:21-27.

64 Allergy and asthma: glossary. Nature 1999; 402:B39 .

65 Behrman RE, Kliegman R, Arvin AM, Nelson WE. Nelson textbook of Pediatrics. 15 ed. 1996.

66 Dezateux C, Fletcher ME, Dundas I, Stocks J. Infant respiratory function after RSV-proven bronchiolitis. Am J Respir Crit Care Med 1997; 155:1349-1355.

67 Welliver RC, Kaul A, Ogra PL. Cell-mediated immune response to respiratory syncytial virus infection: relationship to the development of reactive airway disease. J Pediatr 1979; 94:370-375.

4 Methods

4.1 Study population

Children were included prospectively during two winter epidemic in 6 hospitals in the Netherlands: (1) Wilhelmina Children's Hospital, University Medical Center, Utrecht, (2) Beatrix Children's Hospital, Academic Hospital, Groningen, (3) Rijnstate Hospital, Arnhem, (4) St. Elisabeth Hospital, Tilburg, (5) St. Antonius Hospital, Nieuwegein, (6) Diakonessen Hospital, Utrecht.
Inclusion criteria were: hospital admission, lower respiratory tract symptoms, age < 13 months and immunofluorescence for RSV infection of epithelial cells in nasopharyngeal secretions. Infants with wheezing illness prior to RSV bronchiolitis were not included. One investigator (L.B.) took the history of atopy of parents, grandparents and siblings (asthma, documented food allergy, eczema, hay fever) and inquired whether either parent had smoked in the presence of the infant during the follow-up period. Control children aged < 13 months without evidence of atopy or infection were selected for this study during the same winter season. Included were infants prior to minor surgery, prior to cardiac surgery in the absence of hemodynamic compromise, healthy prematurely born infants, healthy infants screened for congenital disorders and infants with mild anemia. The study was approved by the Medical Ethical Committee in all participating centers. Parents of subjects and controls gave written informed consent.
In **Chapter 5**, patients were included retrospectively. All infants < 13 months of age that were mechanically ventilated for RSV infection between during a 5-year period were included.

4.2 Severity of disease

Patients with severe illness and with non-severe illness were distinguished by the need for mechanical ventilation (**Chapter 6 and 7**).

4.3 Collection of materials

4.3.1 Blood

Within 24 hours after admission venous or arterial blood was taken from all patients. From all subjects blood was obtained in tubes containing ethylenediamine tetracetic acid (EDTA). Tubes were directly put on ice, plasma was separated and stored at -80°C. In addition white cell counts were performed (Technicon H1, Bayer Technicon, New York). From subjects of University Medical Center, Utrecht and Diakonessen Hospital, Utrecht heparinized blood was taken simultaneously for whole blood cultures. Three weeks later, in the convalescent phase, heparinized blood and plasma were taken from all subjects.

4.3.2 Nasopharyngeal aspirates (NPA)

Nasopharyngeal secretions were aspirated using a 3,3 mm suction catheter (Vygon, Ecouen, France). Aspirates were placed on ice immediately and stored at -80°C. Before cytokine measurement, the amount of NPA was weighed, diluted in dilution buffer (4°C) of the ELISA kits that were used for the cytokine assays (CLB, Amsterdam, The Netherlands), sonicated on ice for 2x6 seconds (amplitude 6 μm) and subsequently centrifuged

at 13,000 rpm for 10 minutes at 4°C. For IL-8 measurement, NPA was diluted 1:15000, for measurement of other cytokines NPA was diluted 1:15.
Cytokine levels in NPA were used as an approximation for cytokine levels in fluids of the lower airways. The validity of cytokine measurements in NPA can not be estimated accurately, because real measurements of cytokine levels in the lower airways can not be obtained. However, the potential systematic error caused by the sampling site of NPA was estimated in a subset of mechanically ventilated RSV bronchiolitis patients. In these infants the correlation between cytokine levels in NPA and tracheobronchial aspirates (TBA), collected simultaneously and using the same methods of aspiration and subsequent analysis, was determined[1;2].

4.4 Virus preparation

Long strain RSV was cultured in Hep-2 cells (courtesy of Dr. A.M. van Loon, dept of Virology, University Medical Center, Utrecht,) and 1:1 diluted in sucrose-gelatone solution Z7725a (Laboratory of Vaccine Research, National Institute of Public Health and Environment, Bilthoven, The Netherlands). Titers were determined in Hep-2 cells using the TCID50 method described by Karber (TCID50 10^5)[3]. A control antigen was prepared similarly from uninfected Hep-2 cultures. Virus and control antigen were stored in aliquots at -80°C.

4.5 Whole blood cultures

Freshly taken heparinized blood was diluted 1:10 in RPMI 1640 medium (Life Technologies, Grand Island, NY) and aliquoted (150 µl) into 96-well culture plates (Nunc International, Denmark). The whole blood culture stimulated is a suitable *ex vivo* method to study cytokine production under conditions in which many of the physiologically relevant cellular interactions remain intact (23). All cultures were performed in quadruplicate. Pooled supernatants were kept at -70°C.

4.5.1 Monocyte cytokine responses

To induce monocyte IL-10 and IL-12 production, lipopolysaccharide (LPS) (100ng/ml)+IFNγ (20 ng/ml) was added and cultures were incubated for 48 hours at 37°C in 5% CO_2. It has been shown that maximal monocyte IL-10 production is observed after 48 hours, which is relatively late compared to that of monocyte pro-inflammatory cytokines (20). Also monocyte IL-12 production is (sub)optimally induced after 48 hours stimulation (24). Furthermore, it has been established that monocytes are the main producers of IL-10 and IL-12 in LPS stimulated whole blood cultures (25).

4.5.2 T-cell cytokine responses

To induce lymphocyte cytokine production, phytohaemagglutinin (PHA) (50µg/ml) or anti-CD2,1 (1:12000)+anti-CD2,2 (1:12000)+anti-CD28 (1:3000) monoclonal antibodies (anti-CD2/28 Moabs, CLB, Amsterdam, The Netherlands) were added and cultures were incubated for 48 hours at 37°C in 5% CO_2.

4.5.3 RSV-specific immune responses

Whole blood was infected with RSV at a multiplicity of infection (MOI) of 0.1 - 1.0 or control suspension added and cultures were incubated for 6 days at 37°C in 5% CO_2 (**Chapter 9**). Forty-eight hours after infection, pooled supernatants were collected for cytokine measurement. Five days after infection lymphocytes were pulsed with 0.25 μCi ^{3}H-thymidine for 18 hours and thymidine incorporation was measured (lymphoproliferative response). Stimulation index was defined as the ratio between lymphoproliferative responses in cultures stimulated with RSV and control antigen. A memory response was defined as a stimulation index ≥ 2.0.

4.6 Cytokines

4.6.1 Cytokines measured

In NPA and plasma levels of IL-4, IL-8, IL-10, IL-12 and IFN-γ were determined.
In supernatants of LPS+IFNγ stimulated blood cultures IL-12 and IL-10 were measured. In supernatants of PHA and αCD2+αCD28-stimulated cultures IFNγ and IL-4 were measured. Cytokines that were measured in supernatants of RSV-stimulated whole blood cultures were IL-4, IL-10, IL-12 and IFN-γ.

4.6.2 Cytokine assays

Concentrations of IL-4, IL-8, IL-10 and IFNγ were determined using ELISA kits supplied by the Dutch Laboratory for Blood Transfusion (CLB, Amsterdam, the Netherlands). The detection limit for IL-4 was 1 pg/ml, for IL-8 2.5 pg/ml, for IL-10 2.5 pg/ml and for IFNγ 25 pg/ml. Concentrations of IL-12 were determined using ELISA kit from R&D (Oxon, United Kingdom), the detection limit was 7.8 pg/ml. This assay recognizes only the IL-12 heterodimer and not the individual sub-units of the dimer. When cytokine values were not detectable, for statistical analysis the minimum detectable level was used. When IL-10 responses were above the maximum detectable level (300 pg/ml), this level was used.
To study possible skewing of the Th1-Th2 cytokine balance, IFN-γ/IL-4 ratios (**Chapter 6 and 11**) ratios were calculated.
When cytokine values were not detectable, the minimum detectable level was used for statistical analysis.

4.7 Follow-up evaluations

Follow-up data were collected during one year following discharge (**Chapter 10 and 11**). Follow-up was performed using diaries, which were developed for this study. One investigator instructed parents how to use the diary. Starting three weeks after discharge from the hospital, parents noted the presence of "coughing" and "wheezing" on a daily base. A disease episode was defined as the presence of symptoms for two or more consecutive days. At the end of the follow-up period, patients were classified as "recurrent wheezing" if more than one episode of wheezing was noted following discharge. In addition, at the end of the study period, one investigator contacted the general practitioners of the patients by telephone and inquired if "asthma" had been diagnosed.

4.8 Statistical analysis

Methods used to describe and analyze data are described in the "Statistical analysis" section of each study.

References

1 Van Schaik SM, Tristam DA, Nagpal IS, Hintz KM, Welliver RC, Welliver RC. Increased production of interferon gamma and cysteinyl leukotrienes in virus-induced wheezing. J Allergy Clin Immunol 1999; 103:630-636.

2 Sherran P, Jafri H, Carubelli C, Saavedra J, Johnson C, Krisher K, Sanchez PJ, Ramilo O. Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease. Pediatr Infect Dis J 1999; 18:115-122.

3 Lennette EH, Schmidt NJ. Diagnostic prodecures for viral, rickettsial and chlamydial infections. 5 ed. Washington: 1979.

5 Post-conceptional age in mechanically ventilated infants with respiratory syncytial virus lower respiratory tract infection*

L Bont, AJ van Vught, JLL Kimpen

** published in part: Prophylaxis against RSV in premature infants (Lancet 1999; 354:1003-1004).*

5.1 Introduction

Prematurity, with or without the presence of chronic lung disease, is considered a risk factor for severe RSV infection, often resulting in the need for mechanical ventilation[1]. In addition, term healthy neonates as well as infants with congenital heart disease may have severe course of disease in case of RSV infection[2;3].
New strategies have become available to prevent severe RSV infection in prematurely born infants, including palivizumab, a humanized monoclonal antibody[4]. For neonates and infants with congenital heart disease no effective prophylaxis is yet available. It is not known for how long prevention of RSV infection in preterm infants should be considered. We hypothesized that the duration of the period that children are at risk for severe RSV infection, is determined by the PCA.

5.2 Methods

We studied PCA (in weeks) on admission in all preterm infants without congenital heart disease admitted to our pediatric intensive care unit (PICU) for mechanical ventilation during a period of 5 years. Infants with chronological age > 12 months were not included. Prematurity was defined as gestational age at birth $\leq$ 36 weeks. Infants with and without a pre-existent condition were distinguished. Pre-existent condition was defined as the requirement of care by a pediatrician at any time prior to RSV infection, other than a single contact following delivery.

5.3 Results

Seventy-three infants were included, 33 were born prematurely. Gestational age in preterm infants ranged from 27-36 weeks (median 32 weeks), 8 infants had gestational age < 30 weeks. Of all preterm infants 6 had chronic lung disease. Interestingly, 29 (88%) infants had a PCA < 45 weeks at the time of intubation for RSV bronchiolitis (Figure 5.1).
Of the four preterm infants with PCA > 44 weeks, 3 had chronic lung disease.
Of 40 healthy term infants 32 (80%) infants had PCA < 45 weeks (Figure 5.2). Medical conditions found in 9 (26%) of 35 preterm infants and 11 (22%) of 51 term infants (table 5.1). Six healthy term infants had chronological age >4 weeks, who were therefore not considered neonates. PCA distribution of 11 term infants with pre-existent conditions was markedly different: only 1 infant (9%) had PCA < 45 weeks.

Figure 5.1 *Distribution of post-conceptional age (weeks) of mechanically ventilated preterm infants with RSV bronchiolitis (n=33).*

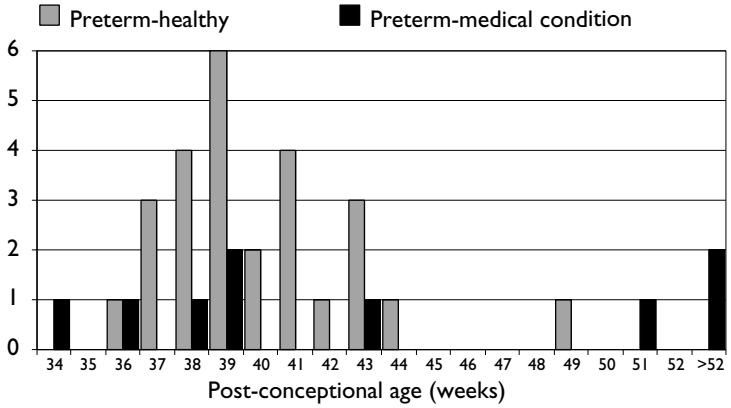
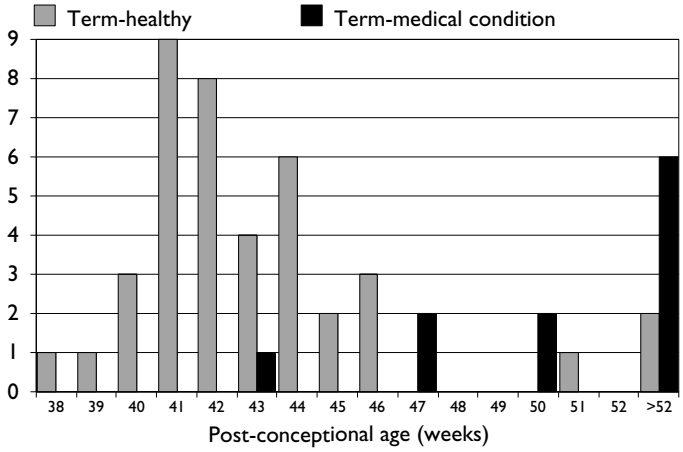


Figure 5.2 *Distribution of post-conceptional age (weeks) of mechanically ventilated term infants with RSV bronchiolitis (n= 40).*



5.4 Conclusions

The main finding of this study was that previously healthy term and preterm infants who require mechanical ventilation for RSV infection were characterized by PCA < 45 weeks. In particular, preterm infants without chronic lung disease, appear to have increased risk for severe RSV infections resulting in respiratory insufficiency, until the PCA reaches 44 weeks. Thus, the risk for severe RSV infection in preterm infants without chronic lung disease with PCA > 44 weeks is relatively low.

This study underscores the importance of maturation-related factors in the pathogenesis of severe RSV infection in infants without pre-existent conditions (reviewed in

Chapter 2). The distribution of PCA of infants with pre-existent conditions emphasized that the pathogenesis of severe RSV infection in these infants is essentially different. This group of infants is characterized by a variety of cardiac, pulmonary and other diseases, which apparently facilitate the development of severe disease in case of RSV infection.
The most important consequence of this study is that prevention of RSV infection in preterm infants without chronic lung disease is most effective in children with PCA < 45 weeks. It remains to be studied whether current prophylactic strategies can effectively prevent severe RSV in healthy term infants with PCA < 45 weeks. We conclude that future strategies to prevent severe RSV infection during infancy should focus on a small subgroup of infants, with PCA < 45 weeks.

Table 5.1 *Pre-existent medical conditions in mechanically ventilated RSV-infected infants. Pre-existent medical condition was defined as pediatric care before RSV infection, except single contact following delivery.*

Diagnosis	Preterm infants (n=9)	Term infants (n=11)
Chronic lung disease	6	
Congenital heart disease	2	3
Down syndrome	1	2
Pierre-Robin malformation		1
Diaphragm paralysis (previously diagnosed)		2
Klebsiella urosepsis 2 weeks prior to RSV infection		1
Neurologic disease after severe birth asphyxia		1
Transfusions for severe thrombocytopenia		1

References

1 Wang EEL, Law BJ, Stephens D. Pediatric Investigators Collaborative Network on infections in Canada (PICNIC) prospective study of risk factors and outcomes in patients hospitalized with respiratory syncytial viral lower respiratory tract infection. J Pediatr 1995; 126:212-219.
2 Hall CB, Kopelman AE, Douglas RG, Geiman JM, Meagher MP. Neonatal respiratory syncytial virus infection. N Engl J Med 1979; 300:393-396.
3 McDonald NE, Hall CB, Suffin SC, Alexon C, Harris PJ, Manning JA. Respiratory syncytial virus infection in infants with congenital heart disease. N Engl J Med 1982; 307:397-400.
4 The IMpact-RSV study group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 1998; 102(3):531-537.

6 Association of peripheral blood cytokine responses with disease severity in respiratory syncytial virus bronchiolitis

L Bont, CJ Heijnen, A Kavelaars, WMC van Aalderen, F Brus, JThM Draaisma, SM Geelen, HJ van Vught, JLL Kimpen

Eur Respir J 1999;14:144-149

6.1 Abstract

The role of cellular immunity in disease severity in RSV bronchiolitis is largely unknown. We investigated the association between disease severity and systemic cytokine responses in hospitalized ventilated and non-ventilated RSV bronchiolitis patients.
In whole blood cultures stimulated with phytohemagglutinin (PHA), we measured lymphoproliferative responses as well as interferon-γ (IFNγ) and Interleukin-4 (IL-4) production during acute illness. In addition plasma cytokines were measured. Measurements were repeated in the convalescent phase, 3-4 weeks after admission.
Fifty patients were included. The median age in ventilated patients was significantly lower than in non-ventilated patients (1 vs. 4 months, $p<0.05$). In comparison with non-ventilated patients, the ventilated patients had significantly lower lymphoproliferative responses and lower production of IFNγ and IL-4. In fact, IFNγ and IL-4 production in ventilated patients was almost completely undetectable. Plasma IL-8 levels in ventilated patients were significantly higher than in non-ventilated patients. In the convalescent phase lymphoproliferative and cytokine responses as well as plasma IL-8 levels were normal in both patient groups. Since RSV bronchiolitis is associated with the subsequent development of asthma, we investigated possible skewing of the Th1-Th2 cytokine balance. This was found neither in the acute nor in the convalescent phase.
Our data indicate that depressed lymphocyte function and elevated plasma IL-8 levels are markers of severe disease. We suggest that age and maturation related immune mechanisms could explain the occurrence of severe RSV bronchiolitis requiring mechanical ventilation in young infants.

6.2 Introduction

Respiratory syncytial virus (RSV) bronchiolitis accounts for a considerable number of pediatric intensive care admissions during yearly winter epidemics. Risk factors predicting a complicated course in RSV lower respiratory tract illness, eventually necessitating ventilatory support, include congenital heart disease, prematurity, age under six weeks and chronic lung disease [1-3].

Although knowledge of the immunopathogenesis of RSV bronchiolitis has improved over the last decades, little is known about the association between immunological parameters and disease severity [4,5]. To our knowledge RSV-specific IgE titers, histamine release and eosinophil cationic protein titers in respiratory secretions and eosinophil blood counts are the only immunological phenomena associated with disease severity [6,7].

Cellular immunity is the classical defense mechanism against viral infections. However, the role of cellular immunity in the immunopathogenesis of RSV bronchiolitis is unclear. In murine models cytotoxic T cells have been associated with both clearance of virus from the lungs and augmentation of lung pathology [8,9]. Others have suggested CD4 cells are required for the development of immune mediated lung disease in RSV infected BALB/c mice [10]. In the human population even less is known about cellular immunity in RSV infection than in animal models. Virus-specific cytotoxicity has been demonstrated in primary acute infections and appeared to be an important parameter in recovery [11]. Furthermore, it has been suggested that in RSV infected patients with lower respiratory tract symptoms, lymphoproliferative responses to phytohemagglutinin (PHA) are depressed, as are IL-4, IFNα and IFNγ production in vitro [12-14]. In particular these depressed lymphocyte functions suggest that suboptimal cellular immunity might play a role in the outcome of RSV infections. However, thus far no association has been demonstrated between cell-mediated immune responses and disease severity.

RSV bronchiolitis has been related to the subsequent development of asthma [15]. Welliver et al showed that RSV can induce virus-specific IgE, which correlates with recurrent episodes of wheezing after RSV infection [6,16]. More recently it has been shown that subjects with asthma have increased release of Th2 cytokines, including IL-4, and normal or low production of the Th1 cytokine IFNγ [17]. Increased IL-4/IFNγ ratios have also been mentioned in the RSV infection and could therefore explain the association between RSV infection and the subsequent development of asthma [13].

We compared lymphoproliferative and cytokine responses in ventilated and non-ventilated children admitted with RSV bronchiolitis in order to determine a possible role for cellular immunity in disease severity. In addition we investigated possible skewing of the Th1-Th2 cytokine balance in the course of the disease.

6.3 Methods

6.3.1 Selection of patients

Children were included during one winter epidemic in 4 hospitals in the Netherlands (Wilhelmina Children's Hospital, Utrecht; Beatrix Children's Hospital, Groningen; St. Elisabeth Hospital, Tilburg; Rijnstate Hospital, Arnhem). Inclusion criteria were: hospital admission, lower respiratory tract symptoms, age < 13 months and positive direct

immunofluorescence for RSV. Patients with severe illness and with non-severe illness were distinguished by the need for mechanical ventilation. Control children aged < 13 months without evidence of allergy or infection were selected for this study during the same winter season. Included were infants prior to minor surgery, prior to cardiac surgery in the absence of hemodynamic compromise, healthy prematurely born infants, healthy infants screened for congenital disorders and infants with mild anemia. This study was approved by the Medical Ethical Committee in all participating centers and parents of subjects and controls gave written informed consent.

6.3.2 Collection of materials

Within 24 hours after admission venous or arterial blood was taken from all patients. From all subjects blood was obtained in tubes containing ethylenediamine tetracetic acid (EDTA). Tubes were directly put on ice, plasma was separated and stored at -70°C. In addition white cell counts were performed (Technicon H1, Bayer Technicon, New York). From subjects in the Wilhelmina Children's Hospital, Utrecht, heparinized blood was taken simultaneously. Three weeks later, in the convalescent phase, heparinized blood and plasma were taken from all subjects.

6.3.3 Cell cultures

Heparinized blood (0.5 ml) was diluted in 4.5 ml RPMI 1640 medium (Life Technologies, Grand Island, NY) and whole blood cultures were performed. Diluted blood was cultured in four-fold in 96-well culture plates (Nunc International, Denmark) in the presence of PHA (50μg/ml) for 48 hours at 37°C with 5% CO_2. Pooled supernatants were kept at -70°C. Subsequently lymphocytes were pulsed with 0.25 μCi ^{3}H-thymidine for 18 hours and thymidine incorporation was measured (lymphoproliferative response).

6.3.4 Cytokine assays

In plasma IL-4, IL-8, IL-12 and IFNγ were measured. In supernatants IL-4 and IFNγ were measured. Concentrations of IL-4 (in supernatants), IL-8 and IFNγ were determined using ELISA kits supplied by the Dutch Laboratory for Blood Transfusion (CLB, Amsterdam, the Netherlands), the detection limit for IL-4 was 4.7 pg/ml, for IL-8 2.5 pg/ml and for IFNγ 25 pg/ml. Plasma concentrations of IL-4 and IL-12 were determined using "high sensitivity" ELISA kits from R& D (Oxon, United Kingdom), the detection limit for IL-4 was 1.0 pg/ml and for IL-12 0.78 pg/ml. When cytokines were not detectable, the minimum detectable level was used in the calculations.

6.3.5 Statistical analysis

Lymphoproliferative responses are expressed as mean ± SEM. Mann-Whitney U analysis was used to compare median age of patients and controls and of ventilated and non-ventilated patients. Chi square test was used to compare relative proportions of prematurely born children. One-way ANOVA was used to compare lymphoproliferative responses. Cytokine levels were analyzed by one-way ANOVA after logarithmic transformation. If one-way ANOVA analysis showed significant differences, unpaired T-tests using Bonferroni correction were performed in order to analyze which groups have significantly different values and to calculate p-values. Pearson's correlation coefficient

was used to evaluate the relation between age and lymphoproliferative responses and cytokine responses.

6.4 Results

6.4.1 Study subjects

Characteristics of patients and controls are shown in table 6.1. A total of 50 patients were studied, 27 subjects were admitted to Wilhelmina Children's Hospital and 23 to one of the other hospitals. Twenty-nine (58%) were boys, median age was 3 months. Fourteen subjects (28%) needed mechanical ventilation. Median age of ventilated patients was 1 month, of non-ventilated patients 4 months ($p<0.05$). Eleven patients (22%), including 3 pairs of twins, were born prematurely (range: 29 4/7 – 36 5/7 weeks). Eight of these required ventilatory support, whereas in one of these children chronic lung disease was diagnosed. None of the patients had cardiac disease. None of the patients received ribavirin or systemic corticosteroids. All patients survived. Twenty-seven controls were included, median age was 4 months. Sixty-three per cent were boys. Two controls were born prematurely.

Table 6.1 *Study population characteristics*

	Patients			Controls
	ventilated (n=14)	non-ventilated (n=36)	total (n=50)	(n=27)
median (range) age (months)	1 (0-11)	4 (0-12)*	3 (0-12)	4 (0-12)
male/female	10/4	19/17	29/21	17/10
prematurely born	8	3*	11	2

* ventilated vs non-ventilated $p<0.05$

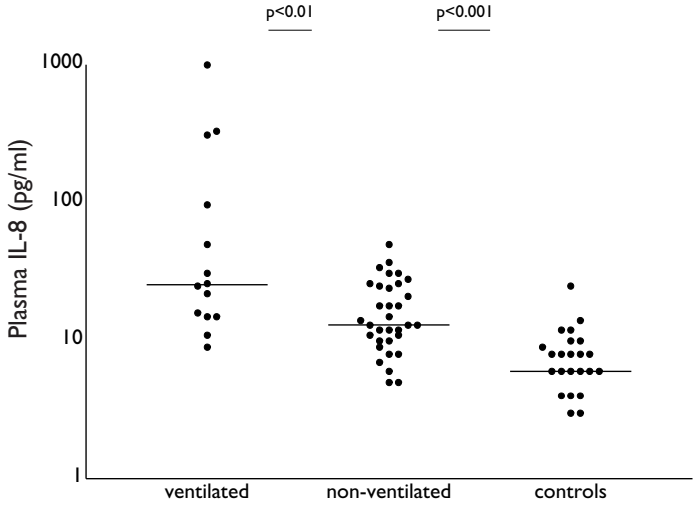
6.4.2 Cytokine levels in plasma

Plasma IL-8 levels were measured in 50 patients and in 27 controls. In the acute phase IL-8 levels were detectable in 48 patients (96%) and in 25 controls (93%). Plasma IL-8 levels in both ventilated and non-ventilated patients were higher than in controls ($p<0.001$) (Figure 6.1). In ventilated patients IL-8 levels were higher than in non-ventilated patients ($p<0.01$). Interleukin-8 levels in the convalescent phase were not significantly different from controls. Interleukin-4, IL-12 and IFNγ levels in plasma were below the detection level.

6.4.3 Lymphoproliferative response PHA stimulated whole blood cultures

White blood cell counts, relative and absolute lymphocyte numbers of ventilated patients did not differ significantly from non-ventilated patients, although absolute lymphocyte numbers in ventilated patients tended to be lower than in non-ventilated patients ($3{\cdot}10^9$/L vs. $4.2{\cdot}10^9$/L). Lymphoproliferative responses in the acute phase are shown in Figure 6.2. Mean lymphoproliferative responses in ventilated patients and non-ventilated

Figure 6.1 *Plasma IL-8 levels within 24 hours after admission in ventilated patients, non-ventilated patients and controls*

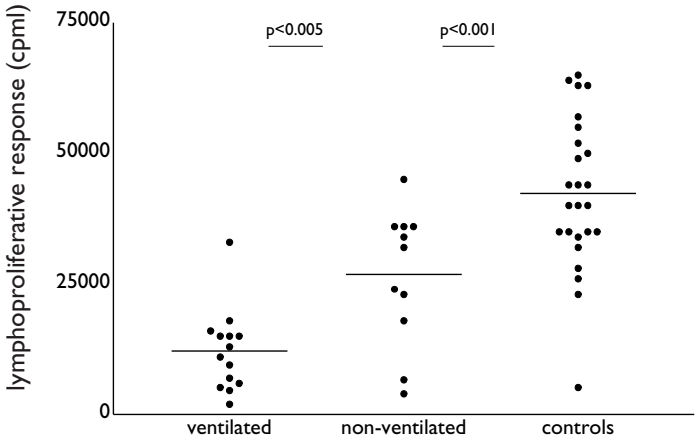


patients were lower than in controls (14000±2500 and 29000±4500 vs 42000±2800 cpm, $p<0.001$). Lymphoproliferative responses in ventilated patients were lower than in non-ventilated patients ($p<0.005$). In the convalescent phase lymphoproliferative responses returned to normal values in both ventilated and non-ventilated patients.
We related age to lymphoproliferative responses in the control infants, no association was found (data not shown).

6.4.4 Cytokine production in PHA stimulated whole blood cultures

Levels of IL-4 and IFN$_\gamma$ in the acute phase in supernatants of PHA stimulated whole blood cultures are shown in Figure 6.3a and 6.3b. In ventilated patients IFNγ and IL-4 levels were below detection level in 11 out of 13 cases (85%), in non-ventilated patients and in controls cytokine levels could be measured in all samples ($p<0.001$). In non-ventilated patients no significant differences with controls were found for both and IL-4. In the convalescent phase IFN$_\gamma$ and IL-4 levels were normal in both patients groups.
We related age to IL-4 and IFNγ responses in control infants (data not shown). Age was not related to IL-4 responses. A positive correlation was found between age and IFNγ responses ($r=0.52$, $p<0.01$). However, nearly all IFNγ responses in ventilated patients were below the minimum IFNγ response in non-ventilated patients and control infants. We could not determine IL-4/IFNγ ratios in the acute phase in ventilated patients, since in practically all patients IL-4 and/or IFNγ levels were below detection level. IL-4/IFNγ ratios in non-ventilated patients were not different from controls. In the convalescent phase IL-4/IFNγ ratios remained normal.

Figure 6.2 *Lymphoproliferative responses induced by PHA within 24 hours after admission of ventilated patients, non-ventilated patients and controls*



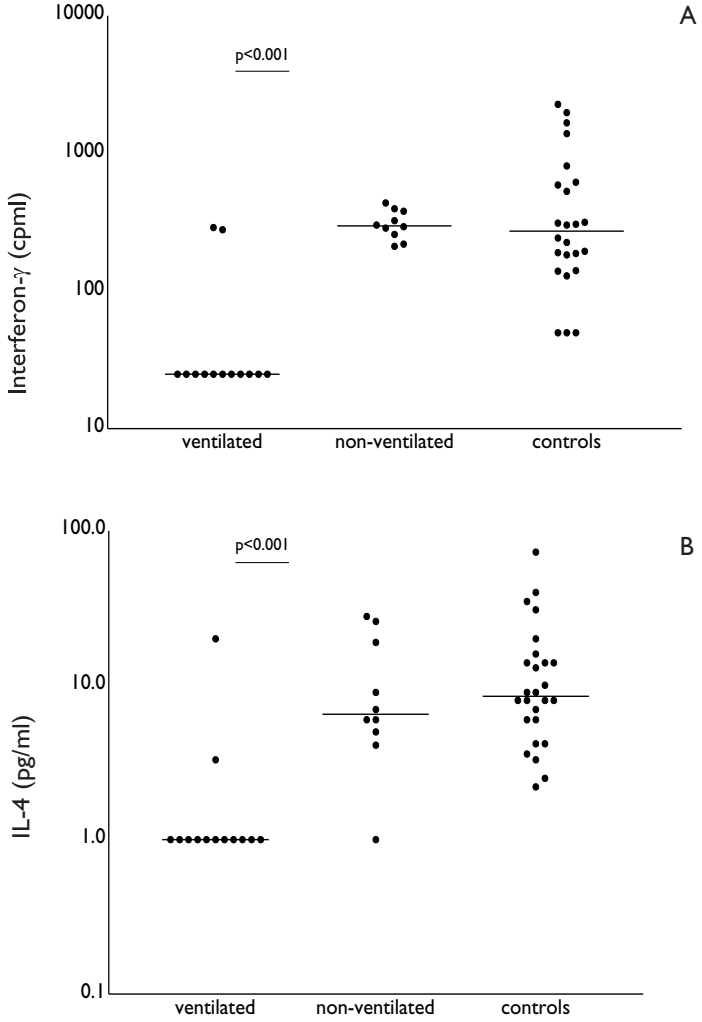
6.5 Discussion

In this study, IL-8 plasma levels, lymphoproliferative responses and lymphocyte cytokine responses in vitro were associated with disease severity in hospitalized patients with RSV bronchiolitis. Lymphoproliferative responses during acute disease are lower in ventilated patients than in non-ventilated patients. Most striking is the almost completely negative response of IFNγ and IL-4 in ventilated patients. In the convalescent phase plasma IL-8, lymphoproliferative responses and cytokine responses normalize in both ventilated and non-ventilated patients.

Young age and prematurity appeared to be important factors determining disease severity. Median age of the ventilated patients was significantly lower than that of the non-ventilated patients; except for 1 patient all ventilated patients were less than 3 months old. Furthermore, we found that more ventilated patients than non-ventilated patients were born prematurely. Both young age and premature birth are known risk factors for the development of severe bronchiolitis [18].

Age and maturation-related immune mechanisms could explain differences between ventilated and non-ventilated patients. It is generally acknowledged that neonates can experience severe morbidity from infections with viral and other intracellular pathogens, including RSV bronchiolitis [2,19-22]. In the neonatal period antiviral immunity appears to be diminished and this is accompanied by a general failure of neonatal anti-viral T cells to mature into protective virus-specific memory [23,24]. In premature neonates this maturational deficiency is even more pronounced. Severely decreased expression of CD40 ligand on activated T cells in neonates could play a role in this maturational failure [25]. Moreover it was demonstrated that neonatal T cells have lower capacity for IFNγ production after polyclonal stimulation than adult T cells [26]. We confirmed this finding in our control group in which we found a significant correlation between IFNγ production (but not IL-4 production) and age ($r = 0.75$, $p < 0.001$) (data not shown). To our knowledge, the development of antigen-specific immune responses in human

Figure 6.3 *Interferon-γ and IL-4 production in 48 hours PHA-stimulated whole blood cultures within 24 hours after admission of ventilated patients, non-ventilated patients and controls*



neonates, including cytokine responses, has only been documented in neonatal herpes simplex virus (HSV) infection; the first 3-6 weeks after delivery, diminished antigen-stimulated IFNγ and lymphoproliferative responses were reported in HSV-infected neonates [20]. As far as RSV is concerned, in vitro studies have demonstrated that RSV-infected human adult lymphocytes show depressed proliferative responses after polyclonal stimulation [27]. Taking together these observations and our results, it appears that RSV suppresses neonatal lymphocyte function also in vivo, preventing adequate immune response to RSV proteins. Moreover we speculate that proliferation and cytokine

responses in neonatal lymphocytes may be more sensitive than more mature lymphocytes to the suppressive effect of RSV. Immature antiviral immunity and increased sensitivity to suppressive influences of RSV on lymphocyte function may explain why lymphoproliferative and cytokine responses in (younger) ventilated patients are more suppressed than in non-ventilated patients.

Our two patient groups were distinguished by the need for mechanical ventilation. The effects of mechanical ventilation on immune responses is largely unknown, but reports in laboratory animals indicate that it can change local as well as systemic inflammatory responses [28]. As a result we cannot exclude a possible influence of mechanical ventilation on the immune responses found in ventilated patients in our study. However, samples taken in the acute phase were collected within the first 24 hours after mechanical ventilation was initiated, in the majority of cases even within 2-6 hours. To our knowledge, in literature no data are available, addressing the kinetics of immune responses during the first hours of mechanical ventilation. However, it is unlikely that mechanical ventilation induced changes in immune responses in our patient group, already, during this short time interval between initiation of mechanical ventilation and blood sampling.

Asthma is characterized by a relative increase in production of Th2 cytokines (e.g. IL-4) and a relative suppression of production of Th1 cytokines (e.g. IFNγ) [15,17]. Considering the association between RSV bronchiolitis and the subsequent development of asthma we investigated possible skewing of the Th1-Th2 cytokine balance by calculating IL-4/IFNγ ratios [29]. IL-4/IFNγ ratios in both patient groups were normal in the acute and convalescent phase. However IL-4/IFNγ ratios could not be calculated for ventilated patients in the acute phase. Roman et al. reported increased IL-4/IFNγ ratios after in vitro stimulation with pokeweed mitogen in RSV infected children with lower respiratory tract symptoms [13]. This apparent contradiction may be due to different culture conditions and a higher median age in their patient group.

The association found between increased plasma IL-8 levels and severe RSV bronchiolitis, is similar to the findings by Shute, who demonstrated an association between increased serum IL-8 levels and severe asthma [30]. However, increased IL-8 plasma levels are not specific for RSV disease, since increased plasma IL-8 levels have been suggested to play a role in various respiratory diseases, possibly by similar mechanisms, including neutrophil and eosinophil attraction, IgE mediated lung disease and virus-induced respiratory inflammation [4,31-35].

During the last decades, data have become available indicating RSV bronchiolitis can be considered an immune-mediated disease [4]. Early trials with a formalin-inactivated RSV vaccine resulting in enhanced disease support this hypothesis. However, this theory does not explain the higher incidence of severe disease observed in young and/or prematurely born infants. Our findings suggest that an immunological process is not the sole factor in the pathogenesis of severe disease in RSV bronchiolitis. In young and prematurely born infants, insufficient antiviral immunity seems to play an important role, particularly with respect to disease severity.

In summary, we have shown suppressed lymphocyte function and increased plasma IL-8 levels to be markers of severe disease in RSV bronchiolitis. In addition, we propose that maturation of virus-specific immunity plays a crucial role in the outcome of RSV infection in young infants. In order to understand the pathogenesis of RSV bronchiolitis and immunological factors contributing to severity of this disease, we need to focus on the

development of the specific immune responses to RSV in preterm and term infants. Elucidating these mechanisms will be important for the development of treatment strategies and vaccines.

References

1 McDonald NE, Hall CB, Suffin SC, Alexon C, Harris PJ, Manning JA: Respiratory syncytial virus infection in infants with congenital heart disease. N Engl J Med 1982;307:397-400.

2 Hall CB, Kopelman AE, Douglas RG, Geiman JM, Meagher MP: Neonatal respiratory syncytial virus infection. N Engl J Med 1979;300:393-6.

3 Opavski MA, Stephens d, Wang EEL: Testing models predicting severity of respiratory syncytial virus infections on the PICNIC RSV database. Pediatric Investigators Collaborative Network on Infections in Canada. Arch Pediatr Adolsc Med 1995;149:1217-20.

4 Kimpen JLL: Respiratory syncytial virus immunology. Pediatr Allergy Immunol 1996;7 (suppl 9):86-90.

5 Openshaw PJM: Immunopathological mechanisms in respiratory syncytial virus disease. Springer Semin Immunopathol 1995;17:187-201.

6 Welliver RC, Wong DT, Sun M, Middleton EJR, Vaughan RS, Ogra PL: The development of respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection. N Engl J Med 1981;305:841-6.

7 Garofalo RP, Ahlstedt DA, Welliver RC: Peripheral blood eosinophil counts and eosinophil cationic protein content of respiratory secretions in bronchiolitis: relationship to severity of disease. Pediatr Allergy Immunol 1994;5:111-7.

8 Graham BS, Bunton LA, Wright PF, Karzon DT: Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991;88:1026-33.

9 Hussell T, Spender IC, Georgiou A, O'Garra A, Openshaw PJM: Th1 and Th2 cytokine induction in pulmonary T cells during infection with respiratory syncytial virus. J General Virol 1996;77:2447-55.

10 Connors M, Kulkarni AB, Firestone CY, Holmes KL, Morse HC, Sotnikov AV, Murphy BR: Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formaline-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J Virol 1992;66:7444-50.

11 Isaacs D, Bangham CRM, McMichael AJ: Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet 1987;769-71.

12 Welliver RC, Kaul A, Ogra PL: Cell-mediated immune response to respiratory syncytial virus infection: relationship to the development of reactive airway disease. J Pediatr 1979;94:370-5.

13 Roman M, Calhoun WJ, Himton KL, et al. respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am J Respir Crit Care Med 1997;156:190-5.

14 Isaacs D: Production of interferon in respiratory syncytial virus bronchiolitis. Arch Dis Child 1989;64:92-5.

15 Folkerts G, Busse WW, Nijkamp FP, Sorkness RL, Gern JE: Virus-induced airway hyperresponsiveness and asthma. Am J Respir Crit Care Med 1998;157:1708-20.

16 Welliver RC, Duff AL: The relationship of RSV-specific immunoglobulin E-antibody responses in infancy, recurrent wheezing and pulmonary function at age 7-8 years. Pediatr Pulmonol 1993;15:19-27.

17 Umetsu dt, DeKruyff RH: Th1 and Th2 cd4+ cells in human allergic diseases. J Allergy Clin Immunol 1997;100:1-6.

18 Madge P, Paton JY, McColl JH, Mackie PLK: Prospective controlled study of four infection-control procedures to prevent nosocomial infection with respiratory syncytial virus. Lancet 1992;340:1079-83.

19 Wilson CB: Immunological basis for increased susceptibility of the neonate to infection. J Pediatr 1986;108:1-12.

20 Burchett SK, Corey L, Mohan KM, Westall J, Ashley R, Wilson CB: Diminished interferon-gamma and lymphocyte proliferation in neonatal and postpartum primary herpes simplex virus infection. J Infect Dis 1992;165:813-8.

21 Starr SE, Toplin MD, Friedman HM: Impaired cellular immunity to cytomegalovirus in congenitally infected children and their mothers. J Infect Dis 1979;140:500-5.

22 Smith S, Jacobs RF, Wilson CB: Immunobiology of childhood tuberculosis: a window on the ontogeny of cellular immunity. J Pediatr 1997;131:16-26.

23 Holt PG: Postnatal maturation of immune competence during infancy and childhood. Pediatr Allergy Immunol 1995;6:59-70.
24 Hayward AR, Groothuis JR: Development of T cells with memory phenotype in infancy. Adv Exp Med Biol 1991;310:71-6.
25 Nonoyama S, Penix LA, Edwards CE: Dimished expression of CD40 ligand by activated neonatal T cells. J Clin Invest 1995;95:66-75.
26 Lewis DB, Yu CC, Meyer J, English BK, Kahn SJ, Wilson CB: Cellular and molecular mechanisms for reduced interleukin 4 and interferon gamma production by neonatal T cells. J Clin Invest 1991;87:194-202.
27 Roberts NJ: Different effects of influenza virus, respiratory syncytial virus, and sendai virus on human lymphocytes and macropohages. Infection and Immunity 1982;35:1142-6.
28 Tremblay L, Valenza F, Ribeiro SP, Li J, Slutsky AS: Injurious ventilatory strategies increases cytokines and c-fos mRNA expression in an isolated rat model. J Clin Invest 1997;99:944-52.
29 Sims DG, Downham MA, Gardner PS: Study of 8-year-old children with a history of respiratory syncytial virus bronchiolitis in infancy. BMJ 1978;1:11-7.
30 Shute J, Vrugt B, Lindley IJD, Holgate ST, Bron A, Aalbers R, Djukanovic R: Free and complexed interleukin-8 in blood and bronchial mucosa in asthma. Am J Respir Crit Care Med 1997;153:1877-83.
31 Kotecha S, Chan B, Azam N, Silverman M, Shaw RJ: Increase in interleukin-8 and soluble intercellular adhesion molecule-1 in bronchoalveolar lavage fluid from premature infants who develop chronic lung disease. Arch Dis Child 1995;72:F90-6.
32 Erger RA, Casale T, B. Interleukin-8 is a potent mediator of eosinophil chemotaxis through endothelium and epithelium. Am J Physiol 1995;268:L117-22.
33 Erger RA, Casale T, B. Interleukin-8 plays a significant role in IgE-mediated lung inflammation. Eur Respir J 1998;11:299-305.
34 Teran LM, Johnston SL, Schroder JM, Church MK, Holgate ST: Role of nasal interleukin-8 in neutrophil recruitment and activation in children with virus-induced asthma. Am J Respir Crit Care Med 1997;155:1362-6.
35 Arnold R, Werner F, Humbert B, Werchau H, Konig W: Effect of respiratory syncytial virus-antibody complexes on cytokine (IL-8, IL-6, TNF alpha) release and respiratory burst in human granulocytes. Immunol 1994;82:184-91.

7 Local interferon-γ levels during respiratory syncytial virus lower respiratory tract infection are associated with disease severity

L Bont, CJ Heijnen, A Kavelaars, WMC van Aalderen, F Brus, JMTh Draaisma, M Pekelharing-Berghuis, RAAM van Diemen-Steenvoorde, JLL Kimpen

J Infect Dis, in press

7.1 Abstract

To investigate the role of cell-mediated immunity during respiratory syncytial virus (RSV) infection interferon (IFN)-γ and interleukin (IL)-10 levels in nasopharyngeal secretions were measured in infants with lower respiratory tract infection (LRTI) caused by RSV. A novel technique was used to measure *in vivo c*ytokine levels in nasopharyngeal aspirates (NPA). Cytokine levels in NPA of 17 mechanically ventilated infants and 43 non-ventilated hospitalized infants were compared. As expected, mechanically ventilated infants were significantly younger than non-ventilated infants (7 vs 14 weeks). IFN-g levels in NPA of mechanically ventilated infants were above detection limit in 3 infants (18%), but in 26 non-ventilated infants (60%). IL-10 levels in NPA in mechanically ventilated and non-ventilated infants were comparable. It is hypothesized that maturation-related mechanisms play a key role in the development of RSV LRTI resulting in mechanical ventilation.

7.2 Introduction

Interferon (IFN)-γ, a type II interferon, is produced by T cells and natural killer (NK) cells and has pleiotropic biological effects[1]. The properties of IFN-γ include a direct antiviral activity, help in the generation and activation of cytotoxic T cells, stimulation of antigen-presentation through induction of expression of major histocompatibility class I and II molecules and activation of NK cells[2]. In addition, IFN-γ plays a role in the regulation of the switch of antibody isotypes, including the switch in expression by B cells from IgM to IgG2a[2]. Taken together these properties, IFN-γ is considered a key cytokine in inducing protective responses against viral pathogens.
A role for IFN-γ has been mentioned in the pathogenesis of RSV infections. We have shown that severe RSV LRTI resulting in the need for mechanical ventilation is associated with low systemic proliferative responses and IFN-γ production[3]. In addition, the degree of oxygen saturation in the blood during RSV bronchiolitis was shown to be associated with decreased mRNA IFN-γ expression in the blood[4]. However, it is not certain how accurately these measurements in the blood reflect *in vivo IFN-γ* levels in the respiratory tract during RSV infection.
A few studies have investigated cytokine profiles in nasopharyngeal lavages (NPL) during RSV LRTI[5-7]. However, one of the difficulties of studying cytokine levels (NPL) is the unpredictable recovery of volumes of secretions[8]. Consequently, the precise dilution factor in NPL can not be known. In addition, NPL may lead to dilution of secretions to an extent that does not allow for detection of immune mediators that are present in low concentrations in NPL, including IFN-γ. To overcome these disadvantages of NPL, in the present study we developed a novel method to measure cytokine levels the respiratory tract. This technique was used to investigated IFN-γ levels in the nasopharynx during RSV LRTI. We tested the hypothesis that IFN-γ levels in the respiratory tract during RSV LRTI are associated with disease severity.

7.3 Methods

7.3.1 Study population

Children were included during one winter epidemic in 5 hospitals in the Netherlands. Inclusion criteria were: hospital admission, lower respiratory tract symptoms, age < 13 months and immunofluorescence for RSV infection of epithelial cells in nasopharyngeal secretions. Symptoms of LRTI were severe chest cough, wheezing, hoarseness, stridor, shortness of breath[9] as well as cyanosis and apnea. Prematurely born infants with congenital heart disease, chronic lung disease and infants with wheezing illness prior to RSV bronchiolitis were not included. Patients with severe and non-severe illness were distinguished by the need for mechanical ventilation. The study was approved by the Medical Ethical Committee in all participating centers. Parents of subjects gave written informed consent.

7.3.2 Nasopharyngeal aspirates (NPA)

Within 24 hours after admission nasopharyngeal secretions were aspirated using a 3.3 mm suction catheter (Vygon, Ecouen, France). Aspirates were placed on ice immediately and stored at -80°C. If NPA could not be obtained due to little amount of secretion

present in the nasopharynx, no second attempt was done later in the course of disease. Before cytokine measurement, the amount of NPA was weighed, diluted in dilution buffer (4°C) of the ELISA kits that were used for the cytokine assays (CLB, Amsterdam, The Netherlands), sonicated on ice for 2x6 seconds (amplitude 6 μm) and subsequently centrifuged at 13,000 rpm for 10 minutes at 4°C. For IL-8 measurement, NPA was diluted 1:15,000, for measurement of other cytokines NPA was diluted 1:15.
Cytokine levels in NPA were used as an approximation for cytokine levels in fluids of the lower airways. The potential systematic error caused by sampling secretions from the upper airways was evaluated by correlating cytokine levels (IL-8 and IL-10) in NPA and tracheobronchial aspirates (TBA)[5]. In a subset of 10 mechanically ventilated RSV bronchiolitis patients NPA and TBA were collected simultaneously. Identical methods of aspiration and subsequent analysis were used for NPA and TBA.

7.3.3 Cytokine assays

Concentrations of IL-4, IL-8, IL-10 and IFN-γ were determined using ELISA kits supplied by the Dutch Laboratory for Blood Transfusion (CLB, Amsterdam, the Netherlands). The detection limit of the assay for IL-4 was 2 pg/ml, for IL-8 2.5 pg/ml, for IL-10 2.5 pg/ml, and for IFN-γ 4 pg/ml. IL-12 concentrations were determined using ELISA kits from R&D (Oxon, United Kingdom), the detection limit for IL-12 was 7.8 pg/ml.

7.3.4 Statistical analysis

Cytokine levels in NPA had non-parametric distributions. They are expressed as median values and range. Pearson's correlation coefficient was used to analyze the relation between cytokine levels in NPA and TBA. Spearman's correlation coefficient was used to analyze the relation between IFN-γ in NPA and age. Mann-Whitney U-test was used to analyze differences in cytokine levels between ventilated and non-ventilated infants. All tests of significance were two-sided. A $p<0.05$ was considered statistically significant.

7.4 Results

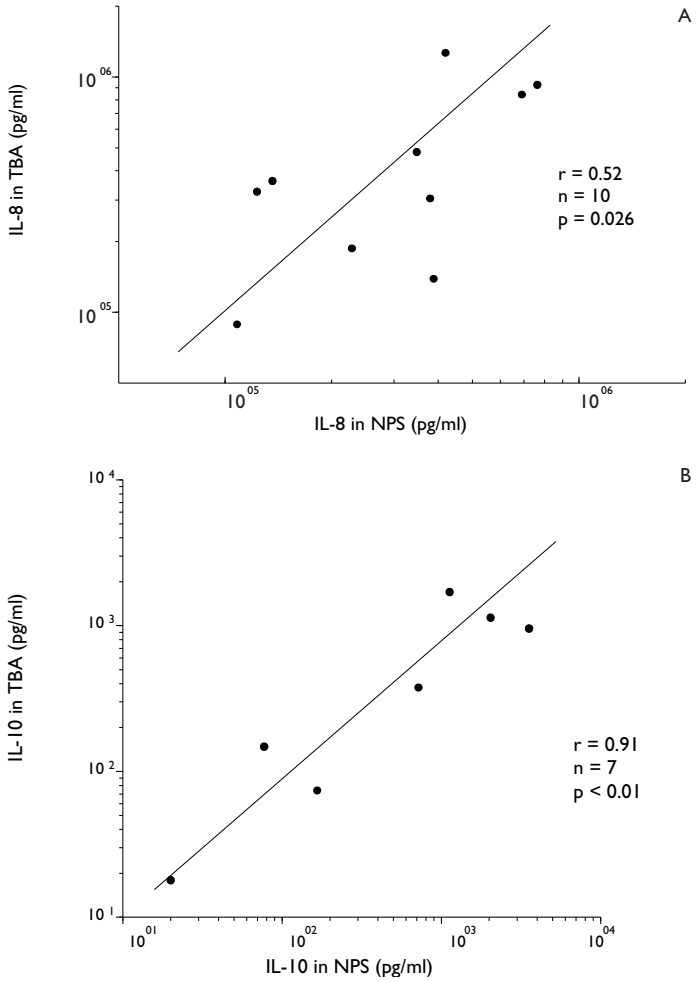
7.4.1 Subject characteristics

The investigated population consisted of 75 patients. Forty-four patients (59%) were boys, median age at the time of RSV bronchiolitis was 9 weeks. Seventeen (23%) infants were born prematurely (median gestational age 34 weeks, range: 27 - 36 weeks). Seventeen subjects (23%) needed mechanical ventilation. As expected, median age in mechanically ventilated infants was lower than in non-ventilated infants (7 vs. 14 weeks, $p=0.002$) and a significantly higher percentage was born prematurely (47 vs. 14%, $p=0.006$). None of the children had immunodeficiency. None of the prematurely born infants had received RSV prophylaxis. None of the patients received ribavirin or systemic anti-inflammatory agents, including corticosteroids. All patients survived.

7.4.2 Cytokine levels in nasopharyngeal aspirates

NPA was collected in 17 mechanically ventilated patients and in 43 non-ventilated patients. In 15 patients (20%) no NPA was obtained. In these cases insufficient amount of secretion was present in the nasopharynx to be aspirated. These 15 infants were all non-ventilated infants. IL-8 (range 5-1300 ng/ml) and IL-10 levels (range 40-3600 pg/ml) in.

Figure 7.1 *Correlation between cytokine levels in nasopharyngeal aspirates and tracheobronchial aspirates in mechanically ventilated RSV bronchiolitis patients. Pearson's correlation coefficients are shown. Correlation for IL-8 is shown in Figure 7.1a, IL-10 in Figure 7.1b. Aspirates were taken simultaneously within 24 hours after initiation of mechanical ventilation.*

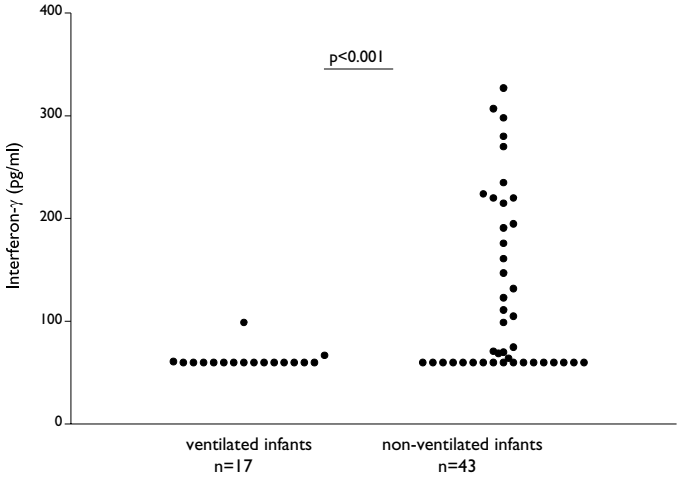


NPA were detectable in all patients. IFN-γ could be measured in NPA of 28 infants (53%). IL-4 and IL-12 levels in NPA were below detection limits in all NPA samples.

In a subgroup of 10 mechanically ventilated infants with RSV infection NPA and TBA were collected simultaneously. In 3 patients, the amount of TBA was not sufficient for IL-10 measurement. The correlation coefficient for IL-8 levels in NPA and TBA was 0.5 ($p=0.03$) ($n=10$), for IL-10 the correlation coefficient was 0.91 ($p<0.01$) ($n=7$) (Figure 7.1a and 7.1b).

Differences in cytokine levels in NPA between ventilated and non-ventilated patients were analyzed. The geometric mean of IL-8 levels in ventilated and non-ventilated patients was 214 (95% CI 148-309) respectively 166 (95% CI 129-209) ng/ml (not significant). The geometric mean of IL-10 levels in ventilated and non-ventilated patients was 347 (95% CI 159-794) respectively 447 (95% CI 309-660) pg/ml (not significant). IFN-γ levels in NPA were below detection limit in 14 of 17 mechanically ventilated patients (82%) and in 17 of 43 non-ventilated patients (40%) ($p<0.001$) (Figure 7.2).

Figure 7.2 *IFN-γ levels in nasopharyngeal aspirates of infants with (n=17) and without mechanical ventilation during RSV bronchiolitis (n=43). Aspirates were taken within 24 hours after admission. Mann-Whitney U-test was used to analyze differences in IFN-γ levels in NPA.*



To investigate whether differences in IFN-γ in NPA were explained by differences in age we analyzed the correlation between age and IFN-γ in NPA in non-ventilated infants. No correlation was found (r=-0.02, p=0.87). Therefore, we consider it unlikely that differences in age explain differences in IFN-γ in NPA.

7.5 Discussion

The main finding of this study was that *in vivo* IFN-γ levels in NPA were severely decreased in mechanically ventilated infants with RSV LRTI as compared with hospitalized non-ventilated infants with RSV LRTI who did not required mechanical ventilation. This is the first study to actually measure local IFN-γ levels during RSV LRTI.
The method to measure cytokine profiles in nasopharyngeal secretion that has been used most widely is the nasopharyngeal lavage[10]. As a result of the unpredictable recovery of volumes of secretions the dilution factor in NPL is not known. In a group of 52 infants with upper respiratory tract infections, estimated dilution factors in nasal lavage fluid varied widely from 1.8 to 432[8]. Correction for the dilution by normalizing cytokine levels to albumin concentrations in nasopharyngeal lavage appears no option, because

albumin levels also increase during upper respiratory tract illness due to increased vascular permeability[10]. This implies that only ratios between cytokines, but not cytokine concentrations, can accurately be established in nasopharyngeal lavages. Moreover, large dilutions potentially result in undetectable levels of cytokines which are present in relatively low concentrations, including IL-10 and IFN-γ[7]. In the present study this drawback of nasopharyngeal lavages was overcome, although IFN-γ levels in NPA were still below detection limits in practically all mechanically ventilated infants. Other disadvantages of NPA can be considered. It is conceivable that nasopharyngeal lavage contains cytokines from the epithelial fluid lining the nasopharynx, which may not be found in NPA. These cytokines could potentially be most relevant. Another drawback of NPA is that it can only be performed if nasal secretions are present in amounts that can be aspirated. Consequently, in the present study NPA could not be taken from infants with small amounts of nasal secretions. In addition, a pilot study in control infants showed that the amount of secretions in healthy infants was insufficient to be aspirated. The validity of NPA was estimated by comparing cytokine levels in NPA and TBA[7]. A high correlation between cytokine levels in NPA and TBA was found, indicating the similarity between cytokine profiles in NPA and the lower respiratory tract. Taken into account its disadvantages, NPA appears a suitable and easy method to study cytokine levels which are present at low levels in the respiratory tract.

Evidence exists that insufficient antiviral immunity plays a role in the pathogenesis of severe RSV LRTI. It was shown that viral titers found in nasopharyngeal samples from RSV infected infants are associated with disease severity and are highest in mechanically ventilated infants[11;12]. Hall and colleagues already speculated that direct antiviral activity of interferons as well as cell-mediated immunity may be important in the pathogenesis of RSV LRTI. Indirect evidence for this hypothesis came from more recent studies that showed that RSV LRTI is associated with decreased IFN-γ production by peripheral blood mononuclear cells[3;4]. The results of the present study directly confirm that severe RSV LRTI is associated with reduced local levels of IFN-γ.

Mechanically ventilated infants with RSV LRTI were significantly younger than non-ventilated infants. Therefore, it is conceivable that maturation-related mechanisms may explaining low systemic as well as local IFN-γ production in the respiratory tract of mechanically ventilated infants during RSV LRTI. Indeed, T cell-mediated responses are delayed during the first 4 to 8 weeks of postnatal age, accompanied by impaired capacity to produce IFN-γ[13]. In addition to maturation-related mechanisms, reduced IL-12 production by antigen-presenting cells may explain low IFN-γ levels in NPA of mechanically ventilated infants during RSV LRTI. IL-12 is a prerequisite for IFN-γ production by T cells and NK cells[1]. In line with this hypothesis, we have previously shown that monocyte IL-12 production is inversely related to duration of mechanical ventilation during RSV LRTI[14].

In summary, NPA is a novel technique to detect *in vivo* cytokine levels in the respiratory tract during RSV LRTI. We have shown decreased IFN-g levels in NPA during RSV LRTI in infants with most severe disease. This finding strongly suggests an important role for IFN-γ in the pathogenesis of RSV LRTI.

References

1 Young HA, Hardy KJ. Role of interferon-gamma in immune cell regulation. J Leukoc Biol 1995; 58:373-381.

2 Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol 1997; 15:749-795.

3 Bont L, Heijnen CJ, Kavelaars A, Aalderen vWMC, Brus F, Draaisma JMTh, Geelen SM, Vught vHJ, Kimpen JLL. Association of peripheral blood cytokine responses with disease severity in respiratory syncytial virus bronchiolitis. Eur Respir J 1999; 14:144-149.

4 Bendelja K, Gagro A, Bace A, Lokar-Kolbas R, Krsulovic-Hresic V, Drazenovic V, Mlinaric-Galinovic G, Rabatic S. Predominant type-2 response in infants with respiratory syncytial virus (RSV) infection demonstrated by cytokine flow cytometry. Clin Exp Immunol 2000; 121:332-338.

5 Sherran P, Jafri H, Carubelli C, Saavedra J, Johnson C, Krisher K, Sanchez PJ, Ramilo O. Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease. Pediatr Infect Dis J 1999; 18:115-122.

6 Lindgren C, Grogaard J. Reflex apnoea response and inflammatory mediators in infants with respiratory tract infection. Acta Paediatr 1996; 85:798-803.

7 Van Schaik SM, Tristam DA, Nagpal IS, Hintz KM, Welliver RC, Welliver RC. Increased production of interferon gamma and cysteinyl leukotrienes in virus-induced wheezing. J Allergy Clin Immunol 1999; 103:630-636.

8 Heikkinen T, Shenoy M, Goldblum RM, Chonmaitree T. Quantification of cytokines and inflammatory mediators in samples of nasopharyngeal secretions with unknown dilution. Pediatr Res 1999; 45:230-234.

9 Stein RT, Sherill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13. Lancet 1999; 354:541-545.

10 Noah TL, Henderson FW, Wortman IA, Devlin RB, Handy H, Koren HS, Becker S. Nasal cytokine production in viral acute upper respiratory infection of childhood. J Infect Dis 1995; 171:584-592.

11 Buckingham SC, Bush AJ, Devincenzo JP. Nasal quantity of respiratory syncytial virus correlates with disease severity in hospitalized infants. Pediatr Infect Dis J 2000; 19:113-117.

12 Hall CB, Douglas-RG J, Geiman JM. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. J Pediatr 1976; 89:11-15.

13 Chandwani S, Borkowski W, Krasinski K, Lawrence R, Welliver RC. Respiratory syncytial virus infection in human immunodeficiency virus-infected children. J Pediatr 1990; 117:251-254.

14 Bont L, Kavelaars A, Heijnen CJ, van Vught AJ, Kimpen JL. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. J Infect Dis 2000; 181:1772-1775.

8 Monocyte interleukin-12 production inversely related to duration of respiratory failure in RSV bronchiolitis

L Bont, A Kavelaars, CJ Heijnen, AJ van Vught, JLL Kimpen

J Infect Dis 2000;181:1772-1775

8.1 Abstract

To estimate factors determining the clinical outcome of respiratory syncytial virus (RSV) bronchiolitis necessitating mechanical ventilation, it was attempted to correlate clinical and immunological parameters to duration of respiratory failure. At initiation of mechanical ventilation interleukin (IL)-12 and IL-10 production was measured in 48-hour peripheral blood cell cultures, stimulated with lipopolysaccharide (LPS) + interferon (IFN)-γ in 30 RSV patients. The ventilation index (VI) correlated with the duration of mechanical ventilation ($r = 0.47$ days/(mmHg•cmH20/min), $p = 0.013$). Age was not associated with duration of mechanical ventilation. A highly significant inverse correlation was found between IL-12 production at admission and duration of mechanical ventilation ($r = -0.62$ days/pg/ml, $p<0.001$). This correlation was independent of ventilation index. No correlation was found between IL-10 production and duration of mechanical ventilation. It is hypothesized that low monocyte IL-12 response during initial RSV infection adversely affects clinical outcome of patients with severe RSV bronchiolitis.

8.2 Introduction

Severe RSV bronchiolitis, resulting in respiratory insufficiency, is an important cause of admission to Pediatric Intensive Care Units (PICU). Although the mortality rate has been reduced considerably, the clinical course of RSV bronchiolitis on PICU varies. Analysis of factors contributing to duration of mechanical ventilation may yield predictors of the clinical course of RSV bronchiolitis on PICU.

Age < 6 weeks, prematurity with or without chronic lung disease and congenital heart disease are risk factors for admission to an intensive care unit and for respiratory insufficiency during an episode of RSV-induced bronchiolitis[1]. We have recently shown that prematurity without chronic lung disease is a risk factor for respiratory insufficiency until post-conceptional age reaches 44 weeks[2]. The ventilation index, a measure of ventilatory disorder has been shown to have prognostic value in mechanically ventilated infants with ARDS[3]. It is not known whether ventilation index is related to duration of mechanical ventilation in RSV bronchiolitis. In addition, it has not been described that monocyte cytokine responses, which probably play a role in the pathogenesis of viral infections, have prognostic value in the prediction of the duration of respiratory insufficiency during RSV bronchiolitis.

IL-12 is a cytokine produced primarily by antigen-presenting cells (APC)[4]. It strongly promotes the differentiation of naive CD4+ T cells to the Th1 phenotype suggesting a critical role for IL-12 as regulator of Th1-driven immune responses, including lytic functions of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. Furthermore, IL-12 greatly enhances the capacity of T cells and NK cells to produce IFNγ and IL-2. Finally, in animal models endogenous IL-12 has been shown to induce antiviral immune responses. In this study we investigated the relationship between clinical parameters and duration of mechanical ventilation in RSV bronchiolitis patients. In addition, we explored the relationship between monocyte IL-12 production and duration of mechanical ventilation in RSV bronchiolitis.

8.3 Methods

8.3.1 Clinical characteristics

Infants <13 months old with respiratory insufficiency, resulting in the need for mechanical ventilation and proven RSV infection (by immunofluorescence on nasopharyngeal secretions) were included during two winter epidemics. Post-conceptional age (weeks) was defined as gestational age at birth (weeks) plus chronological age (weeks). Underlying diseases, including chronic lung disease and cardiac disease, were registered. Ventilation indices (VI) were calculated every eight hours during the first 24 hours of mechanical ventilation, starting eight hours after initiation of mechanical ventilation. The following formula was used to calculate VI: VI=partial pressure of arterial CO_2 (mm Hg) • peak airway pressure (cm H_2O) • respiratory rate (breaths/min)/1000)[3]. The mean VI was defined as the arithmetic mean of the VI at 8, 16 and 24 hours after initiation of mechanical ventilation. Duration of mechanical ventilation was the main outcome parameter. This study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht, The Netherlands. Parents of subjects gave written informed consent.

8.3.2 Collection of materials

Within 24 hours after admission heparinized venous or arterial blood was taken from all patients for whole blood cultures. Simultaneously, routine leucocyte counts were performed. In addition, blood was obtained in tubes containing ethylenediamine tetracetic acid (EDTA). Tubes were directly put on ice, plasma was separated and stored at -70°C.

8.3.3 Whole blood cultures

Freshly taken heparinized blood was diluted 1:10 in RPMI 1640 medium (Life Technologies, Grand Island, NY) and aliquoted (150 µl) into 96-well culture plates (Nunc International, Denmark). To induce monocyte IL-10, IL-12 and IL-8 production, lipopolysaccharide (LPS) (100ng/ml)+IFNγ (20 ng/ml) was added and cultures were incubated for 48 hours at 37°C in 5% CO_2. It has been established that monocytes are the main producers of IL-10 and IL-12 in LPS stimulated whole blood cultures[5]. The number of monocytes per well was defined as the product of total number of leucocytes per well and the percentage monocytes of total leucocytes. All cultures were performed in quadruplicate. Pooled supernatants were kept at -70°C.

8.3.4 Cytokine assays

Interleukin-10 and IL-12 were measured in supernatants of LPS+IFNγ stimulated blood cultures. In plasma IL-8 levels were measured. Concentrations of IL-10 and IL-8 were determined with ELISA kits supplied by the Dutch Laboratory for Blood Transfusion (CLB, Amsterdam, the Netherlands). The detection limit for IL-10 was 2.5 pg/ml, for IL-8 1.2 pg/ml. Concentrations of IL-12 were determined with an ELISA kit from R&D (Oxon, United Kingdom), the detection limit was 7.8 pg/ml. When IL-12 concentrations were below detection limit, the lower limit of detection was used for statistical analysis.

8.3.5 Statistical analysis

Cytokine production, plasma IL-8 levels and duration of mechanical ventilation were analyzed after logarithmic transformation. Cytokine production per monocyte was defined as the ratio between cytokine production per well and number of monocytes per well.

Chronological age, post-conceptional age, mean VI, production of separate cytokines and plasma IL-8 levels were analyzed separately in relation to duration of mechanical ventilation by univariate linear regression.

Three parameters with the highest regression coefficients were analyzed with multiple linear regression analysis by a stepwise forward-entry procedure. Selection criterion for entry was $p<0.10$. No other parameters were entered in the multiple linear regression model because of the limited number of patients. The goodness of fit of the model was estimated by the square of the multiple correlation coefficient (R^2).

8.4 Results

8.4.1 Patient characteristics

The patient population consisted of 30 infants. Nineteen patients (63%) were boys, median age was 5 weeks (range 0-50 weeks). Median post-conceptional age was 41 weeks.

Thirteen infants (43%) were born prematurely, all had post-term age < 5 weeks. One 11 month old girl had chronic lung disease resulting from mechanical ventilation in the neonatal period. None of the patients received ribavirin or systemic corticosteroids. All patients recovered.

8.4.2 Relation between clinical parameters and duration of mechanical ventilation

The main outcome parameter of this study was duration of mechanical ventilation. Median duration of mechanical ventilation was 8 days (range 4-38 days). No relation was found between sex, chronological or post-conceptional age and duration of mechanical ventilation (table 8.1). The mean ventilation index VI was 39 (range 21-75) mmHg•cmH_2O/min (n=28). A positive correlation was found between mean VI and duration of mechanical ventilation (r = 0.47 days/(mmHg•cmH20/min), p = 0.013).

Table 8.1 *Univariate linear regression analyis of candidate predictors for duration of mechanical ventilation (days)**

Parameter	Regression coefficient	p-value
Chronological age (weeks)	0.13	0.51
Post-conceptional age (weeks)	0.10	0.59
Mean VI (mmHg•H2O/min)	0.47	0.013
Plasma IL-8 (pg/ml)*	0.11	0.67
IL-12 production (pg/ml)*	-0.62	<0.001
IL-10 production (pg/ml)*	-0.09	0.63

* analysis after logarithmic transformation

8.4.3 Relation between immunological parameters and duration of mechanical ventilation

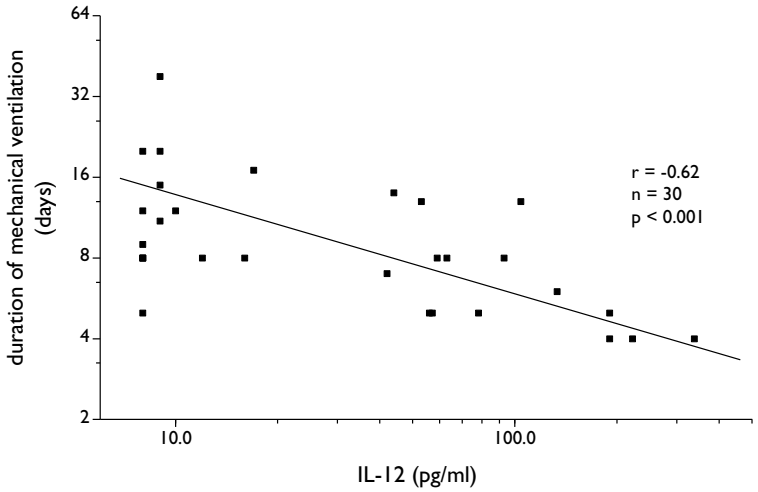
IL-12 production in whole blood cell culture after stimulation with LPS+IFNγ was below the detection limit in five patients. No correlation was found between IL-12 production and chronological age, post-conceptional age or sex. The relation between IL-12 production and duration of mechanical ventilation is shown in Figure 8.1. Interleukin-12 production predicted duration of mechanical ventilation (r = -0.62 days/(pg/ml), p < 0.001) (n=30). Interleukin-12 production per monocyte had comparable predictive value for duration of mechanical ventilation (r = -0.58 days/(pg/ml/monocyte), p = 0.003). Interleukin-10 production and plasma IL-8 did not predict duration of mechanical ventilation (data not shown).

Chronological age, mean VI and IL-12 production were analyzed with multiple regression analysis, which demonstrated that mean VI and IL-12 production were independently associated with duration of mechanical ventilation (R^2 = 0.46).

8.5 Discussion

The main finding of this study was that IL-12 production at initiation of mechanical ventilation is inversely correlated with the duration of mechanical ventilation in RSV bronchiolitis. In addition, the mean VI during the first 24 hours after initiation of

Figure 8.1 *Relation between ex vivo IL-12 production and duration of mechanical ventilation.*
Blood was obtained from RSV bronchiolitis patients within 24 hours after initiation of mechanical ventilation. Whole blood cultures were stimulated for 48 hours with LPS (100ng/ml) and IFNγ (20 ng/ml). Data represent individual values and were analyzed after log transformation.



mechanical ventilation appeared to be an independent predictor of duration of mechanical ventilation. This is the first study to specifically assess the value of clinical and immunological parameters in predicting duration of mechanical ventilation in RSV bronchiolitis.

Wang et al. described clinical parameters, related to increased risk of admission to an intensive care unit and of respiratory insufficiency[1]. These parameters included age < 6 weeks, prematurity with or without chronic lung disease and congenital heart disease. In the present study, no association was found between chronological or post-conceptional age and duration of mechanical ventilation. Whether chronic lung disease and congenital heart disease are associated with prolonged duration of mechanical ventilation could not be studied in our patient population, because only one patient had chronic lung disease and none had congenital heart disease.

The VI is a measure of ventilatory disorder, which takes into account variations in ventilator management by incorporating both therapy (ventilatory rate and peak inspiratory pressure) and response to therapy ($PaCO_2$). The correlation between mean VI and duration of mechanical ventilation is in line with findings of Paret et al., who demonstrated the prognostic value of VI in children with ARDS at day 3-5 after initiation of mechanical ventilation[3]. It is of interest that in the present study the mean VI during the first 24 hours after initiation of mechanical ventilation already corresponds with duration of mechanical ventilation.

Shute et al., but also other investigators have shown that in general plasma IL-8 levels are markers of disease severity in inflammatory diseases of the lung, including asthma[6].

The same was found recently in a RSV study by our group, in which plasma IL-8 levels were significantly higher in mechanically ventilated patients than non-ventilated patients[7]. The present study, however, shows that plasma IL-8 does not predict the duration of mechanical ventilation in RSV bronchiolitis.

In the present study we did not study IL-12 production in mechanically ventilated infants without RSV infection. To our knowledge, no data are available describing the relation between duration of mechanical ventilation and ex vivo IL-12 responses in very young infants. As a result, we can not exclude the possibility that an illness severe enough to require prolonged duration of mechanical ventilation, regardless of the cause, may be associated with low IL-12 production. However, the inverse relation between IL-12 responses and duration of mechanical ventilation suggests a protective role for IL-12 in RSV infection in infants with respiratory failure. The mechanisms by which IL-12 exerts this protective effect could be the initiation of an antiviral immune response by induction of differentiation of naive CD4+ cells into the Th1 phenotype[4]. Cytokines secreted by Th1 cells, including IFNγ, are a prerequisite to generate protective responses against viruses and other intracellular pathogens.

Both patient- and virus-dependent factors could have influenced IL-12 responses in the patients of this study. A patient-dependent factor for IL-12 production is age. In a separate study in healthy infants, we found a significant correlation between IL-12 response to LPS+IFNγ and age in the first months of life (data not shown). Together with decreased *in vitro* IFNγ responses in neonates, this could explain the high frequency of RSV bronchiolitis, resulting in mechanical ventilation, among infants < 6 weeks[8].

On the other hand, other viruses, including the measles virus, have been shown to inhibit IL-12 production in vitro by monocytes/ macrophages[9;10]. Although the effect of RSV on IL-12 production by monocytes/macrophages has not been investigated, it is conceivable that, by inhibition of IL-12 production, RSV itself effectively prevents the initiation of adequate antiviral immune responses.

RSV bronchiolitis is often considered as an immune-mediated disease[11;12]. This theory is supported by the absence of clinical symptoms in T-cell-depleted mice that were infected with RSV[13]. Furthermore, the vaccination trial with inactivated RSV, that resulted in augmentation of disease manifestations, provides additional evidence for an immune-mediated pathogenesis of RSV bronchiolitis[14]. However, is has not been established whether vaccine trials or RSV infection in mice are reliable models for natural human RSV infection. In addition, it was already remarked by Hall et al., that an immune-mediated pathogenesis of RSV bronchiolitis does not explain the high incidence of life-threatening RSV infection among neonates[15]. Although cell-mediated immunity might be involved in the induction of RSV bronchiolitis, the relation between IL-12 production and duration of mechanical ventilation in this study indicates that cell-mediated immunity is also needed for convalescence from respiratory insufficiency during RSV infection.

In summary, this study provides the first model with predictive value for the duration of mechanical ventilation in RSV bronchiolitis. In addition, the inverse correlation between IL-12 production and duration of mechanical ventilation suggests a critical role for the anti-viral immune response in the convalescence from respiratory failure during RSV infection.

References

1 Wang EEL, Law BJ, Stephens D. Pediatric Investigators Collaborative Network on infections in Canada (PICNIC) prospective study of risk factors and outcomes in patients hospitalized with respiratory syncytial viral lower respiratory tract infection. J Pediatr 1995; 126:212-219.

2 Bont L, Van Vught HJ, Kimpen JLL. Prophylaxis against respiratory syncytial virus (RSV) in premature infants. Lancet 1999; 354(9183):1003-1004.

3 Paret G, Ziv T, Barzilai A, Ben-Abraham R, Vardi A, Manisterki Y, Barzilay Z. Ventilation index and outcome in children with acute respiratory distress syndrome. Pediatr Pulmonol 1998; 26:125-128.

4 Ramshaw IA, Ramsay AJ, Karupiah G, Rolph MS, Mahalingam S, Ruby JC. Cytokines and immunity to viral infections. Immunol Rev 1997; 159:119-135.

5 Elenkov IJ, Webster E, Papanicolaou DA, Fleisher TA, Chrousos GP, Wilder RL. Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. J Immunol 1998; 161:2586-2593.

6 Shute J, Vrugt B, Lindley IJD, Holgate ST, Bron A, Aalbers R, Djukanovic R. Free and complexed interleukin-8 in blood and bronchial mucosa in asthma. Am J Respir Crit Care Med 1997; 153:1877-1883.

7 Bont L, Heijnen CJ, Kavelaars A, Aalderen vWMC, Brus F, Draaisma JMTh, Geelen SM, Vught vHJ, Kimpen JLL. Association of peripheral blood cytokine responses with disease severity in respiratory syncytial virus bronchiolitis. Eur Respir J 1999; 14:144-149.

8 Lewis DB, Yu CC, Meyer J, English BK, Kahn SJ, Wilson CB. Cellular and molecular mechanisms for reduced interleukin 4 and interferon gamma production by neonatal T cells. J Clin Invest 1991; 87:194-202.

9 Karp CL, Wysocka M, Wahl LM, Ahearn JM, Cuomo PJ, Sherry B. Mechanisms of suppresion of cell-mediated immunity by measles virus. Science 1996; 273:228-231.

10 McDyer JF, Wu CY, Seder RA. The regulation of IL-12: its role in infectious, autoimmune, and allergic diseases. J Allergy Clin Immunol 1998; 102:11-15.

11 Openshaw PJM. Immunopathological mechanisms in respiratory syncytial virus disease. Springer Semin Immunopathol 1995; 17:187-201.

12 Kimpen JLL. Respiratory syncytial virus immunology. Pediatr Allergy Immunol 1996; 7:S86-90.

13 Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991; 88:1026-1033.

14 Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 1969; 89(4):405-421.

15 Hall CB, Kopelman AE, Douglas RG, Geiman JM, Meagher MP. Neonatal respiratory syncytial virus infection. N Engl J Med 1979; 300:393-396.

9 Natural reinfection with respiratory syncytial virus does not boost virus-specific T cell immunity

L Bont, J Versteegh, WTN Swelsen, A Kavelaars, CJ Heijnen, F Brus, JMTh Draaisma, M Pekelharing-Berghuis, RAAM van Diemen-Steenvoorde, JLL Kimpen

Submitted for publication

9.1 Abstract

To determine the role of respiratory syncytial virus (RSV)-specific cell-mediated immunity (CMI) during natural reinfection we investigated whether RSV-specific T cell responses protect against reinfection and, subsequently, whether reinfection boosts virus-specific memory. In a cohort of 55 infants, who were hospitalized for RSV bronchiolitis, RSV-specific lymphoproliferative responses (LPR) in the peripheral blood were measured at three time-points: on admission, 4 weeks after admission and one year later, following the second winter season. Memory was defined as a stimulation-index (SI) >2. During the second winter season, nasal secretions were collected in every case of a runny nose. Reinfection was diagnosed if immunofluorescence or PCR was positive for RSV. Virus-specific memory was found in 1 child on admission for primary RSV infection, whereas 4 weeks later 44 infants (80%) had memory. Reinfection with RSV was found in 23 infants (43%) during the second winter season. After the second season memory was found in 20 infants (38%). No differences in SI following the second winter season were found between infants with and without reinfection (2.3 versus 2.1). However, a highly significant correlation was found between SI measured 4 weeks after primary RSV infection and LPR following the second winter season ($r=0.40$, $p=0.001$). In conclusion, RSV-specific T cell responses did not provide protection against reinfection. Moreover, reinfection did not boost RSV-specific T cell proliferation. To explain both findings, it is hypothesized that RSV-specific T cells fail to expand *in vivo* upon reinfection.

9.2 Introduction

Respiratory syncytial virus (RSV) is one of the most important respiratory pathogens in infancy causing the majority of lower respiratory tract infections (LRTI) during the winter season. Hospitalization rates for RSV illness are 1-30 cases per 1000 infants < 1 year of age[1-3]. In hospitalized infants with RSV bronchiolitis mechanical ventilation is required in 7-21% of cases[4-6]. Mortality in RSV infected infants with lower respiratory tract symptoms is <1%[7].
Reinfection with RSV occurs frequently and usually has a mild character with symptoms of uncomplicated upper respiratory tract infection [8]. Neutralizing antibodies induced by primary RSV infection appear to provide only partial protection for a limited period of time, which does probably not last until the subsequent RSV season[8;9]. Evidence that intact cell-mediated responses play a role in clearance of the virus and protection against reinfection was derived from animal studies[10]. In humans, little information is available on the role of virus-specific CMI induced during primary infection in the protection against reinfection with RSV.
Currently, no vaccine for RSV is available. In the 1960s a formalin-inactivated vaccine was used in infants[11]. No protection against naturally-acquired RSV was observed. In contrast, enhanced disease and increased mortality were observed during RSV infection following vaccination. CMI has been implicated in the pathogenesis of this phenomenon[12]. Several strategies for safe and effective vaccination, including immunization with attenuated strains and sub-unit vaccines are under investigation at the moment[13-16]. The immune response to a RSV vaccine should be protective, persistent and not disease enhancing upon subsequent contact with the virus. Similar to other vaccines strategies, such as measles and pertussis, it is conceivable that boosting is required in order to maintain virus-specific CMI. For this reason it is important to study the characteristics of the memory response in relation to recurrent infection.
In this prospective follow-up study we investigated the development over time of RSV-specific T cell responses in a cohort of infants hospitalized for RSV bronchiolitis. The first aim of study was to determine whether RSV-specific T cell responses induced during primary RSV bronchiolitis protect against reinfection during the subsequent epidemic. The second aim of the study was to investigate whether virus-specific T cell responses acquired during primary infection are boosted by natural reinfection with RSV.

9.3 Methods

9.3.1 Study population

Infants were included during one winter epidemic in 5 hospitals in the Netherlands. Inclusion criteria were: hospital admission, lower respiratory tract symptoms, age < 13 months and positive immunofluorescence for RSV infection of epithelial cells in nasopharyngeal secretions. Lower respiratory tract symptoms were severe chest cough, wheezing, hoarseness, stridor, shortness of breath[17] as well as cyanosis and apnea. Prematurely born infants with chronic lung disease and infants with wheezing illness prior to RSV bronchiolitis were not included. The study was approved by the Medical Ethical Committee in all participating centers. Parents of subjects gave written informed consent.

9.3.2 Documentation of reinfection

During the second winter season the occurrence of reinfection was studied. Parents received written and oral information during a home visit during the fall season prior to this part of this study. During the winter season parents contacted the investigators (J.V.) in case of respiratory symptoms. To ascertain cooperation by parents a telephone call was made every 3 weeks. Within 48 hours after onset of respiratory symptoms home visits were made to perform nasal washes. No physical examination was performed.
RSV reinfection was confirmed by a commercially available direct immunofluorescence assay using fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (DAKO, Imagen, The Netherlands) and by reverse transcriptase polymerase chain reaction (RT-PCR). Reinfection was defined as a positive immunofluorescence or PCR on nasal washings.

9.3.3 RT-PCR

RNA extraction was performed according to the method described by Boom et al[18]. Briefly, 10-100µl respiratory specimen was mixed with 900µl lysis buffer and 50µl silica and incubated for 10 minutes at room temperature in order to bind the nucleic acid to the silica particles. Unbound material was then removed by several washing steps. The RNA was subsequently eluted in 100µl 40 ng/µl polyA RNA before performing a one-tube reverse transcription (RT)-PCR.
After viral RNA isolation, an equivalent of 1-10µl of respiratory specimen was used to reverse transcribe and amplify the NP gene. A one tube RT-PCR was performed essentially as described by Nijhuis et al., using 1.5 mM $MgCl_2$, 0.4 µM of primer RS-1 (5'-GGA TTG TTT ATG AAT GCC TAT GGT-3' (Pharmacia)) and primer RS-2 (5'-TTC TTC TGC TGT YAA GTC TAR TAC AC-3'). The amount of amplified product was increased further in a nested amplification.
Five µl of first PCR product is further amplified in a nested amplification essentially as described by Nijhuis et al.[19], using 4.5mM of $MgCl_2$ and 0.4µM of primer RS-3 (5'-GGA TTC TAC CAT ATA TTG AAC AA-3') and primer RS-4 (5'-CTR TAC TCT CCC ATT ATG CCT AG-3').
Five µl of nested PCR products were visualized on an ethidium-bromide stained agarose gel using UV illumination. A 100-bp marker was used to control fragment lengths.

9.3.4 Virus preparation

Long strain RSV was cultured in Hep-2 cells (courtesy of Dr. A.M. van Loon, dept of Virology, University Medical Center, Utrecht,) and 1:1 diluted in sucrose-gelatone solution Z7725a (Laboratory of Vaccine Research, National Institute of Public Health and Environment, Bilthoven, The Netherlands). Titers were determined in Hep-2 cells using the TCID50 method described by Karber (TCID50 $2x10^5$)[20]. A control antigen was prepared similarly from uninfected Hep-2 cultures. Virus and control antigen were stored in aliquots at -80°C.

9.3.5 RSV-specific T cell responses

At three time points heparinized blood was taken from subjects for whole blood cultures. The first blood sample was taken within 24 hours after admission (t=1). Only in two of five participating hospitals a blood sample was taken at this time point. The second blood

sample was taken three to four weeks after the initial admission (t=2). The third sample was taken immediately following the second RSV epidemic (t=3). At t=3 RSV-specific LPR, but no cytokine profiles were determined.
Freshly taken heparinized blood was diluted 1:10 in RPMI 1640 medium (Life Technologies, Grand Island, NY) and aliquoted (150 µl) into 96-well culture plates (Nunc International, Denmark). Whole blood was infected with RSV at a multiplicity of infection (MOI) of 0.1 - 1.0 or control suspension. Cultures were incubated for 6 days at 37°C in 5% CO_2. Forty-eight hours after infection, pooled supernatants were collected for cytokine measurement. Five days after infection lymphocytes were pulsed with 0.25 µCi ^{3}H-thymidine for 18 hours and thymidine incorporation was measured (lymphoproliferative response(LPR)). Stimulation index was defined as the ratio between LPR in cultures stimulated with RSV and control antigen. A memory response was defined as a stimulation index ≥ 2.0. All cultures were performed in quadruplicate. Pooled supernatants were kept at -80°C.

9.3.6 Cytokine assays

Cytokines that were measured in supernatants of RSV-stimulated whole blood cultures were IL-4, IL-10, IL-12 and IFN-γ. Concentrations of IL-4, IL-8, IL-10 and IFN-γ were determined using ELISA kits supplied by the Dutch Laboratory for Blood Transfusion (CLB, Amsterdam, the Netherlands). The detection limit of the assay for IL-4 was 2 pg/ml, for IL-8 2.5 pg/ml, for IL-10 2.5 pg/ml, and for IFN-γ 4 pg/ml. Concentrations of IL-12 were determined using ELISA kits from R&D (Oxon, United Kingdom), the detection limit was 7.8 pg/ml. When cytokine values were not detectable, the minimum detectable level was used for statistical analysis.

9.3.7 Statistical analysis

Cytokine production and stimulation indices in RSV-stimulated cultures were logarithmically transformed and expressed as geometric mean and 95% confidence interval. All tests of significance were two-sided. A $p<0.05$ was considered statistically significant. Pearson correlation coefficient was used to describe the correlation between LPR at t=2 and t=3. Spearman's correlation coefficient was used to analyze the correlation between RSV-specific LPR or cytokine responses and age. Paired Student's t-test was used to analyze differences in (log transformed) values between different time points. Unpaired Student's t-test was used to analyze differences in (log transformed) values between infants with and without reinfection. All tests of significance were two-sided. A $p<0.05$ was considered statistically significant.

9.4 Results

9.4.1 Subject characteristics

The investigated population consisted of 55 patients. Thirty patients (55%) were boys, median age at the time of RSV bronchiolitis was 7 weeks. Ten infants (18%) were born prematurely (range: 29–36 weeks). Ten infants (18%) needed mechanical ventilation. One child had cardiac disease, none of the children had immunodeficiency. None of the prematurely born infants had received RSV prophylaxis. None of the patients received

ribavirin or systemic anti-inflammatory agents, including corticosteroids. Patients did not receive inhaled corticosteroids during RSV bronchiolitis. All patients survived.

9.4.2 RSV-specific T cell responses

A pilot study was performed in samples taken on admission (t=1) and following primary RSV infection (t=2) in order to determine kinetics of LPR and cytokine responses. At t=2 maximum LPR to RSV were found after 5 days of culture. Maximum IL-10 production was found at 48 hours. The pattern and magnitude of IL-10 responses at t=1 and t=2 were comparable and unrelated to stimulation indices. Maximum IFN-γ production at t=1 and t=2 were found after 48 hours and 120 hours, respectively. IFN-γ and IL-10 responses in control antigen-stimulated cultures were undetectable or relatively low at any time point as compared to RSV-stimulated cultures. IL-4 production in RSV-stimulated cultures remained low (<10 pg/ml) and was not higher than IL-4 production in cultures stimulated with control antigen. IL-12 production was not detectable at either t=1 or t=2. We therefore concluded there is no RSV-specific IL-4 or IL-12 production under these culture conditions. (data not shown)

At t=1, during the acute phase of RSV bronchiolitis, whole blood cultures were performed in 22 infants. In RSV stimulated whole blood cultures at t=1, only one patient (4.5%) had a memory response suggesting earlier infection. RSV-induced IFN-γ and IL-10 responses at t=1 were 13 (95% CI: 4-199) respectively 25.7 (95% CI: 12.5-52.7) pg/ml.

At t=2, during the convalescent phase of RSV bronchiolitis, whole blood culture were performed in 55 infants. Forty-four patients (80%) had a memory response at t=2, the SI was 5.1(95% CI: 3.7-8.4). RSV-induced IFN-γ production at t=2 was 60 (95% CI: 48–79) pg/ml, significantly higher than at t=1 ($p<0.01$). RSV-induced IFN-γ production was highly correlated with the RSV-specific LPR ($r=0.80$, $p<0.001$). RSV-induced IL-10 production at t=2 was 16 (95% CI: 11-23) pg/ml, not significantly different from t=1. At t=2 no significant correlation was found between age and RSV-specific LPR or cytokine responses. Mechanically ventilated infants had higher LPR than non-ventilated infants (8.5 resp 3.9, $p=0.02$), which is in line previous reports[21].

At t=3, following the second winter season, RSV-specific lymphoproliferation was measured in 53 infants. No blood was drawn in two infants (4%). A memory response was found in 20 infants (38%). RSV-specific LPR was significantly lower than following the primary RSV infection (SI 2.2 vs. 5.1, $p<0.001$). RSV-specific LPR at t=2 and t=3 were highly correlated ($r=0.40$, $p=0.001$) (Figure 9.1).

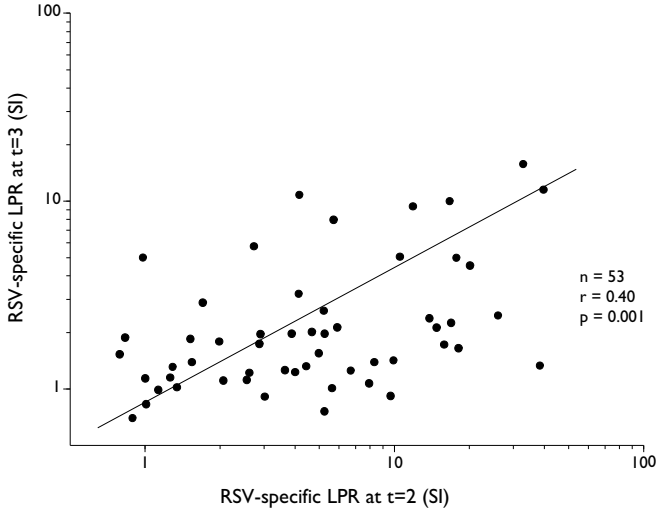
9.4.3 Reinfection during second winter season

Eighty-five episodes of respiratory tract symptoms were reported, reinfection with RSV was diagnosed in 23 infants (42%). In all 23 cases PCR was positive, whereas immunofluorescence was positive in only 9 of 23 cases of RSV infection (39%).

RSV-specific cellular immune responses at t=2 was compared between infants with and without reinfection to assess whether RSV-specific T cell responses protected against reinfection. No differences in LPR were found between infants with and without reinfection (Figure 9.2). In addition, infants with and without reinfection had comparable RSV-specific IFN-γ (70 vs. 54 pg/ml) and IL-10 production (13 vs. 16 pg/ml) at t=2.

RSV-specific LPR at t=3 was compared between infants with and without reinfection to assess whether RSV-specific T cell proliferation was boosted by reinfection. No difference

Figure 9.1 *Correlation between RSV-specific lymphoproliferative responses during primary RSV infection and following the subsequent winter season*
Relation RSV-specific lymphoproliferative responses (SI) measured 3-4 weeks after hospitalization for RSV bronchiolitis (t=2) and following the subsequent winter season (t=3). Lymphoproliferative responses were determined by thymidine-incorporation in whole blood cultures stimulated with RSV for 5 days. Pearson's correlation coefficient is shown.

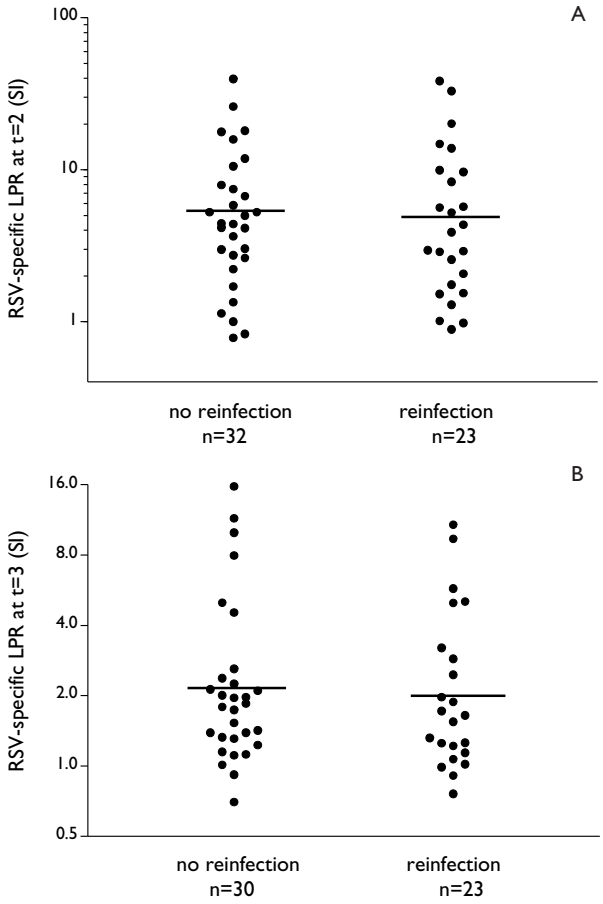


in LPR at t=3 were found between infants with and without reinfection (2.1 resp 2.3)(Figure 9.3). The correlation between LPR at t=2 and t=3 was separately analyzed for infants with and without reinfection. In infants with and without reinfection the correlation between LPR at t=2 and t=3 was similar ($r=0.41$, $p<0.05$ resp. $r=0.40$, $p<0.05$).

9.5 Discussion

The results of this study show that virus-specific T cell responses induced during primary RSV infection does not protect completely against subsequent reinfection. In addition, reinfection with RSV did not boost RSV-specific LPR.
Reinfections with RSV occur throughout life[22], implying that immunity induced by natural infection provides little long-term protection against reinfection. Naturally-induced and exogenously-administered RSV-specific antibodies provide incomplete protection, which is of short duration[9;23-25]. To date, sparse data exist on the protection against reinfection provided by naturally acquired CMI[23;26]. In a follow-up study from the early 80s no relationship was found between RSV-specific lymphoproliferative responses induced during primary infection and the risk for subsequent culture-proven reinfection[23]. However, it can be doubted whether the sensitivity of viral cultures in this study was suitable to diagnose (usually mild) reinfection[27]. Assuming positive PCR for RSV indicates RSV infection, the present study shows that the sensitivity of

Figure 9.2 *RSV-specific lymphoproliferative responses in infants with and without reinfection with RSV*
RSV-specific lymphoproliferative responses (SI) measured 3-4 weeks after hospitalization for RSV bronchiolitis (t=2) (Figure 9.2a) and following the second winter season (t=3) (Figure 9.2b) in infants with and without reinfection with RSV. Lymphoproliferative responses were determined by thymidine-incorporation in whole blood cultures stimulated with RSV for 5 days. Geometric means are shown.



immunofluorescence for RSV infection in this patient group was only 39%. Thus PCR, and not culture or immunofluorescence, is required to adequately establish mild reinfection with RSV.
One of the limitations of the present study is that we did not attempt to measure the degree of respiratory disease. Therefore, we cannot exclude that RSV-specific cell-mediated responses decrease the severity of disease in case of reinfection. In addition, in the present study RSV-specific CMI is represented by virus-specific LPR. Although this is in line with previous studies[21;26], it is not known what part of CMI is reflected by RSV-

specific LPR. The assay measures proliferation in a pool of heterogeneous cells. Therefore, LPR may reflect proliferation of cytotoxic T cells (CTL), cytokine-producing cells or non-specific cells that respond to IL-2 produced in the culture system. Cytotoxicity assays measure CTL responses by CD8+ cells and reflect a more accurate effector function of CMI. However little data are available on CTL responses in infants during RSV infection[28;29].

To address the role of RSV-specific CTL in protection against infection in the mouse model, vaccination studies with vaccinia virus expressing the M2 protein of RSV were performed. It was shown that protection against RSV infection largely depended on CTL formation[30]. CTL responses waned within 45 days after vaccination, which was paralleled by a loss in protection.

In the present study RSV-specific LPR was induced by primary RSV infection in the majority of infants, which is in line with previous studies[21]. Memory was found in one child during the acute phase of disease, whereas 3 to 4 weeks later memory to RSV was found in 80% of the infants. To our surprise we did not find evidence that reinfection boosted virus-specific CMI, since infants with and without reinfection had similar RSV-specific LPR at t=2 and t=3. Moreover, RSV-specific LPR at t=2 and t=3 were highly correlated. Therefore, it can be concluded that RSV-specific LPR induced during primary RSV bronchiolitis partially persists for more than a year and is not boosted by naturally acquired reinfection.

The physiological role of RSV-specific T cells during reinfection is not well understood. First, RSV-specific T cell responses did not protect against reinfection. Second, virus-specific LPR were induced by primary RSV infection, but not boosted by reinfection. Both findings may be explained by an absence of *in vivo* expansion of RSV-specific T cells during a re-encounter with RSV. It is uncertain, however, why RSV-specific memory T cells are formed during primary infection, but do not proliferate in vivo during reinfection.

The immune response during reinfection with RSV is apparently effective, since symptoms are usually mild and last for a short period. If RSV-specific T cells do indeed fail to expand *in vivo* during reinfection, it can be questioned whether T cell memory plays a major role in the immune response during reinfection. B cell memory may be more important during reinfection. This is supported by a prominent rise in neutralizing antibodies observed following reinfection with RSV [8]. In addition, non-adaptive immunity could have a function in the elimination of RSV during reinfection. Natural killer (NK) cells are important in the defense against viruses because the are capable of providing cytotoxicity and producing cytokines, including IFN-g[31]. Although in humans no data exist on the role of NK cells during RSV infection, this cell type has been implicated in the immune response during RSV infection in mice[32;33]. In addition, type I interferons (IFN-α/β) could play a role in the anti-viral immune response. These cytokines with direct anti-viral properties are produced in high amounts by airway epithelium upon infection with RSV [34] and they are found in the airways of RSV infected infants[35].

An important aim in the development of a vaccine against viruses is the induction of virus-specific memory. Subsequent encounter with the virus should result in expansion of at least part the pool of memory T cells. The present study clearly shows that memory cells do not necessarily expand during reinfection. If a future RSV vaccine for humans induces RSV-specific T cells, which lack the potential to expand *in vivo* upon natural

infection, this could have implications for the effectiveness of the vaccine. Limited protection by memory T cells would be expected, the effect of the vaccine would than largely depend on the formation of B cell memory. It remains to be seen whether B cell memory is sufficient for protection against reinfection

In conclusion, the present study shows that RSV-specific T cell responses do not protect against reinfection. Moreover, reinfection does not boost RSV-specific LPR. Together, these findings suggest that RSV-specific T cells do not expand *in vivo* upon reinfection with RSV, which could bear relevance for the development of an effective vaccine.

References

1 Karron RA, Singleton RJ, Bulkow L, Parkinson A, Kruse D, DeSmet I, Indorf C, Petersen KM, Leombruno D, Hurlburt D, Santosham M, Harrison LH. Severe respiratory syncytial virus disease in Alaska native children. RSV Alaska Study Group. J Infect Dis 1999; 180:41-49.

2 Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 1999; 282:1440-1446.

3 Wang EEL, Law BJ. Respiratory syncytial virus infection in pediatric patients. Seminars in Pediatric Infectious Diseases 1998; 9:146-153.

4 Outwater KM, Crone RK. Management of respiratory failure in infants with acute viral bronchiolitis. Am J Dis Child 1984; 138:1071-1075.

5 Frankel LR, Lewiston NJ, Smith DW, Stevenson DK. Clinical observations on mechanical ventilation for respiratory failure in bronchiolitis. Pediatr Pulmonol 1986; 2:307-311.

6 Tissing WJE, Van Steensel-Moll HA, Offringa M. Risk factors for mechanical ventilation in respiratory syncytial virus infection. Eur J Pediatr 1993; 152:125-127.

7 Shay DK, Holman RC, Roosevelt GE, Clarke MJ, Anderson LJ. Bronchiolitis-Associated Mortality and Estimates of Respiratory Syncytial Virus-Associated Deaths among US Children, 1979-1997. J Infect Dis 2000; 183.

8 Henderson FW, Collier AM, Clyde WA, Jr., Denny FW. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N Engl J Med 1979; 300:530-534.

9 Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis 1991; 163:693-698.

10 Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991; 88:1026-1033.

11 Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 1969; 89(4):405-421.

12 Graham BS. Pathogenesis of respiratory syncytial virus vaccine-augmented pathology. Am J Respir Crit Care Med 1995; 152:S63-S66.

13 Plotnicky-Gilquin H, Huss T, Aubry JP, Haeuw JF, Beck A, Bonnefoy JY, Nguyen TN, Power UF. Absence of lung immunopathology following respiratory syncytial virus (RSV) challenge in mice immunized with a recombinant RSV G protein fragment. Virology 1999; 258:128-140.

14 Groothuis JR, King SJ, Hogerman DA, Paradiso PR, Simoes EAF. Safety and immunogenicity of a purified F protein respiratory syncytial virus (PFP-2) vaccine in seropositive children with bronchopulmonary dysplasia. J Infect Dis 1998; 177:467-469.

15 Piedra PA, Grace S, Jewell A, Spinelli S, Hogerman DA, Malinoski F, Hiatt PW. Sequential annual administration of purified protein vaccine against respiratory syncytial virus in children with cystic fibrosis. Pediatr Infect Dis J 1998; 17:217-224.

16 Waris ME, tsou c, Erdman DD, Day DB, Anderson LJ. Priming with live respiratory syncytial virus (RSV) prevents the enhanced pulmonary inflammatory response seen after RSV challenge in BALB/c mice immunized with formalin-inactivated RSV. J Virol 1997; 71:6935-6939.

17 Stein RT, Sherill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13. Lancet 1999; 354:541-545.

18 Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der NJ. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 1990; 28(3):495-503.
19 Nijhuis M, Boucher CA, Schuurman R. Sensitive procedure for the amplification of HIV-1 RNA using a combined reverse-transcription and amplification reaction. Biotechniques 1995; 19(2):178-80, 182.
20 Lennette EH, Schmidt NJ. Diagnostic prodecures for viral, rickettsial and chlamydial infections. 5 ed. Washington: 1979.
21 Welliver RC, Kaul A, Ogra PL. Cell-mediated immune response to respiratory syncytial virus infection: relationship to the development of reactive airway disease. J Pediatr 1979; 94:370-375.
22 Hall CB, Geiman JM, Biggar R, Kotok DI, Hogan PM, Douglas GR, Jr. Respiratory syncytial virus infections within families. N Engl J Med 1976; 294:414-419.
23 Fernald GW, Almond JR, Henderson FW. Cellular and humoral immunity in recurrent respiratory syncytial virus infection. Pediatr Res 1983; 17:753-758.
24 The IMpact-RSV study group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 1998; 102(3):531-537.
25 Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. The PREVENT Study Group. Pediatrics 1997; 99:93-99.
26 Welliver RC, Tristram DA, Batt K, Sun M, Hogerman DA, Hildreth S. Respiratory syncytial virus-specific cell-mediated immune responses after vaccination with a purified fusion protein subunit vaccine. J Infect Dis 1994; 170:425-428.
27 Michaels MG, Serdy C, Barbadora K, Green M, Apalsch A, Wald ER. Respiratory syncytial virus: a comparison of diagnostic modalities. Pediatr Infect Dis J 1992; 11:613-616.
28 Bangham CR, McMichael AJ. Specific human cytotoxic T cells recognize B-cell lines persistently infected with respiratory syncytial virus. Proc Natl Acad Sci U S A 1986; 83:9183-9187.
29 Isaacs D, Bangham CRM, McMichael AJ. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet 1987;769-771.
30 Kulkarni AB, Connors M, Firestone CY, Morse HC, III, Murphy BR. The cytolytic activity of pulmonary CD8+ lymphocytes, induced by infection with a vaccinia virus recombinant expressing the M2 protein of respiratory syncytial virus (RSV), correlates with resistance to RSV infection in mice. J Virol 1993; 67:1044-1049.
31 Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999; 17:189-220.
32 Anderson JJ, Serin M, Harrop J, Amin S, Toms GL, Scott R. Natural killer cell response to respiratory syncytial virus in the Balb/c mouse model. Adv Exp Med Biol 1989; 257:211-220.
33 Hussell T, Openshaw PJ. Intracellular IFN-gamma expression in natural killer cells precedes lung CD8+ T cell recruitment during respiratory syncytial virus infection. J Gen Virol 1998; 79 (Pt 11):2593-2601.
34 Garofalo RP, Mei F, Espejeo R, Ye G, Haeberle H, Baron S, Ogra PL, Reyes VE. Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class 1 MHC Expression through the induction of IFN-beta and IL-1-alpha. J Immunol 1996; 157:2506-2513.
35 Nakayama T, Sonoda S, Urano T, Sasaki K, Maehara N, Makino S. Detection of alpha-interferon in nasopharyngeal secretions and sera in children infected with respiratory syncytial virus. Pediatr Infect Dis J 1993; 12:925-929.

10 Airflow limitation during respiratory syncytial virus lower respiratory tract infection predicts recurrent wheezing

L Bont, WMC van Aalderen, J Versteegh, F Brus, JThM Draaisma, M Pekelharing-Berghuis, RAAM van Diemen-Steenvoorde, JLL Kimpen

Pediatr Infect Dis J 2001;20:277-282

10.1 Abstract

Respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) is frequently followed by recurrent wheezing. Thus far, no clinical risk factors have been identified to predict which infants will have wheezing episodes subsequent to RSV LRTI. To objective of the present study was to determine clinical predictors for airway morbidity following RSV LRTI. In a one year follow-up study, we investigated the predictive value of auscultatory findings characteristic of airflow limitation (wheezing) during RSV LRTI for subsequent airway morbidity. Clinical characteristics, including the presence or absence of signs of airflow limitation, of hospitalized infants with RSV LRTI were prospectively recorded during two winter epidemics. During a one year follow-up period parents of 130 infants recorded daily airway symptoms. Recurrent wheezing defined as ≥2 episodes of wheezing. Signs of airflow limitation during RSV LRTI were absent in 47 (36%) infants and present in 83 (64%) infants. Recurrent wheezing was recorded in 10 (21%) infants without signs of airflow limitation and in 51 (61%) with signs of airflow limitation during initial RSV LRTI (relative risk 0.29, $p<0.001$). In a multiple logistic regression model airflow limitation during initial RSV LRTI proved independent from other clinical parameters, including age, parental history of asthma and smoke exposure. We conclude that signs of airflow limitation during RSV LRTI is the first useful clinical predictor for subsequent recurrent wheezing.

10.2 Introduction

Acute RSV LRTI, one of the most common respiratory diseases in infancy, occurs in winter epidemics. Numerous epidemiological studies have suggested an increased risk for recurrent episodes of wheezing following acute RSV bronchiolitis.[1-5] In addition to respiratory symptoms, abnormalities of infant respiratory function subsequent to RSV bronchiolitis have been reported in many but not all studies. [6-10] Recently, a prospective study showed that during the first three years of life, RSV LRTI is a risk factor for recurrent wheezing up to age 11 years, but not at age 13.[11]
Several studies have attempted to delineate clinical risk factors for airway morbidity following RSV LRTI.[3;4] However, no clinical risk factors have thus far been identified. Most earlier reports have used standardized questionnaires filled in by parents to obtained information on airway morbidity. An important limitation of questionnaires is that parents might have forgotten earlier occurrence of respiratory symptoms (recall bias). Signs of airflow limitation, as evidenced by wheezing on auscultation of the chest during the acute phase of RSV LRTI, as a risk factor for recurrent wheezing following RSV LRTI has not been investigated.
In the present study, we prospectively obtained detailed quantitative information on respiratory symptoms following RSV LRTI using a simple diary during a one year follow-up period. We tested the hypothesis that the absence of physical sounds of airflow limitation in infants with RSV LRTI predicts a lower incidence of airway morbidity during a one year follow-up period than in infants with the more classical picture of RSV bronchiolitis with airflow limitation.

10.3 Methods

10.3.1 Study population

Children at 6 hospitals in the Netherlands were prospectively included during two winter epidemics. Two hospitals had a pediatric intensive care unit (PICU). Inclusion criteria were: hospital admission, lower respiratory tract symptoms, age < 13 months and immunofluorescence for RSV in epithelial cells from nasopharyngeal secretions. There were no standard criteria used for hospitalization. Symptoms of LRTI were severe chest cough, wheezing, hoarseness, stridor, shortness of breath[11] as well as cyanosis and apnea. Infants with wheezing respiration prior to RSV infection were excluded. A history of premature birth, chronic lung disease, congenital heart disease as well as the presence of fever >38.5 ^{o}C and the presence of physical sounds of airflow limitation on admission (expiratory wheeze or prolonged expiration) were prospectively recorded during hospitalization. On admission all infants were examined by a pediatrician. Standardized forms were used to record findings on physical examination on admission, including the presence of respiratory wheeze. During subsequent hospitalization a resident daily performed physical examination as part of clinical routine.
A single investigator (J.V.) retrospectively analyzed the main presenting symptom (as noted by the pediatrician) from the patient histories, without having information on follow-up data. Pediatricians did not always chart one main presenting symptom. The main presenting symptom was subjectively categorized into apnea, respiratory insufficiency resulting in mechanical ventilation, feeding problem, dyspnea or "RSV infection

not specified" on admission. In general, when "dyspnea" was noted as the main presenting symptom, this was followed by supplemental oxygen treatment during hospitalization. When "feeding problems" were noted as the main presenting symptom, feeding measures were subsequently taken.
This study was approved by the Medical Ethical Committee in all participating centers. Parents of subjects gave written informed consent.

10.3.2 Follow-up

Diaries – Starting three weeks after hospital discharge parents recorded respiratory symptoms, airway medication and doctor's visits on a daily basis in a diary. A single investigator (L.B.) instructed parents how to use the diary. Respiratory symptoms that were recorded were "runny nose", "coughing" and "wheezing". Symptoms were graded in severity on a scale from 0-3 (absent, moderate, severe, very severe). The absence of respiratory symptoms was also noted. In order to enhance compliance telephone contact was maintained every three months. The completed diaries were returned to the investigators by mail every three months.
In order to quantify respiratory symptoms during the first year following RSV bronchiolitis, the number of episodes the child experienced coughing and/or wheezing were counted. A disease episode was defined as the presence of respiratory symptoms for two or more consecutive days. In addition, the number of days the child experienced coughing and/or wheezing during the first year were counted. To estimate the role of upper respiratory tract infections during episodes of coughing and/or wheezing, the occurrence of preceding or concurring symptoms of a "runny nose" were analyzed.
Physician diagnosis of asthma – In order to evaluate respiratory morbidity with a different method less dependent on the perception of parents, we contacted the general practitioner of all infants by telephone one year following discharge and inquired whether an asthma diagnosis had been made following RSV LRTI.

10.3.3 Statistical analysis

The number of episodes and days of wheezing or coughing were expressed as median values. Diffences in sex, prematurity, requirement of mechanical ventilation, parental history of asthma, presence of fever, main presenting symptom, frequency of recurrent wheezing and physician-diagnosed asthma between infants with and without signs of airflow limitation during the initial RSV LRTI were analyzed using Chi-square. Differences in age, number of episodes of wheezing or coughing and number of days of coughing or wheezing between infants with and without signs of airflow limitation during the initial RSV LRTI were analyzed using Mann-Whitney U test. Univariate logistic regression was used to analyze predictive values for recurrent wheezing. The predictive value of chronological age was analyzed after log transformation, although the distribution remained skewed to the right after transformation. Forward stepwise logistic regression was used to separately analyze the predictive value of signs of airflow limitation, age, sex, respiratory insufficiency and parental history of asthma as candidate predictors during RSV LRTI for recurrent wheezing in one prediction model. No other parameters were entered in the multiple logistic regression model because of the limited number of patients. The goodness of fit of the model was estimated by the square of the multiple correlation coefficient (R^2).

10.4 Results

10.4.1 Patient characteristics

One-hundred-thirty-six children with RSV LRTI were enrolled, 130 were followed for one year. Six children did not return one or more 3-month diaries and were considered lost to follow-up. Seventy-one (55%) were boys, median age was 2 months. Thirty-nine patients (30%), were born prematurely (median: 34 weeks, range: 25-36 weeks). Twenty-nine subjects (22%) needed mechanical ventilation. Four patients (3%) had cardiac disease, 2 (2%) had chronic lung disease. Signs of airflow limitation were noted in 83 (64%) patients. Clinical characteristics and main presenting symptom of patients with and without signs of airflow limitation are shown in table 10.1. The main presenting symptom noted by the pediatrician in infants with signs of airflow limitation during initial RSV LRTI was dyspnea in 42 infants (51%), whereas respiratory insufficiency resulting in mechanical ventilation was the main presenting symptom in 40% of infants without signs of airflow limitation during initial RSV LRTI. None of the patients received ribavirin or systemic corticosteroids during their initial RSV LRTI. All patients survived.

Table 10.1 *Clinical characteristics of infants with RSV LRTI with and without signs of airflow limitation (n=130)*

Variable	Airflow limitation during initial RSV LRTI (*n*=83)	No airflow limitation during initial RSV LRTI (*n*=47)	*p*-value
Male (%)	47 (57)	24 (51)	NS*
Prematurity (%)	22 (27)	17 (36)	NS*
Median chronological age in weeks (range)	11 (0-52)	6 (1-50)	<0.001#
Median post-conceptional age in weeks (range)	50 (37-91)	42 (34-92)	0.002#
Mechanical ventilation during RSV(%)	10 (12)	19 (40)	0.004*
Paternal history of asthma	10 (12)	1(2)	0.05*
Maternal history of asthma	4 (5)	3 (6)	NS*
Parental history of asthma	14 (17)	4 (9)	NS*
Fever > 38.5 °C during initial infection (%)	20 (24)	9 (19)	NS*
Smoke exposure (%)	18 (22)	4 (9)	NS*
Main presenting symptom apnoea (%)	0	3 (7)	NS*
respiratory insufficiency (%)	7 (8)	19 (40)	<0.001*
feeding problems (%)	14 (17)	8 (16)	NS*
dyspnea (%)	42 (51)	9 (18)	<0.001*
not specified (%)	20 (24)	8(16)	NS*

* Chi square test

\# Mann-Whitney U test

NS not significant

10.4.2 Wheezing and coughing symptoms during follow-up

Follow-up characteristics are shown in table 10.2 and 10.3. In total, 241 episodes of wheezing were reported. All but two episodes were accompanied by a runny nose and coughing. Recurrent wheezing was reported in 51 (61%) of the infants with signs of airflow limitation and in 10 (21%) of the infants without signs of airflow limitation during the initial RSV LRTI ($p<0.001$).

Table 10.2 *Respiratory symptoms during first year following RSV LRTI (n=130)*

Follow-up findings	Median	Range
Number of disease episodes	6	1–12
Number of episodes of wheezing	2	0–11
Number of days of wheezing	11	0–135
Number of episodes of coughing	6	0–12
Number of days of coughing	75	0–316
Mean during of episode of coughing (days)	14	1–31

One year after hospitalization for RSV LRTI the family doctors of the infants were contacted by telephone. An asthma diagnosis had been made by the general practitioner in 42 (32%) infants. Recurrent wheezing was noted in 37 (88%) of these cases with physician-diagnosed asthma. Physician-diagnosed asthma was reported in 35 (42%) of the infants with signs of airflow limitation and in 7 (15%) of the infants without signs of airflow limitation during the initial RSV LRTI ($p=0.003$).

During the initial RSV LRTI mechanical ventilation was required in significantly more infants without signs of airflow limitation than in infants with signs of airflow limitation (table 10.1). We therefore separately investigated the relation between signs of airflow limitation during initial RSV LRTI and recurrent wheezing or physician-diagnosed asthma in non-ventilated infants. Recurrent wheezing was reported in 39 (53%) of the 73 infants with signs of airflow limitation and in 4 (14%) of the 28 infants without signs of airflow limitation during the initial RSV LRTI ($p<0.001$). Physician-diagnosed asthma was found in 28 (38%) infants of the 73 infants with signs of airflow limitation and in 2 (7%) of the 28 infants without signs of airflow limitation during the initial RSV LRTI ($p<0.001$).

During follow-up 8 (7%) children had to be readmitted to the hospital for wheezing respiratory illness. All had signs of airflow limitation during initial RSV LRTI. One child had to be readmitted twice. Two of the re-admissions occurred during the first month following discharge.

Four children (3%)HT did not have any episode of coughing during follow-up, 75 children (58%) had between one and five episodes of coughing and 51 children (39%) had six or more episodes of coughing during the follow-up period. All episodes of coughing were accompanied by a runny nose. Striking was that in 36 children (28%) ≥100 days of coughing were reported during the follow-up period. Of these children, 6 (17%) never wheezed during follow-up.

Table 10.3 *Airway morbidity during a one year follow-up period following RSV LRTI with and without signs of airflow limitation (n=130)*

Follow-up findings	Signs of airflow limitation during initial RSV LRTI (*n*=83)	No signs of airflow limitation during initial RSV LRTI (*n*=47)	*p*-value
Number of disease episodes per infant (median)	6	5	0.013#
Recurrent wheezing (%)	51 (61)	10 (21)	<0.001*
Number of wheezing episodes (median)	2	1	<0.001#
Number of days of wheezing per infant (median)	15	3	0.007#
Number of coughing episodes (median)	5	4	0.031#
Number of days of coughing (median)	82	54	NS#
Use of inhalation steroids (%)	33 (40)	10 (21)	0.024*
Use of bronchodilating inhalants (%)	42 (51)	12 (25)	0.004*
Hospitalization for airway symptoms during follow-up (%)	8 (10)	6 (13)	NS*
Physician diagnosis of asthma (%)	35 (42)	7 (15)	0.003*

* Chi square test

\# Mann-Whitney U test

NS not significant

10.4.3 Prediction of recurrent wheezing and physician-diagnosed asthma following RSV LRTI

The relation between separate clinical characteristics or symptoms during initial RSV LRTI and recurrent wheezing or physician-diagnosed asthma are shown in table 10.4a. A statistically significant relation was only found between signs of airflow limitation during the initial RSV LRTI and recurrent wheezing during the first year of follow-up. A comparable association was found between signs of airflow limitation during the initial RSV LRTI and physician-diagnosed asthma. Signs of airflow limitation during the initial RSV LRTI appeared an independent predictor of recurrent wheezing and physician-diagnosed asthma using multiple linear regression analysis (R^2 = 0.21 respectively 0.13) (table 10.4b). The relative risk of recurrent wheezing in infants without signs of airflow limitation during initial RSV LRTI was 0.29 (95% CI 0.16 – 0.56). For non-ventilated infants without signs of airflow limitation this was 0.20 (95% CI 0.08 – 0.52). The relative risk of physician-diagnosed asthma in infants without signs of airflow limitation during initial RSV LRTI was 0.35 (95% CI 0.16 – 0.75). For non-ventilated infants without signs of airflow limitation this was 0.18 (95% CI 0.05 – 0.65).

Table 10.4a *Predictive value of separate candidate predictors for recurrent wheezing and physician diagnosed asthma following RSV LRTI (n=130) using univariate logistic regression analysis*

Candidate predictor	*p* value	
	Recurrent wheezing	Physician diagnosed asthma
Signs of airflow limitation during initial RSV LRTI	0.001	0.006
Paternal history of asthma	0.26	0.89
Maternal history of asthma	0.37	0.53
Parental history of asthma	0.22	0.72
Mechanical ventilation during initial RSV LRTI	0.87	0.39
Sex	0.64	0.09
Fever during initial RSV LRTI	0.76	0.49
Prematurity	0.39	0.32
Smoke exposure	0.82	0.71
Chronological age (in weeks)*	0.30	0.22

* analysis after log transformation

Table 10.4b *Forward stepwise logistic regression analysis of candidate predictors for recurrent wheezing following RSV LRTI (n=130)*

Step	Candidate predictor	*p* value	
		Recurrent wheezing	Physician-diagnosed asthma
1	Signs of airflow limiation during initial RSV LRTI	<0.001	<0.001
	Not included		
	Mechanical ventilation during initial RSV LRTI	0.10	0.10
	Sex	0.31	0.40
	Parental history of asthma	0.36	0.72
	Smoke exposure	0.45	0.80
	Age (weeks)*	0.92	0.10

* analysis after log transformation

10.5 Discussion

In the present study we show that 47% of infants hospitalized for RSV LRTI reported recurrent wheezing during a one year follow-up. The incidence of recurrent wheezing during follow-up was significantly lower for infants without signs of airflow limitation (21%) than for infants with signs of airflow limitation during the acute RSV infection (61%). Signs of airflow limitation during initial RSV LRTI appeared to be an

independent predictor for subsequent recurrent wheezing. The same pattern was seen for physician-diagnosed asthma. To our knowledge, this is the first longitudinal report estimating the risk for recurrent wheezing and physician-diagnosed asthma in a cohort of children hospitalized for RSV LRTI without signs of airflow limitation. Moreover, the inverse relation between the absence of signs of airflow limitation and subsequent recurrent wheezing is the first clinical predictor for the development of recurrent wheezing following RSV LRTI.

It has been difficult to quantify respiratory symptoms following RSV bronchiolitis. Some studies were performed retrospectively, inquiring about initial respiratory symptoms years later.[1;3;4] Thus far, prospective studies have assessed respiratory symptoms at various time intervals using standardized questionnaires, which were filled in by parents. In these studies respiratory symptoms are collected closer to the period when the symptoms occurred than in retrospective studies.[6] However, it is conceivable that even parental recall of symptoms over a one-year period is biased. In the present study these limitations were overcome by the daily information on airway symptoms from the diaries.

A limitation of the present study is that physical examination during initial RSV LRTI was performed by different physicians. In addition, there are no follow-up data of healthy age-matched controls. Therefore, we can not determine whether infants without signs of airflow limitation during RSV LRTI have normal or increased incidence of recurrent wheezing.

Several studies attempted to delineate clinical predictors for airway morbidity following RSV LRTI. In these studies different inclusion criteria were used. In some studies proven RSV infection during LRTI was required for inclusion,[3-5;12;13] while in others wheezing on auscultation was a prerequisite for inclusion.[6;8;14;15] Attempts to associate recurrent wheezing following RSV LRTI with clinical and social parameters, including sex, age and disease severity were not successful.[3;4;6;14;15] In the present study we confirmed that these clinical parameters have no predictive value for airway morbidity following RSV LRTI.

In the present study parental history of asthma and airway morbidity following RSV LRTI were not associated. The prevalence of recurrent wheezing and lung function changes following RSV LRTI have been shown to be unrelated to atopy.[11;13;15] For this reason, we studied the predictive value for airway morbidity following RSV LRTI of maternal and paternal history of asthma but not eczema, rhinitis, allergy or other atopy related symptoms in family members. It has been suggested recently that asthma in the mother, but not in the father, is a risk factor for asthma in infants who were not sensitized to *Alternaria* in the U.S. Southwest desert environment.[16] In a different study it was also suggested that childhood asthma might be more related to maternal than paternal factors. This form of probably non-atopic asthma could be closely related to asthmatic symptoms following RSV LRTI.[11] In the present study we did not find an association between parental history of asthma and recurrent wheezing following RSV LRTI, which is in line with most previous studies.[3;14;15] The different findings with respect to the role of maternal asthma as a risk factor for childhood asthma could be explained by climate or other environmental differences between the studies. On the other hand, airway morbidity following RSV LRTI could be a separate disease with a different pathogenesis from other closely related forms of early onset non-atopic asthma.

Exposure to cigarette smoke before or after RSV LRTI was no risk factor for subsequent recurrent wheezing, which is in line with previous studies.[6;17] In the present study we did not investigate maternal cigarette smoking during pregnancy. Recently it was shown that maternal cigarette smoking during pregnancy is a risk factor for wheezing during early childhood[18]. Whether this is also true for wheezing following RSV LRTI is not known. The absence of a relation between cigarette smoke exposure and airway morbidity following RSV LRTI is in striking contrast with findings in studies on risk factors for asthma in infants who were not hospitalized for RSV.[19] This discrepancy might be explained by a smaller number of follow-up studies on RSV than childhood asthma. However, it is conceivable, again, that airway morbidity following RSV LRTI is a separate disease with a different pathogenesis from other closely related forms of early onset non-atopic asthma.

It has been shown by Martinez and colleagues[20] that lower levels of lung function in newborns are associated with subsequent incidence of lower respiratory tract illness with or without wheezing in the first year of life. It was suggested that RSV lower respiratory tract illness might also result from congenital lower levels of lung function, but this still needs to be established.[11]

Detailed quantitative information of respiratory symptoms during follow-up was obtained. Among infants with recurrent wheezing, large differences in frequency of airway symptoms were shown. Parents of some infants only reported one or two episodes for which they did not consult a physician whereas others reported frequent episodes of airway symptoms for which even hospitalization was required. Our study also shows that some children report more than 100 days of coughing during a follow-up period of one year without any report of wheezing respiration. It has been shown recently that a large proportion of children with cough as a sole symptom can be diagnosed as having asthma.[21-23] Although no healthy controls were included in our study, it appears likely that not only recurrent wheezing but also recurrent or persistent cough may be associated with RSV LRTI.

In conclusion, airflow limitation during RSV LRTI is a useful predictor for the development of recurrent wheezing and physician-diagnosed asthma during early childhood. We hypothesize that airway morbidity after RSV LRTI is a separate form of childhood asthma because risk factors well-known in childhood asthma do not appear to play a role in airway morbidity after RSV LRTI.

References

1 Murray M, Webb MSC, O'Callaghan C, Swarbrick AS, Milner AD. Respiratory status and allergy after bronchiolitis. Arch Dis Child 1992; 67:482-487.

2 Hall CB, Hall WJ, Gala CL, McGill FB, Leddy JP. Long-term prospective study in children after respiratory syncytial virus infection. J Pediatr 1984; 105:358-364.

3 Pullan CR, Hey EN. Wheezing, asthma and pulmonary dysfunction 10 years after infection with respiratory syncytial virus infection in infancy. BMJ 1982; 284:1665-1669.

4 Sims DG, Downham MA, Gardner PS. Study of 8-year-old children with a history of respiratory syncytial virus bronchiolitis in infancy. BMJ 1978; 1:11-17.

5 Eisen AH, Bacal HL. The relationship of acute bronchiolitis to bronchial asthma - a 4 tot 14 year follow-up. Pediatrics 1963; 31:859-861.

6 Sly PD, Hibbert ME. Childhood asthma following hospitalization with acute viral bronchiolitis in infancy. Pediatr Pulmonol 1989; 7:153-158.

7 Gurwitz D, Mindorff C, Levinson H. Increased evidence of bronchial reactivity in children with a history of acute bronchiolitis. J Pediatr 1981; 98:551-555.
8 McConnochie KM, Mark JD, McBride JT, Hall WJ, Brooks JG, Klein SJ, Miller RL, McInerny TK, Nazarian LF, McWhinney JB. Normal pulmonary function measurements and airway reactivity in childhood after mild bronchiolitis. J Pediatr 1985; 107:54-58.
9 Dezateux C, Fletcher ME, Dundas I, Stocks J. Infant respiratory function after RSV-proven bronchiolitis. Am J Respir Crit Care Med 1997; 155:1349-1355.
10 Merth IT, de Winter JP, Zonderland HM, Brand R, Quanjer PH. Pulmonary function during the first year of life following acute viral bronchiolitis. Am J Respir Crit Care Med 1996; 154:689-694.
11 Stein RT, Sherill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13. Lancet 1999; 354:541-545.
12 Webb MSC, Henry RL, Milner AD, Stokes GM, Swarbrick AS. Continuing respiratory problems three and a half years after acute viral bronchiolitis. Arch Dis Child 1985; 60:1064-1067.
13 Mok JYQ, Simpson H. Symptoms, atopy, and bronchial reactivity after lower respiratory infection in infancy. Arch Dis Child 1984; 59:299-305.
14 McConnochie KM, Roghmann KJ. Predicting clinically significant lower respiratory tract illness in childhood following mild bronchiolitis. Am J Dis Child 1985; 139:625-630.
15 Sims DG, Garner PS, Weightman D, Turner MW, Soothill JF. Atopy does not predispose to RSV bronchiolitis or postbronchiolitic wheezing. BMJ 1981; 282:2086-2091.
16 Halonen M, Stern DA, Lohman C, Wright AL, Brown MA, Martinez FD. Two subphenotypes of childhood asthma that differ in maternal and paternal influences on asthma risk. Am J Respir Crit Care Med 1999; 160:564-570.
17 Duff AL, Pomeranz ES, Gelber LE, Weyman Price G, Farris H, Hayden FG, Platts-Mills TAE, Heymann PW. Risk factors for acute wheezing in infants and children: viruses, passive smoke, and IgE antibodies to inhalant allergens. Pediatrics 1997; 92:535-540.
18 Stein RT, Holberg CJ, Sherrill D, Wright AL, Morgan WJ, Taussig L, Martinez FD. Influence of parental smoking on respiratory symptoms during the first decade of life: the Tucson Children's Respiratory Study. Am J Epidemiol 1999; 149:1030-1037.
19 Tager I.B., Hanrahan JP, Tosteson TD, Castile RG, Brown RW, Weiss.S.T., Speizer FE. Lung function, pre- and post-natal smoke exposure, and wheezing in the first year of life. Am Rev Respir Dis 1993; 147:811-817.
20 Martinez FD, Morgan WJ, Wright AL, Holberg CJ, Taussig LM. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. N Engl J Med 1988; 319:1112-1117.
21 Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Joseph LK, Symington P, O'Toole S, Myint SH, Tyrell DAJ, Holgate ST. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. BMJ 1995; 310:1225-1228.
22 Pattemore PK, Asher MI, Harrison AC, Mitchell EA, Rea HH, Stewart AW. The interrelationship among bronchial hyperresponsiveness, the diagnosis of asthma, and asthma symptoms. Am Rev Respir Dis 1990; 142:549-554.
23 Kelly YJ, Brabin BJ, Milligan PJM, Reid JA, Heaf D, Pearson MG. Clinical significance of cough and wheeze in the diagnosis of asthma. Arch Dis Child 1996; 75:489-493.

11 Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one year follow-up study

L Bont, CJ Heijnen, A Kavelaars, WMC van Aalderen, F Brus, JThM Draaisma, SM Geelen, JLL Kimpen

Am J Respir Crit Care Med 2000;161:1518-1523

11.1 Abstract

Respiratory syncytial virus (RSV) bronchiolitis is associated with subsequent recurrent wheezing episodes. To determine whether cytokine responses during infection can be of predictive value for the development of recurrent wheezing, we performed a follow-up study in 50 hospitalized children with RSV bronchiolitis. Monocyte and lymphocyte cytokine responses *in vitro* were studied during the acute phase of disease, and again during the convalescent phase, 3-4 weeks later. Monocyte cytokine responses, including interleukin-10 (IL-10), were measured in whole blood cultures, stimulated with lipopolysaccharide and interferon-g (LPS+IFNγ). In addition, T cell cytokine responses, including IFNγ and IL-4 production, were measured in whole blood cultures, stimulated with phytohemaglutinin (PHA) or αCD2+αCD28. Cytokine responses were analysed in relation to the development of recurrent episodes of wheezing, documented by parents in a diary during a one year follow-up period. IL-10 responses during the acute phase of RSV bronchiolitis were comparable to healthy controls. During the convalescent phase, IL-10 responses were significantly increased in patients as compared to healthy controls ($p<0.001$). At follow-up, 27 children (58%) had recurrent episodes of wheezing. IL-10 levels, measured during the convalescent phase, were significantly higher in patients who developped recurrent wheezing during the year following RSV bronchiolitis than in patients without recurrent episodes of wheezing ($p=0.006$). Moreover, IL-10 responses during the convalescent phase correlated significantly with the number of wheezing episodes ($r=0.42$, $n=46$, $p=0.004$). Interestingly, no association was found between IFNγ responses, IL-4 responses or IFNγ/IL-4 ratios and recurrent wheezing. We conclude, that monocyte IL-10 responses *in vitro* upon stimulation with nonspecific stimuli may have predictive value for the development of recurrent wheezing following RSV bronchiolitis. Moreover, our results indicate, that not only allergen-driven Th2 cytokine responses can lead to asthmatic symptoms, but also virus-induced changes in cytokine responses may result in asthmatic symptoms.

11.2 Introduction

The occurrence of recurrent episodes of wheezing in early childhood following respiratory syncytial virus bronchiolitis has been well documented (1-5). Actually, RSV bronchiolitis is followed by recurrent wheezing in 20-80% of the cases (6-8). It is thought, that these wheezing episodes are triggered by viral upper respiratory tract infections and they appear to be independent of atopy (9). In addition, follow-up studies show bronchial hyperresponsiveness 4 to 8 years after hospitalization for RSV bronchiolitis (6,7,10).
Although RSV infection induces cytokine production by a number of cells in vivo and in vitro, the relation of these cytokine responses to recurrent wheezing is largely unknown. CD4+ T cells can be functionally divided into Th1 and Th2 cells (11). This division is based on the profile of cytokine production. Th1 cells selectively secrete IFNγ and promote cell-mediated immunity. In contrast, Th2 cells secrete IL-4, IL-5 and IL-13. These cytokines are involved in humoral immunity and are thought to contribute to allergic asthmatic inflammation. Recent studies suggest that a Th2 cytokine profile during RSV bronchiolitis is associated with wheezing during follow-up (12,13).
To our knowledge, the role of monocytes/macrophages in recurrent wheezing following RSV bronchiolitis is unknown. However, it is evident that alveolar macrophages participate in the immune response during RSV infections. Alveolar macrophages recovered from bronchoalveolar lavage (BAL) fluid from children with severe RSV bronchiolitis are quantitatively the most important cell type (14). *In vitro* data show that monocytes can readily be infected by RSV (15,16). They have the ability to produce a spectrum of cytokines, including IL-12 and IL-10. Interleukin-12 is required for the initiation of the antiviral immune response (17,18), whereas IL-10 has several properties, including down-regulation of cytokine production by Th1-like T cells and inhibition of antigen presentation by antigen presenting cells (APC) (19,20). Alveolar macrophages in BAL fluid from RSV bronchiolitis patients show increased expression of pro-inflammatory cytokines, including IL-1b and TNFα, as compared to healthy controls (21). In addition, *in vitro* data show induction of IL-6, IL-8 and IL-10 production by macrophages infected with RSV (22).
The aim of this study was to determine whether cytokine responses during the acute and convalescent phase of RSV bronchiolitis are associated with the subsequent development of recurrent wheezing. We therefore studied T cell and monocyte cytokine responses in hospitalized children with RSV bronchiolitis and related these cytokine responses to recurrent episodes of wheezing in a one year follow-up period.

11.3 Methods

11.3.1 Study population

Fifty children were included during one winter epidemic in 4 hospitals in the Netherlands (Wilhelmina Children's Hospital, Utrecht; Beatrix Children's Hospital, Groningen; St. Elisabeth Hospital, Tilburg; Rijnstate Hospital, Arnhem). Inclusion criteria were: hospital admission, lower respiratory tract symptoms, age < 13 months and immunofluorescence for RSV infection of epithelial cells in nasopharyngeal secretions. Infants with wheezing illness prior to RSV bronchiolitis were not included. One investigator (L.B.) took the history of atopy of parents, grandparents and siblings (asthma, documented food allergy,

eczema, hay fever) and inquired whether either parent had smoked in the presence of the infant during the follow-up period. Twenty-seven control children aged < 13 months without evidence of atopy or infection were selected for this study during the same winter season. Included were infants prior to minor surgery, prior to cardiac surgery in the absence of hemodynamic compromise, healthy prematurely born infants, healthy infants screened for congenital disorders and infants with mild anemia. The study was approved by the Medical Ethical Committee in all participating centers. Parents of subjects and controls gave written informed consent.

11.3.2 Whole blood cultures

Heparinized venous or arterial blood was taken within 24 hours after admission, from subjects in the Wilhelmina Children's Hospital, Utrecht (n=24). Three to four weeks later, during the convalescent phase, heparinized blood was taken from all subjects (n=50). Freshly taken heparinized blood was diluted 1:10 in RPMI 1640 medium (Life Technologies, Grand Island, NY) and aliquoted (150 µl) into 96-well culture plates (Nunc International, Denmark).

The whole blood culture stimulated with LPS is a suitable *ex vivo* method to study monocyte cytokine production under conditions in which many of the physiologically relevant cellular interactions remain intact (23). To induce monocyte IL-10 and IL-12 production, lipopolysaccharide (LPS) (100ng/ml)+IFNg (20 ng/ml) was added and cultures were incubated for 48 hours at 37°C in 5% CO_2. It has been shown that maximal monocyte IL-10 production is observed after 48 hours, which is relatively late compared to that of monocyte pro-inflammatory cytokines (20). Also monocyte IL-12 production is (sub)optimally induced after 48 hours stimulation (24). Furthermore, it has been established that monocytes are the main producers of IL-10 and IL-12 in LPS stimulated whole blood cultures (25).

To induce lymphocyte cytokine production, phytohaemagglutinin (PHA) (50µg/ml) or anti-CD2,1 (1:12000)+anti-CD2,2 (1:12000)+anti-CD28 (1:3000) monoclonal antibodies (anti-CD2/28 Moabs, CLB, Amsterdam, The Netherlands) were added and cultures were incubated for 48 hours at 37°C in 5% CO_2.

All cultures were performed in quadruplicate. Pooled supernatants were kept at -70°C.

11.3.3 Cytokine assays

In supernatants of LPS+IFNγ stimulated blood cultures IL-12 and IL-10 were measured. In supernatants of PHA stimulated cultures IFNγ and IL-4 were measured. Concentrations of IL-10, IFNγ and IL-4 were determined using ELISA kits supplied by the Dutch Laboratory for Blood Transfusion (CLB, Amsterdam, the Netherlands). The detection limit for IL-10 was 2.5 pg/ml, for IFNγ 25 pg/ml and for IL-4 1.0 pg/ml. Concentrations of IL-12 were determined using ELISA kit from R&D (Oxon, United Kingdom), the detection limit was 7.8 pg/ml. This assay recognizes only the IL-12 heterodimer and not the individual subunits of the dimer. When cytokine values were not detectable, for statistical analysis the minimum detectable level was used. When IL-10 responses were above the maximum detectable level (300 pg/ml), this level was used.

11.3.4 Follow-up evaluations

Follow-up data were collected during one year following discharge. Follow-up was performed using diaries, which were developed for this study. One investigator instructed parents how to use the diary. Starting three weeks after discharge from the hospital, parents noted the presence of "coughing" and "wheezing" on a daily base. A disease episode was defined as the presence of symptoms for two or more consecutive days. At the end of the follow-up period, patients were classified as "recurrent wheezing" if more than one episode of wheezing was noted following discharge. In addition, at the end of the study period, one investigator contacted the general practitioners of the patients by telephone and inquired if "asthma" had been diagnosed.

11.3.5 Statistical analysis

Cytokine production and IFNγ/IL-4 ratios were analyzed after logarithmic transformation. Mean (geometric mean) and standard error of mean (SEM) of cytokine levels are calculated of logarithmically transformed values. Chi-square test was used to evaluate whether gender, the need for mechanical ventilation and the presence of a positive family history of atopy were associated with recurrent wheezing. Differences in age at onset of disease, IL-10 and IL-12 responses and IFNγ/IL-4 ratios between infants with and without recurrent wheezing and infants with and without a family history of atopy were analyzed with unpaired Student's t-test. The relation between cytokine response in the acute and convalescent phase were analyzed with paired Student's t-test. Pearson's correlation coefficient was used to analyze the relation between cytokine levels and the number of reported wheezing episodes. All tests of significance were two-sided.

11.4 Results

11.4.1 Subject characteristics

The investigated population consisted of 50 patients and 27 controls. Twenty-nine patients (58%) were boys, median age was 3 months. Eleven patients (22%), including 3 pairs of twins, were born prematurely (range: 29 4/7 - 36 5/7 weeks). In the control group, 17 children were boys (63%), the median age was 4 months and 2 controls were born prematurely. Thirty-six patients (72%) and 18 controls (67%) had a positive family history of atopy. Respiratory distress was present in all children. Three infants had had apnea prior to admission. Fourteen subjects (28%) needed mechanical ventilation. In one child chronic lung disease was diagnosed, none of the patients had cardiac disease or an immunodeficiency. None of the patients received ribavirin or systemic anti-inflammatory agents, including corticosteroids. Patients did not receive inhaled steroids during RSV bronchiolitis. All patients survived.

11.4.2 Cytokine responses

Cytokine responses in LPS+IFNγ stimulated whole blood cultures from patients during the acute phase (n=24) and convalescent phase (n=50) are shown in Figure 11.1 and 11.2. During the acute phase, IL-12 production was significantly lower in patients than in controls (geometric mean 28 vs 66 pg/ml, $p = 0.007$). During the convalescent phase, IL-12 responses in patients increased to levels that were not significantly different from controls (44 pg/ml). In contrast, the amount of IL-10 produced during the acute phase

was not significantly different from controls (26 vs 38 pg/ml). During the convalescent phase, however, IL-10 production (112 pg/ml) was significantly higher than in the acute phase ($p<0.001$) and higher compared to controls ($p < 0.001$).
In PHA-stimulated whole blood cultures of patients during the acute phase of disease, both IL-4 and IFNγ responses were low (n=24). Actually, in 11 patients, levels of either cytokine were below detection limits. During the acute phase of disease, IL-4 production was lower than in controls (2.9 vs 9.6 pg/ml, $p < 0.001$), as were IFNγ responses (89 vs 602 pg/ml, $p = 0.001$). In the convalescent phase both IL-4 and IFNγ responses returned to values that were not significantly different from controls (n=49). In one patient no supernatant of PHA-stimulated whole blood cultures was collected for technical reasons.
In cultures stimulated with αCD2+αCD28 the same response pattern of IL-4 and IFNγ were seen. During the acute phase, in comparison to controls, we found decreased production of IL-4 (4.2 vs 8.1 pg/ml, $p = 0.03$) and IFNγ (8.1 vs 186 pg/ml) ($p < 0.001$). In the convalescent phase IL-4 response (6.6 pg/ml) and IFNγ response (170 pg/ml) were comparable to controls.

11.4.3 Follow-up data

Diaries were returned of 46 (92%) patients in follow-up, whereas 4 patients were lost in follow-up. Twenty-seven children (59%) had 2 or more episodes of wheezing (range 2-11 wheezing episodes). In patients requiring mechanical ventilation, 43% had recurrent episodes of wheezing in the follow-up period, which was not significantly different from-non-ventilated infants. The number of episodes of wheezing correlated strongly with the number of episodes of coughing ($r = 0.76$, $p<0.001$).
One or more episodes of coughing in follow-up was documented in 43 patients (93%). In 4 patients (9%) one episode of coughing was noted, in 7 children (15%) 2 episodes of coughing were noted and in 32 patients (70%) 3 or more episode of coughing was noted. Asthma was diagnosed by a physician in 16 patients (35%). These patients had all more than one documented episode of wheezing.
A positive history of atopy was noted in 19 infants with recurrent wheezing (70%) and in 15 infants without recurrent wheezing (79%), which was not significantly different. Six infants were exposed to tobacco smoke by at least one parent during follow-up, three had recurrent episodes of wheezing. Age at onset of disease and gender were not associated with the occurrence of recurrent wheezing.
IL-10 responses during the convalescent phase were significantly higher in infants with recurrent wheezing than without recurrent wheezing ($p=0.006$) (Figure 11.3). The difference was found between IL-10 responses in the convalescent phase in infants with and without physician diagnosed asthma ($p=0.004$). Moreover, IL-10 levels during the convalescent phase correlated with the number of episodes of wheezing ($r=0.42$, $p=0.004$) (Figure 11.4).

Figure 11.1 *Ex vivo IL-12 production during the acute and convalescent phase of RSV bronchiolitis. Blood was obtained from RSV bronchiolitis patients within 24 hours after admission to the hospital (acute phase) and 3-4 weeks later (convalescent phase) and controls. Whole blood cultures were stimulated for 48 hours with LPS (100ng/ml) and IFNγ (20 ng/ml). Data represent individual values and mean ± SEM. Data were analyzed after log transformation.*

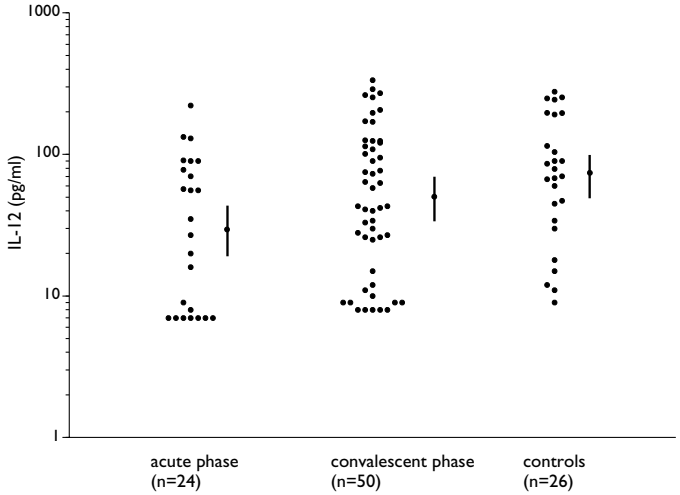


Figure 11.2 *Ex vivo IL-10 production during the acute and convalescent phase of RSV bronchiolitis. Samples and cultures as described in the legends to Figure 11.1. Data represent individual values and mean ± SEM. Data were analyzed after log transformation.*

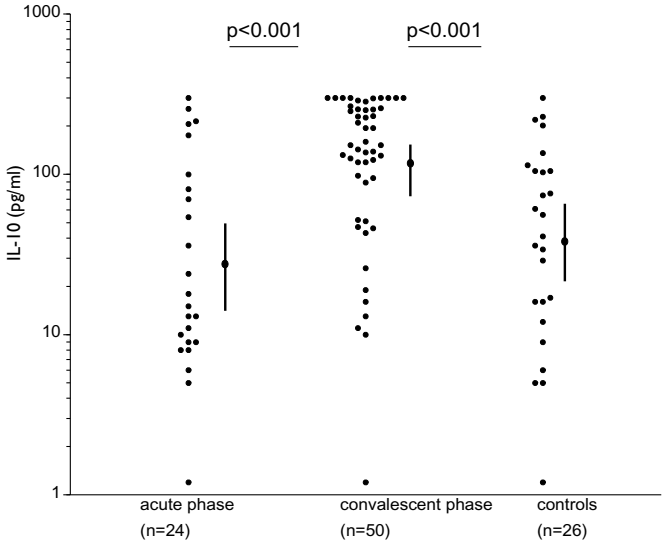
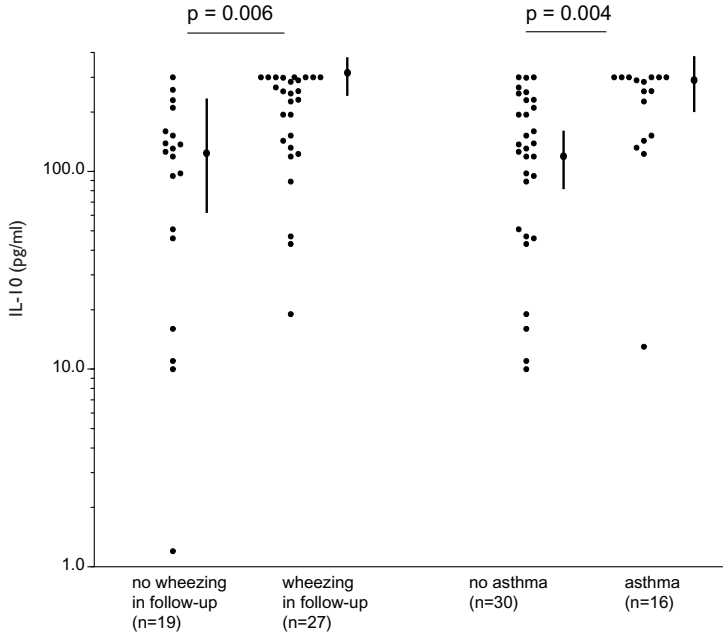


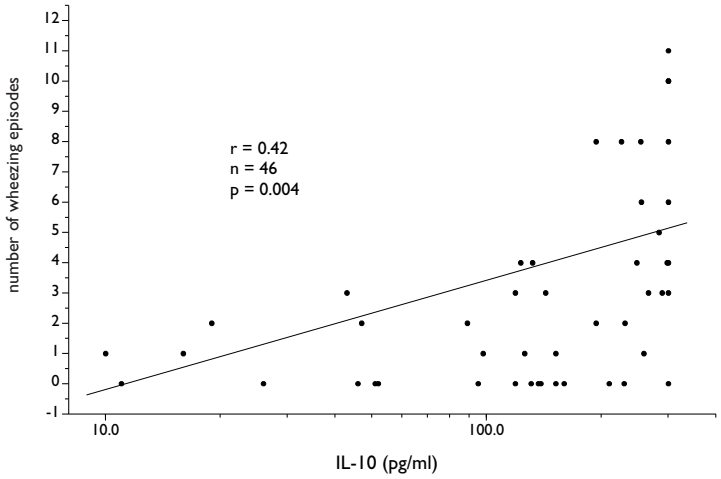
Figure 11.3 *Ex vivo IL-10 production in patients with and without subsequent recurrent wheezing and physician-diagnosed asthma.*
Blood was obtained from RSV bronchiolitis 3-4 weeks after admission (convalescent phase) and controls. Cultures as described in the legends to Figure 11.1. Data represent individual values and mean ± SEM. Data were analyzed after log transformation.



We considered the possibility that the association between IL-10 responses and recurrent wheezing could only be found during the first months of the follow-up period following RSV bronchiolitis. We therefore evaluated the association between IL-10 responses during the convalescent phase and wheezing during the last 3 months of the study period (the winter season). During this period 20 patients had ≥ 1 wheezing episode, and again, a difference was found in IL-10 responses was found between infants with and without a wheezing episode ($p = 0.02$).

Interleukin-12 responses were not associated with recurrent wheezing during follow-up. In addition, no differences in IL-4 and IFNγ responses in both PHA and αCD2+αCD28 stimulated blood cultures were found between wheezing and non-wheezing infants. Moreover, IFNγ/IL-4 ratios in PHA stimulated cultures (Figure 11.5a) during the convalescent phase were comparable for wheezing and non-wheezing infants. As expected, in infants with a positive family history of atopy, decreased IFNγ/IL-4 ratios were found in PHA stimulated cultures ($p<0.05$) (Figure 11.5b). In αCD2+αCD28 stimulated cultures the same association was seen, although this did not reach a significant level. Monocyte cytokine responses, including IL-10 responses during the convalescent phase, were not associated with family history of atopy.

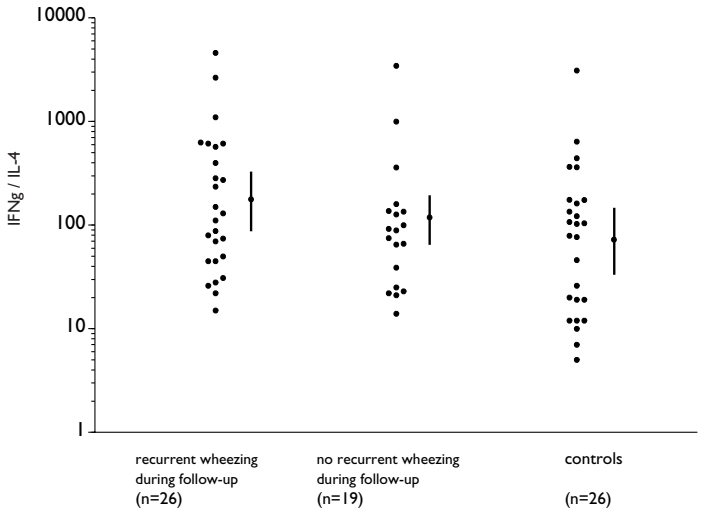
Figure 11.4 *Relation between ex vivo IL-10 production and number of wheezing episodes during a one year follow-up period.*
Blood was obtained from RSV bronchiolitis patients 3-4 weeks after admission (convalescent phase). Cultures as described in the legends to Figure 11.1. Data represent individual values. Linear regression line is drawn.



11.5 Discussion

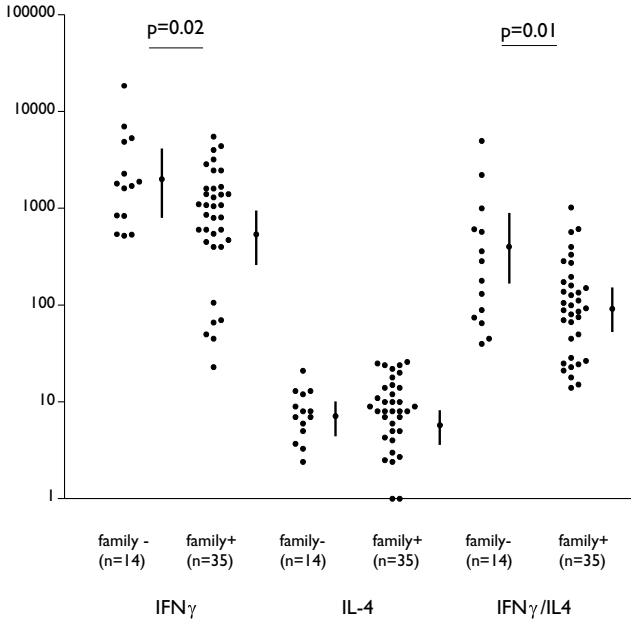
The results of this study demonstrate that increased monocyte IL-10 response *in vitro* upon stimulation with aspecific stimuli during the convalescent phase of RSV bronchiolitis is associated with the development of subsequent recurrent episodes of wheezing, during a one year follow-up period. Moreover, there was a highly significant correlation between IL-10 production in the convalescent phase and the number of wheezing episodes. The same association was found between IL-10 response and physician-diagnosed asthma. Recurrent wheezing during the year following clinical bronchiolitis was not associated with a family history of atopy. In agreement with the latter finding, T cell cytokine responses (IFNg and IL-4) in vitro upon stimulation with nonspecific stimuli were not associated with recurrent wheezing.
Clinical risk factors for the development of recurrent wheezing following RSV bronchiolitis were established by previous investigators (9,26-28). These risk factors include male gender, low age at onset of disease, and disease severity. In our study, boys were not more likely to develop recurrent wheezing than girls, consistent with findings of Sims et al., but not with findings of McConnochie and Roghmann, who found an increased risk for boys (6,29). Furthermore, we did not find a relation between either age at onset or disease severity during RSV bronchiolitis and subsequent recurrent wheezing, consistent with most other studies (3,6,7). Finally, some reports indicate an increased risk for post-bronchiolitis wheezing in children with a positive family history of atopy, but most recent reports, including ours do not support this suggestion (6,8,30).

Figure 11.5a *Relation between ex vivo IFNγ/IL-4 ratios in PHA stimulated whole blood cultures and the development of recurrent wheezing following RSV bronchiolitis.*
Blood was obtained from RSV bronchiolitis patients 3-4 weeks after admission to the hospital (convalescent phase) and from controls. Whole blood cultures were stimulated for 48 hours with PHA (50μg/ml). Data represent individual values and mean ± SEM. Data were analyzed after log transformation.



Ex-vivo IL-10 production was significantly increased during the convalescent phase of RSV infection. Although IL-10 can be produced by different cell types, including monocytes, Th2 cells and B cells, it has been shown that IL-10 in LPS stimulated whole blood cultures, IL-10 is most likely monocyte derived (19,20,25). In the LPS+IFNg stimulated cultures in the present study, IL-8 was decreased significantly (data not shown), indicating that the increased IL-10 response is not explained by a general increase in monocyte cytokine responses.
Two mechanisms can explain changes in cytokine responses by monocytes in peripheral blood during RSV bronchiolitis. The presence of RSV ribonucleic acid (RNA) in the blood and the potential to cause productive infection *in vitro* in monocytes suggest that during RSV bronchiolitis, changes in monocyte function could result from direct infection (15,31). Another explanation is that changes in monocyte cytokine responses are the systemic consequence of local production of cytokines and other mediators by epithelial cells and macrophages in the respiratory tract during RSV infection. Finally, we note that the immune response in respiratory tract and changes in cytokine production by local macrophages are potentially different from what is found in circulating monocytes. More research is required to evaluate whether cytokine responses by circulating monocytes reflect cytokine responses by macrophages in the respiratory tract.

Figure 11.5b *Ex vivo IFNγ and IL-4 production and IFNγ/IL-4 ratios in PHA stimulated whole blood cultures in patients with a positive/negative family history of atopy.*
Samples and cultures as described in the legends to Figure 11.5a. Data represent individual values and mean ± SEM. Data were analyzed after log transformation.



Ex-vivo IL-12 production was significantly decreased during the acute phase of RSV bronchiolitis. Different viruses, including measles virus, have been shown to inhibit IL-12 production in vitro by monocytes/ macrophages (24,32). Although the effect of RSV on IL-12 production by monocytes/macrophages has not been investigated, it is conceivable that, RSV itself effectively inhibits IL-12 production. More study is needed to evaluate whether low IL-12 responses play a role in the pathogenesis of acute RSV bronchiolitis.
We propose two possible mechanisms by which increased production of monocyte IL-10 leads to recurrent wheezing. On the one hand, increased monocyte/macrophage IL-10 responses *in vivo* may result in suppression of Th1 cells and enhancement of Th2 cells by antagonizing IL-12 (33-35). As a result, this could then lead to allergic asthmatic airway inflammation. This latter possibility is not supported by our data, that show the absence of an association between IFNg/IL-4 ratios and recurrent wheezing. On the other hand, it is conceivable that in vivo increased IL-10 production leads to decreased antiviral immunity in the lower airways, as a result of suppression of antigen presentation by pulmonary macrophages. One could then speculate that viral infections in the upper respiratory tract more easily leads to infection and inflammation of the lower respiratory tract, leading to wheezing and bronchial hyperresponsiveness. This explanation is in line with the

clinical picture of wheezing following RSV bronchiolitis, usually associated with upper respiratory symptoms (8).

In this study we found decreased IFNγ and IL-4 responses in patients in the acute phase of RSV bronchiolitis. We recently described this finding and showed in addition, that depressed lymphocyte proliferative responses and T cell cytokine responses are markers of disease severity (36).

Overwhelming evidence is available that a Th2-like cytokine response pattern leads to allergic asthmatic airway inflammation. Therefore, one could hypothesize, that a Th2-like cytokine response pattern also plays a role in recurrent wheezing following RSV bronchiolitis. For example, CD4+ T-cells, specific for the RSV attachment protein (protein G) secrete IL-4 and IL-5, but little IL-2 (i.e. a Th2-like pattern) when stimulated with antigen (37). This hypothesis is supported by the study of Renzi et al. showing an association between a Th2 cytokine response after allergen (*Dermatophagoides farinae)* stimulation 5 months after hospitalization for bronchiolitis, and subsequent wheezing in 26 infants (13). In contrast to our study, in Renzi's study 43% of the patients were negative for RSV and patients requiring mechanical ventilation or with radiographic evidence for bacterial infection were excluded. Interestingly, in our study we did not find evidence for an association between IFNg/IL-4 ratios and the subsequent development of recurrent wheezing. In the present study, IFNγ/IL-4 ratios in both PHA and αCD2+αCD28 stimulated cultures were comparable for infants with and without recurrent wheezing in the follow-up period. We were capable of detecting a lower IFNγ/IL-4 ratio in infants with a family history of atopy, which resulted mainly from differences in IFNγ responses. These data demonstrate that our methods are suitable to detect biologically significant differences in IFNγ/IL-4 ratios. We note, however, that other Th2-like cytokines, including IL-5 and IL-13, have not been measured. When other Th2-like cytokines are used to assess the Th1-Th2 cytokine balance or when other *in vitro* stimuli are used, it is conceivable that other results can be found with respect to role of the Th1-Th2 cytokine balance in recurrent wheezing following RSV bronchiolitis.

We conclude that monocyte IL-10 production increases during the course of RSV bronchiolitis and that increased IL-10 production is associated with the development of recurrent wheezing and physician diagnosed asthma. We did not find support for a role of IFNγ/IL-4 balances in the development of recurrent wheezing following RSV bronchiolitis. This study indicates that not only allergen-driven Th2 cytokine responses can result in asthmatic symptoms, but also virus induced changes in monocyte cytokine responses can lead to asthmatic symptoms.

References

1. Eisen, A.H. and Bacal, H.L. 1963. The relationship of acute bronchiolitis to bronchial asthma - a 4 tot 14 year follow-up. Pediatrics 31:859-861.
2. Gurwitz, D., Mindorff, C., and Levinson, H. 1981. Increased evidence of bronchial reactivity in children with a history of acute bronchiolitis. J. Pediatr. 98:551-555.
3. Hall, C.B., Hall, W.J., Gala, C.L., McGill, F.B., and leddy, j.p. 1984. Long-term prospective study in children after respiratory syncytial virus infection. J. Pediatr. 105:358-364.
4. Henry, R.L., Hodges, I.G.C., Milner, A.D., Stokes, G.M., and Swarbuck, A.S. 1985. Continuing respiratory problems three and a half years after acute viral bronchiolitis. Arch. Dis. Child. 60:1064-1067.

5. McConnochie, K.M., Mark, J.D., McBride, J.T., Hall, W.J., Brooks, J.G., Klein, S.J., Miller, R.L., McInerny, T.K., Nazarian, L.F., and McWhinney, J.B. 1985. Normal pulmonary function measurements and airway reactivity in childhood after mild bronchiolitis. J. Pediatr. 107:54-58.
6. Sims, D.G., Downham, M.A., and Gardner, P.S. 1978. Study of 8-year-old children with a history of respiratory syncytial virus bronchiolitis in infancy. BMJ 1:11-17.
7. Pullan, C.R. and Hey, E.N. 1982. Wheezing, asthma and pulmonary dysfunction 10 years after infection with respiratory syncytial virus infection in infancy. BMJ 284:1665-1669.
8. Murray, M., Webb, M.S.C., O'Callaghan, C., Swarbrick, A.S., and Milner, A.D. 1992. Respiratory status and allergy after bronchiolitis. Arch. Dis. Child. 67:482-487.
9. Sly, P.D. and Hibbert, M.E. 1989. Childhood asthma following hospitalization with acute viral bronchiolitis in infancy. Pediatr. Pulmonol. 7:153-158.
10. Welliver, R.C. and Duff, A.L. 1993. The relationship of RSV-specific immunoglobulin E-antibody responses in infancy, recurrent wheezing and pulmonary function at age 7-8 years. Pediatr. Pulmonol. 15:19-27.
11. Romagnani, S. 1991. Human Th1 and Th2 subsets: doubt no more. Immunol. Today 12:256-257.
12. Roman, M., Calhoun, W.J., Himton, K.L., Avendano, L.F., Simon, V., Escobar, A.M., Gaggero, A., and Diaz, P.V. 1997. respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am. J. Respir. Crit. Care. Med. 156:190-195.
13. Renzi, P.M., Turgeon, J.P., Yang, J.P., Drblik, S.P., Marcotte, J.E., Pedneault, L., and Spier, S. 1997. Cellular immunity is activated and a Th2-response is associated with early wheezing in infants after bronchiolitis. J. Pediatr. 130:584-593.
14. Midulla, F., Villani, a., Panuska, J.R., Dab, I., Kolls, J.K., Merolla, R., and Ronchetti, R. 1993. Respiratory syncytial virus lung infection in infants: immunoregulatory role of infected alveolar macrophages. J. Infect. Dis. 168:1515-1519.
15. Midulla, F., Huang, Y.T., Gilbert, I.A., Cirno, N.M., McFadden, E.R., and Panuska, J.R. 1989. Respiratory syncytial virus infection of human cord and adult blood monocytes and alveolar macrophages. Am. Rev. Respir. Dis. 140:771-777.
16. Panuska, J.R., Cirino, N.M., Midulla, F., Despot, J.E., McFadden, E.R., and Huang, Y.T. 1990. Productive infection of isolated human alveolar macrophages by respiratory syncytial virus. J. Clin. Invest. 86:113-119.
17. Germann, T. and Rude, E. 1995. Interleukin-12. Int. Arch. Allergy. Immunol. 108:103-112.
18. Scott, P. and Trinchieri, G. 1997. IL-12 as an adjuvant for cell mediated immunity. Semin. Immunol. 9:285-291.
19. Rennick, D., Berg, D., and Holland, G. 1993. Interleukin 10: an overview. Progress in Growth Factor Research 4:207-227.
20. De Waal Malefyt, R., Yssel, H., Roncarolo, M.G., Spits, H., and De Vries, J.E. 1992. Interleukin 10. Curr. opinion immunol. 4:314-320.
21. Becker, S., Quay, J., and soukup, j. 1991. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. J. Immunol. 147:4307-4312.
22. Panuska, J.R., Merolla, R., and Rebert, N.A. 1995. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages. J. Clin. Invest. 96:2445-2453.
23. Wilson, B.M., Severn, A., Rapson, N.T., and Hopkins, P. 1991. A convenient whole blood culture system for studying the regulation of tumor necrosis factor release by bacterial lipopolysaccharide. J. Immunol. Methods 139:223-240.
24. McDyer, J.F., Wu, C.Y., and Seder, R.A. 1998. The regulation of IL-12: its role in infectious, autoimmune, and allergic diseases. J. Allergy. Clin. Immunol. 102:11-15.
25. Elenkov, I.J., Webster, E., Papanicolaou, D.A., Fleisher, T.A., Chrousos, G.P., and Wilder, R.L. 1998. Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. J. Immunol. 161:2586-2593.
26. Wang, E.E.L., Law, B.J., and Stephens, D. 1995. Pediatric Investigators Collaborative Network on infections in Canada (PICNIC) prospective study of risk factors and outcomes in patients hospitalized with respiratory syncytial viral lower respiratory tract infection. J. Pediatr. 126:212-219.
27. Opavski, M.A., Stephens, D., and Wang, E.E.L. 1995. Testing models predicting severity of respiratory syncytial virus infections on the PICNIC RSV database. Pediatric Investigators Collaborative Network on Infections in Canada. Arch. Pediatr. Adolsc. Med. 149:1217-1220.
28. Dezateux, C., Fletcher, M.E., Dundas, I., and Stocks, J. 1997. Infant respiratory function after RSV-proven bronchiolitis. Am. J. Respir. Crit. Care. Med. 155:1349-1355.

29. McConnochie, K.M. and Roghmann, K.J. 1984. Bronchiolitis as a possible cause of wheezing in childhood: new evidence. Pediatrics 74:1-10.
30. Mok, J.Y.Q. and Simpson, H. 1984. Symptoms, atopy, and bronchial reactivity after lower respiratory infection in infancy. Arch. Dis. Child. 59:299-305.
31. O'Donnell, D.R., McGarvey, M.J., Tully, J.M., Balfour-Lynn, I.M., and Openshaw, P.J.M. 1998. Respiratory syncytial virus RNA in cells from the peripheral blood during acute infection. J. Pediatr. 133:272-274.
32. Karp, C.L., Wysocka, M., Wahl, L.M., Ahearn, J.M., Cuomo, P.J., and Sherry, B. 1996. Mechanisms of suppresion of cell-mediated immunity by measles virus. Science 273:228-231.
33. Fiorentino, W.F., Bond, M.W., and Mossmann, T.R. 1989. Two types of mouse helper T cells. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. 170:2081-2095.
34. Moore, K.W., Vieira, P., Fiorentino, W.F., Trounstine, M.L., Khan, T.A., and Mossmann, T.R. 1990. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein Barr Virus gene BCRF1. Science 248:1230-1234.
35. De Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M.G., Te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H., and De Vries, J.E. 1991. Interleukin-10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminshing the antigen-presenting capacity of monocytes via downregulation of Class II Major Histocompatibility Complex expression. J. Exp. Med. 174:915-924.
36. Bont, L., Heijnen, C.J., Kavelaars, A., Aalderen, v., W.M.C., Brus, F., Draaisma, J.M.T., Geelen, S.M., Vught, v., H.J., and Kimpen, J.L.L. 1999. Association of peripheral blood cytokine responses with disease severity in respiratory syncytial virus bronchiolitis. Eur. Respir. J.
37. Alwan, W.H., Record, F.M., and Openshaw, P.J.M. 1993. Phenotypic and functional characterization of T cell lines specific for individual respiratory syncyctial virus proteins. J. Immunol. 150:5211-5218.

12 General Discussion

12.1 Introduction

The principal aim of the cohort study described in this report was to investigate the predictive value of clinical and immunological parameters during RSV bronchiolitis for short-term and long-term airway morbidity. The results of the studies have expanded our knowledge on clinical and non-clinical factors during RSV bronchiolitis determining disease outcome (table 12.1).

12.2 Pathogenesis of RSV infection resulting in mechanical ventilation (MV)

The occurrence of RSV bronchiolitis resulting in MV in infants with severe pre-existing pulmonary or cardiac morbidity is likely the direct result of the pre-existing condition (**Chapter 2**). Immunological mechanisms that are either protective or disease-enhancing, are probably of considerably less importance. The most intriguing question, however, has been why neonates (term and preterm) without pre-existent medical conditions are at risk for RSV bronchiolitis resulting in MV.

Airway inflammation is the hallmark of RSV bronchiolitis resulting in MV. Two different mechanisms appear to contribute to airway inflammation. On the one hand, airway inflammation results from necrosis of airway epithelial cells which is directly caused by the cytopathological effect of RSV[1-3]. Decreased cellular immunity in young infants would hereby allow for more widespread viral replication resulting in more extensive damage to the airways. On the other hand, the immune response to RSV may directly damage the airways resulting in inflammation and more airway destruction. Indeed, animal studies have supported the concept that T cells can directly cause airway inflammation[4;5]. In T cell-depleted RSV-infected mice disease was milder as compared to non-depleted infected mice. In addition, transfer of RSV-infected T cells to gamma-irradiated RSV-infected mice resulted in severe respiratory disease and airway inflammation[4]. However, support for a T cell-mediated pathogenesis of naturally-acquired RSV in humans, however, is not available.

Immaturity of the immune system in preterm infants and neonates could play a role in RSV bronchiolitis resulting in MV. Fetal and early post-natal life are associated with a physiological immune deficiency[6-8]. Deficient immune function in neonates is characterized by relatively deficient functioning of innate and antigen-specific immunity[8-10]. Functional deficits of cells of the innate immune system consist of delayed recruitment of neutrophils and monocytes to infected tissue and diminished NK cell cytotoxicity[11]. During the neonatal period antigen-presentation by macrophages and dendritic cells (DC) is less efficient, possibly due to insufficient IL-12 production, resulting in slow development of antigen-specific immunity[7;8;12]. In addition, T cell-mediated responses are delayed during the first 4 to 8 weeks of post-natal age, accompanied by impaired capacity to produce IFN-γ[6;8-10;13]. The results of our study indeed indicate an important role for immature cell-mediated immune responses in RSV resulting in MV (**Chapter 6 to 8**)[14;15]. We found that *ex vivo*-produced IFN-g (**Chapter 6**) as well as IFN-γ levels in the nasopharynx (**Chapter 7**) is practically absent in infants requiring MV for RSV, but less so in other hospitalized infants with RSV LRTI. In addition, the inverse relation between monocyte IL-12 production and duration of MV (**Chapter 8**) points

out that initiation of virus-specific cellular immunity is probably of importance to recover from RSV LRTI resulting in MV.

Table 12.1 *Clinical and immunological factors described to determine short-term and long-term airway morbidity associated with RSV bronchiolitis*

Short term airway morbidity	Long-term airway morbidity
Clinical risk factors	*Clinical predictors*
– Maturation-related: – Neonatal status[87] – Prematurity[88-90] – Post-conceptional age < 45 weeks*[91] Pre-existent medical conditions: – Chronic lung disease[92] – Congenital heart disease[93]	– Signs of airflow limitation during RSV*[94] – Atopy(?)[26]
Immunological correlates	*Immunological predictors*
– RSV-specific IgE in respiratory tract[95;96] – Histamine release in respiratory tract [96] – Leukotriene C_4 in respiratory tract [97;98] – Eosinophilic cationic protein in respiratory tract [99;100] – Low IFN-γ levels in respiratory tract*[15] – Low peripheral blood T cell IFN-γ production*[74] – Low monocyte IL-12 production*[14]	– Elevated allergen-specific serum IgE[26] – RSV-specific IgE in respiratory tract [25;101] – High monocyte IL-10 production*[72]
Virological correlates	
– High viral load[102;103] – RSV type A[104]	
Miscellaneous (short-term and long-term airway morbidity)**	
– Pre-existent low level of lung function[30;105] – Substance P and other neurogenic factors[106-108]	

* described in this thesis

** suggested in literature, but not formally studied

12.3 RSV bronchiolitis in relation to recurrent wheezing

Infants who recover from respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) have an increased risk for the subsequent development of recurrent wheezing during early childhood[16-30]. Most studies have shown that approximately half of infants with RSV LRTI will have recurrent episodes of wheezing during childhood. Both

early and more recently published data indicate that airway morbidity following RSV LRTI is transient and subsides during school age[16;30].
We have shown that the signs of airflow limitation during RSV LRTI is a powerful predictor for subsequent airway morbidity (**Chapter 10**). To date this is the first practically applicable predictor for airway morbidity following RSV LRTI. In addition, this finding emphasises that inclusion criteria used in RSV follow-up studies determine the prevalence of airway morbidity that will be found. Strict inclusion of RSV-infected infants with classical bronchiolitis will result in a higher observed incidence of airway morbidity[16] than in inclusion of RSV-infected infants with any lower respiratory tract symptom[30].
Atopy as well as Th2 responses have been mentioned as potential key mechanisms underlying the development of recurrent wheezing following RSV bronchiolitis[26;28;29]. However, our cohort study did not show an association between family history of atopy and recurrent wheezing during the follow-up period (**Chapter 11**), which is consistent with most other studies (reviewed in **chapter 3**). In addition, we showed that recurrent wheezing following RSV is not associated with Th2-like cytokine production by peripheral blood mononuclear cells upon non-specific stimulation (**Chapter 11**). Moreover, in vivo cytokine profiles in nasopharyngeal secretion did not suggest the involvement of Th2-type immune responses in recurrent wheezing following RSV LRTI (data not shown). The mixed Th1/Th2 cytokine pattern is consistent with most studies on primary RSV infection in the mouse model[31-33], although some studies show a predominant Th1 response[34;35]. To our knowledge, no major role for Th2-type cytokines have been found in primary RSV infection in the mouse model. In conclusion, it appears that atopy and Th2-type immune responses do not play an important role in the development of airway morbidity following RSV bronchiolitis.
One of the major findings in the cohort study presented in this thesis is the association between monocyte IL-10 production during the convalescent phase of RSV infection and subsequent recurrent wheezing (**Chapter 11**). IL-10 was originally described as soluble mediator that inhibits cytokine production by Th1 clones[36]. Later, it was established that IL-10 can also decrease the production of IL-4 and IL-5 by Th2 clones[37]. As a result of its down-regulating effect, IL-10 has even been considered as potential treatment for inflammatory diseases of the airways[38]. However, contrasting data have been reported on the role of IL-10 in lung inflammation. In asthmatics reduced IL-10 expression was found in BAL lymphocytes[39]. Others reported elevated IL-10 levels in BAL fluid from allergic asthmatics[40].

In our study we found an association between monocyte IL-10 response during RSV and recurrent wheezing, that was independent of a family history of atopy or Th2-type responses in the lymphocyte populations (**Chapter 11**). Thus, if monocyte IL-10 plays a role in the development of recurrent wheezing following RSV infection, the underlying mechanism appears to be unrelated to Th2-type responses. Recurrent wheezing following RSV infection is strongly associated with bronchial hyperresponsiveness[30;41;42]. A potential mechanism by which virus-induced IL-10 production could lead to recurrent wheezing is interference with normal antigen-presentation[43]. Infection of monocyte-derived DC with rhinovirus induces IL-10-mediated MHC II down-regulation and a concomitantly decreased capacity to stimulate CD4+ T cells[44]. It is conceivable that this virus-induced suppression of APC function, can result in decreased anti-viral immu-

nity in the lower airways. One could then speculate that viral infections in the upper respiratory tract more easily lead to infection and inflammation of the lower respiratory tract, leading to wheezing and bronchial hyperresponsiveness. This explanation is in line with the clinical picture of wheezing following RSV bronchiolitis, usually associated with upper respiratory symptoms[45]. Other Th2-independent mechanisms involved in bronchial hyperresponsiveness include altered neural regulation of airway tone[46;47], hypertrophy of airway smooth muscle[48], mucus hypersecretion[49] and airway epithelial damage[50]. Recent studies in different animal models have shown that IL-10 is required for the induction of airway hyperresponsiveness by Th2-independent mechanisms[51;52]. Makela and colleagues reported data on airway responsiveness in ovalbumin-induced IL-10 knockout mice[51]. In this established mouse model of allergic airway inflammation, deficiency of IL-10 did not lead to any interference with allergen-induced production of IgE, leukotriene C_4 or Th2-like cytokines. However, bronchial hyperresponsiveness was completely abolished in these mice. Intranasal inoculation of an adenoviral construct with the IL-10 gene reconstituted IL-10 production and also the development of airway hyperresponsiveness. These data strongly suggest the possibility that IL-10 plays an important role in Th2-independent induction of airway hyperresponsiveness.

12.4 Antigen-presenting cells in RSV infection

We have demonstrated that *ex vivo* production of monocyte IL-10 during the convalescent phase of RSV bronchiolitis has predictive value for subsequent recurrent wheezing (**Chapter 11**)[15]. Together with the inverse relation between monocyte IL-12 responses and duration of MV during RSV infection (**Chapter 8**), this cohort study directly points to a critical role for antigen-presenting cell function in the pathogenesis of recurrent wheezing following RSV.

It was shown that monocytes and, to a lesser extent, alveolar macrophages *in vitro* are permissive to RSV infection and subsequent viral replication[53-56]. Subsequent to infection with RSV these APC release of soluble immune mediators, such as prostaglandin E2 (PgE2), tumor necrosis factor (TNF)-a and IL-6[57], but also IL-10[55;58;59]. A recent study reveiled that the cytokine response to RSV by human monocytes is mediated by CD14, the major receptor for lipopolysaccharide (LPS)[60]. Using macrophages from CD14 knock-out mice, it was shown that immune responses to the RSV F protein are fully dependent on the expression of CD14. In addition, it was shown that Toll-like receptor (TLR)-4, which is required for LPS-induced signal transduction, was required for RSV-induced cytokine responses by macrophages *in vitro* as well as elimination of the virus in a mouse model system. The depency of CD14 and TLR-4 expression emphasizes the potential role of the innate immune system in the pathogenesis of RSV infection.

In addition to the ability to infect monocytes and macrophages, it was shown that RSV can infect DC from cattle by endocytosis[61]. For humans or rodents no *in vivo* or *in vitro data* are available on RSV infection of DC.

Alveolar macrophages and DC are the main professional APC in the lung. The role of each of these cell types in presenting RSV antigen to T cells is not known. Alveolar macrophages are found throughout the lung. However, they possess poor antigen-

presenting capacity and may therefore play a minor role in presentation of RSV antigen[62].

DC are not only highly specialized in the induction of primary immune responses, but also form an extensive network above the basement membrane of the airway epithelium that ensures accessibility to inhaled antigens[63]. After encountering antigen the DC actively translocates to secondary lymphoid tissue where they transform to mature DC capable of antigen-presentation[64;65]. Mature DC most effectively present antigen in the context of either MHC I or II molecules[66]. One of the factors enabling mature DC to present antigen is the density of both MHC I and II on the surface of DC, which is higher than on other APC[67]. Taking into account these properties, the DC is an excellent candidate to play an important role in antigen-presentation during RSV infection. Surprisingly, practically no data exist on this subject. One of the potential roles of DC in the pathogenesis of RSV infection is induction of virus-specific cytotoxic T cell generation. For influenza A virus, it was shown in an *in vitro* model, that monocyte-derived mature DC can elicit the generation of CD8+ virus-specific T cells[68]. In this model, help from CD4+ T cells was not required for the induction of anti-viral immunity. Taken together the physiological properties of DC and the available data on the role of DC in other respiratory viral pathogens, DC may well play a prominent role in RSV infection in humans as well as animals.

MHC I molecules are required for antigen-presentation to CD8+ cells resulting in the generation of cytotoxic T cells [69]. MHC-I restricted antigen-presentation could be of relevance in RSV infection. Virus-specific CTL have been implicated, at least in the mouse model, in viral clearance from the lung as well as disease-enhancement[5]. MHC II molecules are involved in priming naive CD4+ T cells, after which the CD4+ will develop into the T-helper phenotype[65;70]. Data from the mouse model have clearly shown an important role for T-helper cells in the pathogenesis of RSV infection [5;71]. As mentioned before, the role of T-helper cells, in particular Th2-cells in human RSV, has been mentioned, but is still subject of debate[15;28;29;72].

Finally, the role of epithelial cells should be discussed. This "non-professional" APC is quantitatively the most important cell type in the lung. Although epithelial cells are capable of expressing MHC II molecules and providing appropriate co-stimulation, they do not usually do so[69]. RSV infection of epithelial cell lines has been shown to result in the up-regulation of MHC I molecules, which potentially results in better recognition by CD8+ cells[73]. As mentioned in the previous paragraph, mouse model studies have shown MHC I-restricted antigen-presentation to CD8+ cells could be most relevant in the pathogenesis of RSV infection[5]. Therefore, the interaction between RSV and epithelium might be important for the induction of disease-enhancing immune responses mediated by CD8+ cells.

12.5 Strategies by RSV to evade immune surveillance

A potentially powerful mechanism by which RSV could decrease anti-viral immunity is interference with the production of cytokines or cytokine signaling. IFN-γ has anti-viral activity, up-regulates expression of MHC I and II molecules, activates macrophages and has a regulatory role in cell-mediated immunity. Decreased production of IFN-γ could allow RSV to escape from anti-viral immunity. Whether this truly occurs in vivo is not

known. However, we and others have shown that severe RSV in humans is associated with decreased IFN-γ production (**Chapter 6**)[29;74]. This could partially be due to a down-regulatory effect of RSV on IFN-γ-producing cells *in vivo*. It is also conceivable that RSV decreases the capacity of APC to produce IL-12 which is required for initiation of cellular immunity. This mechanism would be in line with findings in another paramyxovirus study, in which it was shown that measles virus can interfere with APC IL-12 production[75;76]. It has also been shown that in vitro infection with RSV of alveolar macrophages results in the production of regulatory cytokines such as IL-10[58]. It was suggested that RSV-induced IL-10 production has an immune suppressive effect potentially leading to ineffective elimination of the virus, but this still needs to be shown[58].
A potentially different strategy by RSV to prevent viral destruction is prevention of apoptosis (programmed cell-death)[77]. Replicating virus, in general, may lead to apoptosis of the host cell, for example by inducing TNF-α secretion[77]. Premature cell-death limits the time for virus to produce new virions. It was shown by Krilov that RSV is capable of decreasing DNA strand breaks upon *in vitro* infection of peripheral blood monocytes[78]. Although the mechanism explaining this phenomenon has not net been provided, this finding indicates that RSV may, indeed, prevent monocyte apoptosis. Considering the ability RSV to replicate in monocytes and macrophages[55], prolongation of the life of the infected cell could result in enhanced viral replication.

12.6 Directions for future studies

12.6.1 Development of animal models for neonatal RSV infection

The studies on RSV infection resulting in MV have lead to questions that will not be easily answered by human studies. The association between decreased cellular immunity and RSV infection resulting in MV likely reflects impaired cellular immune function of the immature neonate. To prove this hypothesis pre-existent immune function should be measured. In addition, it could be that RSV enhances the effects of this immaturity through a depressing action on cellular immunity. In order to study further the development of severe RSV in neonates, new animal models should be evaluated for their relevance. As noted before, the mouse model does not appear to be the first candidate[71]. Suitable animal models for neonatal RSV infection may be found among other primates. Of all primates humans are genetically most accurately approached by chimpanzees. The scarcity and the costs involved in caring for these animals make them less practical for research purposes. Limited data are available on primary RSV infection in chimpanzees, mainly obtained in vaccine studies[79-81]. It is not known whether chimpanzees are more affected by RSV during infancy than later in life. Neonatal RSV infection of chimpanzees, however, remains a model that appears worthwhile to explore. Of interest is a 1976 study by Prince and Porter who studied RSV infection in ferrets[81]. Following nasal RSV inoculation no illness was observed, but inflammation and concomitant replicating virus were found in nasal tissue in ferrets of all age. However, inflammation and viral replication in the lungs was only found in infant ferrets during the first 1-2 weeks of life (life expectancy 4 years). To date, this is the only animal model in which an age-dependent pulmonary inflammation has been documented[82]. Disadvantages of ferrets, however, include the lack of inbred strains and immunologic reagents.

12.6.2 The role of DC in RSV infection

The data presented in this study suggest that the role of APC in RSV infection requires further study. The best candidate to fulfill this role is the DC. Human studies do not easily allow for study of airway DC, because a main site of DC are the non-accessible regional lymphoid tissue[65]. Therefore, it appears more feasible to explore the role of DC in RSV primarily in *in vitro* and animal models.

Future study should focus on the role of DC in the initiation of anti-viral immunity during primary RSV infection. More knowledge is required on the exact interaction between RSV, DC and T cells. The potential interference by RSV with normal antigen-presentation by DC, including inhibition of adequate IL-12 production, should be further explored. The mechanism by which RSV-specific CD4+ and CD8+ T cells are generated and activated *in vivo* are largely obscure. However, it is likely that DC play an important role in the process.

Finally, it is conceivable that DC play a role in recurrent wheezing following RSV bronchiolitis. DC-secreted mediators, such as IL-10, might have a direct effect on airway epithelium and smooth muscle[51]. DC are capable to polarize T cell function. For influenza A virus it was shown that infection of DC results in the generation of either IFN-γ or IL-4 secreting T cells (Th1 respectively Th2-response) depending on the dose of infection of the DC[37;83]. In addition to T cell polarization, RSV could interfere with tolerance induction or sensitization to common aero-allergens. In a mouse model of influenza A a potential role of DC in virus-associated induction of allergic responses has been shown [84]. In this model allergic sensitization to ovalbumin during influenza A co-infection was associated with DC migration to airway epithelium and MHC II upregulation. Whether the same interaction between RSV and allergic sensitization exists remains to be investigated. However, these studies stress that interaction between viral infection and DC-mediated immune responses is a promising subject for further study.

12.6.3 Prevention of severe RSV and long-term sequelae

RSV-specific antibodies have been proven to be effective to reduce hospitalization in case of RSV infection in high-risk infants treated until the age of 6-12 months[85;86]. However, no evidence exists whether antibody prophylaxis prevents RSV infection resulting in MV. To address this question, new intervention studies are required. Because of the relative low frequency of RSV infection resulting in MV (even in high-risk infants) a large study population will likely be required. To minimize the size of the study population, study designs should be chosen in which the largest potential effect of prophylaxis is expected. A possible study design could include administration of RSV-specific antibodies to neonates and/or preterm infants until post-conceptional age of 44 weeks is reached and assess reduction in RSV infection resulting in MV (Chapter 5).

To date, one of the major challenges in the area of clinical RSV research is the development of strategies to prevent long-term effects. With the current widespread use of antibody prophylaxis in high-risk infants, data will become available whether prevention of RSV infection requiring hospitalization will reduce the risk for recurrent wheezing during early childhood in this group of infants. However, it is unlikely that antibody prophylaxis will be used in the general pediatric population to prevent long-term airway morbidity. Therefore, alternative interventions should be developed. The follow-up studies described in this thesis do not easily give direction to the nature of intervention stud-

ies. However, the identification of clinical (Chapter 10)[72] predictors can improve selection of infants with the highest risk for long-term airway morbidity.

12.7 Conclusions

The present follow-up study has resulted in the description of the first clinical predictors for RSV associated short-term and long-term airway morbidity. They can be used in clinical practise, but can also be incorporated in predictive models on RSV associated airway morbidity. In addition, we have shown a potential role for APC in both RSV LRTI resulting in MV and recurrent wheezing following RSV LRTI. The exact mechanisms by which APC, in particular DC, are stimulated and activated during RSV infection *in vivo* are challenged to be elucidated in the future.

References

1 Noah TL, Becker S. Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line. Am J Physiol 1993; 265:L472-L478.

2 Tristram DA, Hicks W, Jr., Hard R. Respiratory syncytial virus and human bronchial epithelium. Arch Otolaryngol Head Neck Surg 1998; 124:777-783.

3 Patel JA, Kunimoto M, Sim TC, Garofalo R, Eliott T, Baron S, Ruuskanen O, Chonmaitree T, Ogra PL, Schmalstieg F. Interleukin-1 alpha mediates the enhanced expression of intercellular adhesion molecule-1 in pulmonary epithelial cells infected with respiratory syncytial virus. Am J Respir Cell Mol Biol 1995; 13:602-609.

4 Cannon MJ, Openshaw PJM, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J Exp Med 1988; 168:1163-1168.

5 Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991; 88:1026-1033.

6 Adkins B. T-cell function in newborn mice and humans. Immunol Today 1999; 20:330-335.

7 Murgita RA, Witzel H. Regulation of immune functions in the fetus and newborn. Prog Allergy 1981; 29:54-133.

8 Wilson CB, Lewis DB, English BK. Immunity in the neonate. Semin Pediatr Infect Dis 1999; 10(2):83-90.

9 Adinolfi M. Ontogeny of human natural and acquired immunity. onbekend 1998.

10 Watson W, Oen K, Ramhdahin R, Harman C. Immunoglobulin and cytokine production by neonatal lymphocytes. Clin Exp Immunol 1991; 83:169-174.

11 Dominguez E, Madrigal JA, Layrisse Z, Cohen SB. Fetal natural killer cell function is suppressed. Immunol 1998; 94:109-114.

12 Holt PG. Postnatal maturation of immune competence during infancy and childhood. Pediatr Allergy Immunol 1995; 6:59-70.

13 Coutinho GC, Delassus S, Kourilsky P, Bandeira A, Coutinho A. Developmental shift in the patterns of interleukin production in early post-natal life. Eur J Immunol 1994; 24:1858-1862.

14 Bont L, Kavelaars A, Heijnen CJ, van Vught AJ, Kimpen JL. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. J Infect Dis 2000; 181:1772-1775.

15 Bont L, Heijnen CJ, Kavelaars A, Aalderen vWMC, Brus F, Draaisma JMTh, Pekelharing-Berghuis M, Diemen vJAAM, Kimpen JLL. Local interferon- levels during respiratory syncytial virus lower respiratory tract infection are associated with disease severity. submitted 2001.

16 Eisen AH, Bacal HL. The relationship of acute bronchiolitis to bronchial asthma - a 4 tot 14 year follow-up. Pediatrics 1963; 31:859-861.

17 Sims DG, Downham MA, Gardner PS. Study of 8-year-old children with a history of respiratory syncytial virus bronchiolitis in infancy. BMJ 1978; 1:11-17.

18 Gurwitz D, Mindorff C, Levinson H. Increased evidence of bronchial reactivity in children with a history of acute bronchiolitis. J Pediatr 1981; 98:551-555.

19 Pullan CR, Hey EN. Wheezing, asthma and pulmonary dysfunction 10 years after infection with respiratory syncytial virus infection in infancy. BMJ 1982; 284:1665-1669.
20 Hall CB, Hall WJ, Gala CL, McGill FB, Leddy JP. Long-term prospective study in children after respiratory syncytial virus infection. J Pediatr 1984; 105:358-364.
21 McConnochie KM, Roghmann KJ. Bronchiolitis as a possible cause of wheezing in childhood: new evidence. Pediatrics 1984; 74:1-10.
22 Mok JYQ, Simpson H. Symptoms, atopy, and bronchial reactivity after lower respiratory infection in infancy. Arch Dis Child 1984; 59:299-305.
23 McConnochie KM, Mark JD, McBride JT, Hall WJ, Brooks JG, Klein SJ, Miller RL, McInerny TK, Nazarian LF, McWhinney JB. Normal pulmonary function measurements and airway reactivity in childhood after mild bronchiolitis. J Pediatr 1985; 107:54-58.
24 Sly PD, Hibbert ME. Childhood asthma following hospitalization with acute viral bronchiolitis in infancy. Pediatr Pulmonol 1989; 7:153-158.
25 Welliver RC, Duff AL. The relationship of RSV-specific immunoglobulin E-antibody responses in infancy, recurrent wheezing and pulmonary function at age 7-8 years. Pediatr Pulmonol 1993; 15:19-27.
26 Sigurs N, Bjarnason R, Sigursbergsson F, Kjellman B, Brorksten B. Asthma and immunoglobulin E Antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. Pediatrics 1995; 95:500-505.
27 Reijonen TM, Korppi M, Kleemola M, Savolainen K, Kuikka l, Mononen I, Remes K. Nasopharyngeal eosinophil cationic protein in bronchiolitis: relation to viral findings and subsequent wheezing. Pediatr Pulmonol 1997; 24:35-41.
28 Renzi PM, Turgeon JP, Yang JP, Drblik SP, Marcotte JE, Pedneault L, Spier S. Cellular immunity is activated and a Th2-response is associated with early wheezing in infants after bronchiolitis. J Pediatr 1997; 130:584-593.
29 Roman M, Calhoun WJ, Himton KL, Avendano LF, Simon V, Escobar AM, Gaggero A, Diaz PV. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am J Respir Crit Care Med 1997; 156:190-195.
30 Stein RT, Sherill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13. Lancet 1999; 354:541-545.
31 Tang YW, Neuzil KM, Fischer JE, Robinson FW, PArker RA, Graham BS. Determinants and kinetics of cytokine expression patterns in lungs of vaccinated mice challenged with respiratory syncytial virus. Vaccine 1997; 15:597-602.
32 Waris ME, tsou c, Erdman DD, Day DB, Anderson LJ. Priming with live respiratory syncytial virus (RSV) prevents the enhanced pulmonary inflammatory response seen after RSV challenge in BALB/c mice immunized with formalin-inactivated RSV. J Virol 1997; 71:6935-6939.
33 Tripp RA, Moore D, Anderson LJ. TH(1)- and TH(2)-TYPE cytokine expression by activated t lymphocytes from the lung and spleen during the inflammatory response to respiratory syncytial virus. Cytokine 2000; 12:801-807.
34 Graham BS, Henderson GS, Tang YW, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. J Immunol 1993; 151:2032-2040.
35 Hussell T, Spender IC, Georgiou A, O'Garra A, Openshaw PJM. Th1 and Th2 cytokine induction in pulmonary T cells during infection with respiratory syncytial virus. J General Virol 1996; 77:2447-2455.
36 Fiorentino WF, Bond MW, Mossmann TR. Two types of mouse helper T cells. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 1989; 170:2081-2095.
37 Smyth RL, Fletcher JN, thomas hm, Hart CA. immunological responses to respiratory syncytial virus infection in infancy. Arch Dis Child 1997; 76:210-214.
38 Pretolani M, Goldman M. IL-10: a potential therapy for allergic inflammation? Immunol Today 1997; 18:277-280.
39 Borish L, Aarson A, Rumbyrt J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. J Allergy Clin Immunol 1996; 97:1288-1296.
40 Robinson DS, Tsicopoulos A, Meng Q, Durham S, Kay AB, Hamid Q. Increased interleukin-10 messenger RNA expression in atopic allergy and asthma. Am J Respir Cell Mol Biol 1996; 14:113-117.
41 Dezateux C, Fletcher ME, Dundas I, Stocks J. Infant respiratory function after RSV-proven bronchiolitis. Am J Respir Crit Care Med 1997; 155:1349-1355.

42 Duiverman EJ, Neijens HJ, van Strik R, Affourtit MJ, Kerrebijn KF. Lung function and bronchial responsiveness in children who had infantile bronchiolitis. Pediatr Pulmonol 1987; 3:3-44.

43 Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. Annu Rev Immunol 1999; 17:255-281.

44 Stockl J, Vetr H, Majdic O, Zlabinger G, Kuechler E, Knapp W. Human major group rhinoviruses downmodulate the accessory function of monocytes by inducing IL-10. J Clin Invest 1999; 104:957-965.

45 Murray M, Webb MSC, O'Callaghan C, Swarbrick AS, Milner AD. Respiratory status and allergy after bronchiolitis. Arch Dis Child 1992; 67:482-487.

46 Wills-Karp M, Uchida Y, Lee JY, Jinot J, Hirata A, Hirata F. Organ culture with proinflammatory cytokines reproduces impairment of the beta-adrenoceptor-mediated relaxation in tracheas of a guinea pig antigen model. Am J Respir Cell Mol Biol 1993; 8:153-159.

47 Jacoby DB, Gleich GJ, Fryer AD. Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor. J Clin Invest 1993; 91:1314-1318.

48 Du T, Sapienza S, Wang CG, Renzi PM, Pantano R, Rossi P, Martin JG. Effect of nedocromil sodium on allergen-induced airway responses and changes in the quantity of airway smooth muscle in rats. J Allergy Clin Immunol 1996; 98:400-407.

49 Marom Z, Shelhamer JH, Bach MK, Morton DR, Kaliner M. Slow-reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro. Am Rev Respir Dis 1982; 126:449-451.

50 Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. Am Rev Respir Dis 1988; 137:62-69.

51 Makela MJ, Kanehiro A, Borish L, Dakhama A, Loader J, Joetham A, Xing Z, Jordana M, Larsen GL, Gelfand EW. IL-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. Proc Natl Acad Sci U S A 2000; 97:6007-6012.

52 van Scott MR, Justice JP, Bradfield JF, Enright E, Sigounas A, Sur S. IL-10 reduces Th2 cytokine production and eosinophilia but augments airway reactivity in allergic mice. Am J Physiol Lung Cell Mol Physiol 2000; 278:L667-L674.

53 Midulla F, Huang YT, Gilbert IA, Cirno NM, McFadden ER, Panuska JR. Respiratory syncytial virus infection of human cord and adult blood monocytes and alveolar macrophages. Am Rev Respir Dis 1989; 140:771-777.

54 Domurat F, Roberts NJ, Walsh EE, Dagan R. Respiratory syncytial virus infection of human mononuclear leukocytes in vitro and in vivo. J Infect Dis 1985; 152:895-902.

55 Panuska JR, Cirino NM, Midulla F, Despot JE, McFadden ER, Huang YT. Productive infection of isolated human alveolar macrophages by respiratory syncytial virus. J Clin Invest 1990; 86:113-119.

56 Roberts NJ, Prill AH, Mann TN. Interleukin 1 and interleukin 1 inhibitor production by human macrophages exposed to influenza or respiratory syncytial virus. Respiratory syncytial virus is a potent inducer of inhibitory acitivity. J Exp Med 1986; 163:511-519.

57 Franke-Ullmann G, Pfortner C, Walter P, Steinmuller C, Lohmann-Matthes ML, Kobzik L, Freihorst J. Alteration of pulmonary macrophage function by respiratory syncytial virus infection in vitro. J Immunol 1995; 154:268-280.

58 Panuska JR, Merolla R, Rebert NA. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages. J Clin Invest 1995; 96:2445-2453.

59 Matsuda K, Tsutsumi H, Sone S, Yoto Y, Oya K, Okamoto Y, Ogra PL, Chiba S. Characteristics of IL-6 and TNF-alpha production by respiratory syncytial virus-infected macrophages in the neonate. J Med Virol 1996; 48:199-203.

60 Kurt-Jones EA, Popova L, Kwinn L, Haynes M, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, Finberg RW. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 2000; 1:398-401.

61 Werling D, Hope JC, Chaplin P, Collins RA, Taylor G, Howard CJ. Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells. J Leukoc Biol 1999; 66:50-58.

62 Lee TH, Lane SJ. The role of macrophages in the mechanisms of airway inflammation in asthma. Am Rev Respir Dis 1992; 145:S27-S30.

63 Holt PG, Schon-Hegrad MA, Oliver J, Holt BJ, McMenamin PG. A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. Int Arch Allergy Appl Immunol 1990; 91:155-159.

64 Austyn JM, Kupiec-Weglinski JW, Hankins DF, Morris PJ. Migration patterns of dendritic cells in the mouse. Homing to T cell- dependent areas of spleen, and binding within marginal zone. J Exp Med 1988; 167:646-651.
65 Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu Rev Immunol 2000; 18:767-811.
66 Pamer E, Cresswell P. Mechanisms of MHC class I--restricted antigen processing. Annu Rev Immunol 1998; 16:323-358.
67 Inaba K, Pack M, Inaba M, Sakuta H, Isdell F, Steinman RM. High levels of a major histocompatibility complex II-self peptide complex on dendritic cells from the T cell areas of lymph nodes. J Exp Med 1997; 186:665-672.
68 Larsson M, Messmer D, Somersan S, Fonteneau JF, Donahoe SM, Lee M, Dunbar PR, Cerundolo V, Julkunen I, Nixon DF, Bhardwaj N. Requirement of mature dendritic cells for efficient activation of influenza A-specific memory CD8+ T cells. J Immunol 2000; 165:1182-1190.
69 Nakajima J, Ono M, Takeda M, Kawauchi M, Furuse A, Takizawa H. Role of costimulatory molecules on airway epithelial cells acting as alloantigen-presenting cells. Transplant Proc 1997; 29:2297-2300.
70 Inaba K, Metlay JP, Crowley MT, Steinman RM. Dendritic cells pulsed with protein antigens in vitro can prime antigen- specific, MHC-restricted T cells in situ [published erratum appears in J Exp Med 1990 Oct 1;172(4):1275]. J Exp Med 1990; 172:631-640.
71 Graham BS, Perkins MD, Wright PF, Karzon DT. Primary respiratory syncytial virus infection in mice. J Med Virol 1988; 26:153-162.
72 Bont L, Heijnen CJ, Kavelaars A, van Aalderen WM, Brus F, Draaisma JT, Geelen SM, Kimpen JL. Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one-year follow-up study. Am J Respir Crit Care Med 2000; 161(5):1518-1523.
73 Garofalo RP, Mei F, Espejeo R, Ye G, Haeberle H, Baron S, Ogra PL, Reyes VE. Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class 1 MHC Expression through the induction of IFN-beta and IL-1-alpha. J Immunol 1996; 157:2506-2513.
74 Bont L, Heijnen CJ, Kavelaars A, Aalderen vWMC, Brus F, Draaisma JMTh, Geelen SM, Vught vHJ, Kimpen JLL. Association of peripheral blood cytokine responses with disease severity in respiratory syncytial virus bronchiolitis. Eur Respir J 1999; 14:144-149.
75 Fugier-Vivier I, Servet-Delprat C, Rivailler P, Rossoan MC, Liu YJ, Rabourdin-Combre C. Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and t cells. J Exp Med 1997; 186:813-823.
76 Karp CL, Wysocka M, Wahl LM, Ahearn JM, Cuomo PJ, Sherry B. Mechanisms of suppresion of cell-mediated immunity by measles virus. Science 1996; 273:228-231.
77 Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL. Viral subversion of the immune system. Annu Rev Immunol 2000; 18:861-926.
78 Krilov LR, McCloskey TW, Harkness SH, Pontrelli L, Pahwa S. Alterations in apoptosis of cord and adult peripheral blood mononuclear cells induced by in vitro infection with respiratory syncytial virus. J Infect Dis 2000; 181:349-353.
79 Collins PL, Purcell RH, London WT, Lawrence LA, Chanock RM, Murphy BR. Evaluation in chimpanzees of vaccinia virus recombinants that express the surface glycoproteins of human respiratory syncytial virus. Vaccine 1990; 8:164-168.
80 Belshe RB, Richardson LS, London WT, Sly DL, Lorfeld JH, Camargo E, Prevar DA, Chanock RM. Experimental respiratory syncytial virus infection of four species of primates. J Med Virol 1977; 1:157-162.
81 Prince GA, Porter DD. The pathogenesis of respiratory syncytial virus infection in infant ferrets. Am J Pathol 1976; 82:339-352.
82 Byrd LG, Prince GA. Animal models of respiratory syncytial virus infection. Clin Infect Dis 1997; 25:1363-1368.
83 Kimpen JLL. Respiratory syncytial virus immunology. Pediatr Allergy Immunol 1996; 7:S86-90.
84 Yamamoto N, Suzuki S, Shirai A, Suzuki M, Nakazawa M, Nagashima Y, Okubo T. Dendritic cells are associated with augmentation of antigen sensitization by influenza A virus infection in mice. Eur J Immunol 2000; 30:316-326.
85 Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. The PREVENT Study Group. Pediatrics 1997; 99:93-99.

86 The IMpact-RSV study group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 1998; 102(3):531-537.
87 Hall CB, Kopelman AE, Douglas RG, Geiman JM, Meagher MP. Neonatal respiratory syncytial virus infection. N Engl J Med 1979; 300:393-396.
88 Lebel MH, Gauthier M, Lacroix J, Rousseau E, Buithieu M. Respiratory failure and mechanical ventilation in severe bronchiolitis. Arch Dis Child 1989; 64:1431-1437.
89 Stevens TP, Sinkin RA, Hall CB, Maniscalo WM, McConnochie KM. Respiratory syncytial virus and premature infants born at 32 weeks' gestation or earlier. Arch Pediatr Adolesc Med 2000; 154:55-61.
90 Tissing WJE, Van Steensel-Moll HA, Offringa M. Risk factors for mechanical ventilation in respiratory syncytial virus infection. Eur J Pediatr 1993; 152:125-127.
91 Bont L, Van Vught HJ, Kimpen JLL. Prophylaxis against respiratory syncytial virus (RSV) in premature infants. Lancet 1999; 354(9183):1003-1004.
92 Groothuis JR, Guttierez KM, Lauer b. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 1988; 82:199-203.
93 McDonald NE, Hall CB, Suffin SC, Alexon C, Harris PJ, Manning JA. Respiratory syncytial virus infection in infants with congenital heart disease. N Engl J Med 1982; 307:397-400.
94 Bont L, Aalderen vWMC, Versteegh J, Brus F, Draaisma JMTh, Pekelharing-Berghuis M, Diemen vJAAM, Kimpen JLL. Airflow limitation during respiratory syncytial virus (RSV) lower respiratory tract infection predicts recurrent wheezing. Pediatr Infect Dis J 2000; in press.
95 Welliver RC, Kaul TN, Ogra PL. The appearance of cell-bound IgE in respiratory-tract epithelium after respiratory-syncytial-virus infection. N Engl J Med 1980; 303:1198-1202.
96 Welliver RC, Wong DT, Sun M, Middleton EJR, Vaughan RS, Ogra PL. The development of respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection. N Engl J Med 1981; 305:841-846.
97 Volovitz B, Welliver RC, De Castro G, Krystofik DA, Ogra PL. The release of leukotrienes in the respiratory tract infection with respiratory syncytial virus: role of obstructive airway disease. Pediatr Res 1988; 24:504-507.
98 Van Schaik SM, Tristam DA, Nagpal IS, Hintz KM, Welliver RC, Welliver RC. Increased production of interferon gamma and cysteinyl leukotrienes in virus-induced wheezing. J Allergy Clin Immunol 1999; 103:630-636.
99 Garofalo RP, Kimpen JL, Welliver RC, Ogra PL. Eosinophil degranulation in the respiratory tract during naturally acquired respiratory syncytial virus infection. J Pediatr 1992; 120:28-32.
100 Garofalo RP, Ahlstedt DA, Welliver RC. Peripheral blood eosinophil counts and eosinophil cationic protein content of respiratory secretions in bronchiolitis: relationship to severity of disease. Pediatr Allergy Immunol 1994; 5:111-117.
101 Welliver RC, Sun M, Rinaldo D, Ogra PL. Predictive value of respiratory syncytial virus- specific IgE responses for recurrent wheezing following bronchiolitis. J Pediatr 1986; 109:776-780.
102 Buckingham SC, Bush AJ, Devincenzo JP. Nasal quantity of respiratory syncytial virus correlates with disease severity in hospitalized infants. Pediatr Infect Dis J 2000; 19:113-117.
103 Hall CB, Douglas-RG J, Geiman JM. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. J Pediatr 1976; 89:11-15.

Summary

Respiratory syncytial virus (RSV) infection is the most frequent cause of bronchiolitis during infancy. During the winter season RSV bronchiolitis is one the most common causes of hospitalization as well as mechanical ventilation (MV). Risk factors for RSV bronchiolitis are prematurity with or without chronic lung disease and neonatal status as well as congenital heart disease and immunodeficiencies. The disease typically begins with signs of a common cold, followed after a few days by coughing, dyspnea and an expiratory wheeze. Following RSV bronchiolitis 30-70% infants develop recurrent episodes of wheezing. It is not yet possible to predict which children will go on to have wheezing following RSV bronchiolitis.

Chapter 1 is a general introduction to this thesis. The pathogenesis of RSV bronchiolitis is not well understood. Evidence exists that symptoms during RSV bronchiolitis are caused by a pathological immune response to the virus. In the airways of patients RSV-specific immunoglobulin E (IgE) is found. In addition, there are signs of eosinophil activation during RSV bronchiolitis. Moreover, the relation with recurrent wheezing suggests that possibly atopy is the underlying mechanism of both the acute disease as well as the sequelae. However, an aberrant immune response does not easily explain why preterm infants without chronic lung disease as well as healthy neonates have increased risk for severe course of disease in case of RSV infection.

No treatment for RSV bronchiolitis is available. Anti-viral drugs are not effective. The effectiveness of corticosteroids is controversial, but in general no great benefit can be expected in mild and moderately severe RSV bronchiolitis. Currently, no vaccine for RSV is available. In the 1960s a formalin-inactivated vaccine was used in infants. No protection against naturally-acquired RSV was observed. In contrast, enhanced disease and increased mortality were observed during RSV infection following vaccination. In the absence of a vaccine, passive immunization strategies were developed. Both donor-derived and a humanized RSV-specific monoclonal antibody have been proven to be safe and effective in reducing hospitalization in case or RSV infection in high-risk infants.

Chapter 2 reviews potential mechanisms that play a role in the development of RSV infection resulting in MV. Pre-existent cardiac or pulmonary compromise have been documented as clinical risk factors for severe RSV bronchiolitis. However, a larger proportion of mechanically ventilated RSV bronchiolitis patients are previously healthy preterm or term infants. In general, infants at this early age have maturation-related deficient cell-mediated immunity (CMI). Several studies show an association between decreased CMI and severe RSV bronchiolitis, indeed suggesting that a maturation-related defect of the cellular immune system facilitates severe RSV. In addition, low virus-specific antibody titers prior to RSV bronchiolitis have been shown to be a risk factor for severe RSV bronchiolitis. Studies in the mouse model have demonstrated that the immune system, in particular T cells, can enhance pulmonary inflammation during RSV infection. However, it is not yet clear in what way RSV in the animal model bears relevance to RSV bronchiolitis during infancy.

Chapter 3 is a review of the relation between RSV bronchiolitis and recurrent wheezing. Despite differences in study design, follow-up studies consistently show that approximately half of the patients with RSV bronchiolitis during infancy go on to have recurrent wheezing episodes during childhood. Respiratory symptoms are associated with abnormal lung function, including bronchial hyperresponsiveness. Wheezing symptoms following RSV bronchiolitis gradually decrease and it appears that during school age airway morbidity is no longer related to RSV bronchiolitis during infancy. Mechanisms underlying the association between RSV bronchiolitis and long-term airway morbidity are poorly understood. On the one hand, abnormal airway function that is congenitally present or acquired before RSV bronchiolitis occurs could be the cause of both RSV LRTI and subsequent recurrent wheezing. On the other hand, it is possible that RSV bronchiolitis causes changes in the lower airways or the immune system that result in long-term airway morbidity. Animal models suggest RSV infection can promote the development of allergic sensitization, but most studies in humans do not indicate a role for atopy in the development of recurrent wheezing following RSV infection.

New strategies have become available to prevent severe RSV infection in prematurely born infants, including palivizumab, a humanized monoclonal antibody. It is not known for how long prevention of RSV infection in preterm infants should be considered. We hypothesized that the duration of the period that children are at risk for severe RSV infection, is limited by the post-conceptional age (**Chapter 5**). Post-conceptional age distribution was studied in mechanically ventilated preterm RSV bronchiolitis patients. It was shown that preterm infants, without chronic lung disease have increased risk for severe RSV infection until post-conceptional age reaches 44 weeks.

To study the role of CMI in disease severity during RSV lower respiratory tract infection (LRTI) we compared the immune response in the blood and nasopharyngeal aspirates between RSV-infected infants with and without the requirement of MV. Mechanically ventilated infants were characterized by decreased lymphoproliferative responses and IFN-γ production by peripheral blood cells upon non-specific stimuli (**Chapter 6**). In addition, *in vivo IFN-γ* levels in the nasopharynx were lower in RSV-infected infants requiring MV (**Chapter 7**). To further investigate potential mechanisms underlying decreased CMI in RSV-infected infants requiring MV, we studied monocyte IL-12 production in the blood. IL-12 is a prerequisite for the initiation of CMI. We showed that monocyte IL-12 production was inversely related to duration of MV (**Chapter 8**). In this chapter we present the first model to predict the clinical outcome in infants with RSV LRTI requiring MV. Taken together, these data suggest that severe RSV LRTI is associated with decreased level of CMI and that initiation of CMI is required for the convalescence of MV during RSV LRTI.

In addition to a role for CMI during primary RSV infection, we investigated whether RSV-specific CMI, induced during primary RSV LRTI, protected against reinfection by RSV during the subsequent winter season. (**Chapter 9**). We found that RSV-specific memory is induced in the majority of infants during RSV LRTI, but that RSV-specific memory responses did not protect against reinfection. Moreover, reinfection did not boost CMI, which may have consequences for the development of future vaccines against RSV.

Studies in the past have attempted to relate clinical parameters during RSV LRTI to long term airway morbidity. Sex, age at onset of RSV LRTI and disease severity appeared not

to be associated with subsequent recurrent wheezing of lung function abnormalities. In **Chapter 10** we demonstrate that signs of airflow limitation during RSV LRTI is the first useful clinical predictor for subsequent recurrent wheezing.

In **Chapter 11** we evaluated the predictive of atopy and cytokine profiles during RSV LRTI for subsequent airway morbidity. Family history of atopy as well as Th2-like cytokine profiles in the blood during RSV LRTI were not related to subsequent recurrent wheezing. However, the predictive value of monocyte IL-10 production in the blood during the convalescent phase of RSV LRTI for subsequent recurrent wheezing is one of the major findings presented in this thesis.

This thesis has demonstrated that the majority of previously healthy infants with RSV LRTI requiring MV have a post-conceptional age < 45 weeks. Therefore, it is conceivable that immaturity of the immune system, and possibly also of the airways, plays a key role in the pathogenesis of this severe form of RSV LRTI. We showed that RSV LRTI requiring MV is associated with decreased level of CMI, which may interfere with adequate viral clearance. Furthermore, this thesis has shown that monocyte IL-12 and IL-10 production have predictive value for short-term, respectively long-term airway morbidity. These findings lead to the conclusion that antigen-presenting cells (APC) could play an orchestrating role in the immune response during RSV bronchiolitis. Therefore, we conclude that future research should focus on the role of APC, including dendritic cells, in the pathogenesis in RSV LRTI.

Samenvatting

Respiratoir syncytieel virus (RSV) infectie is de meest voorkomende oorzaak van bronchiolitis bij kinderen tot de leeftijd van 12 maanden. Gedurende de wintermaanden worden in Nederland jaarlijks ongeveer 1000-2000 kinderen opgenomen wegens RSV bronchiolitis. Ongeveer 100-150 kinderen moeten worden beademd op een Pediatrische Intensive Care Afdeling. Risicofactoren voor RSV bronchiolitis zijn prematuriteit en leeftijd onder de 5 weken (neonatale status). Tijdens de wintermaanden is RSV bronchiolitis de meest voorkomende reden van opname op een Algemene Pediatrische Afdeling of een Pediatrische Intensive Care Afdeling. Het typische ziektebeeld begint met neusverkoudheid, na een paar dagen gevolgd door hoesten, benauwdheid en een piepende uitademing. Deze piepende uitademing duidt op vernauwing van de lagere luchtwegen. Na herstel van RSV bronchiolitis ontwikkelt 30-70% van de kinderen terugkerend episodes van piepen. Niet duidelijk is of dit beeld van "post-bronchiolitis piepen" onderdeel is van atopisch astma, waarbij in de regel ook allergie met een familiaire predispositie gezien wordt. Het is niet mogelijk om te voorspellen welke kinderen luchtwegklachten ontwikkelen na RSV bronchiolitis.

Hoofdstuk 1 is een algemene inleiding tot dit proefschrift. De oorzaak van RSV bronchiolitis is niet geheel duidelijk. Er zijn aanwijzingen dat symptomen tijdens RSV infectie worden veroorzaakt door een pathologische afweerreactie tegen het virus. Zo wordt in de luchtwegen van patienten RSV-specifiek immunglobuline E (IgE) gevonden. Ook zijn er tijdens RSV bronchiolitis tekenen van activatie van eosinofiele granulocyten. Bovendien suggereert de relatie met recurrent piepen dat mogelijk atopie de oorzaak is van zowel RSV bronchiolitis als de daaropvolgende luchtwegmorbiditeit. Echter, een aberante afweerreactie verklaart niet eenvoudig waarom gezonde prematuur geboren kinderen en gezonde neonaten een verhoogd risico hebben op een ernstig verlopende RSV infectie.

Er is geen behandeling voor RSV bronchiolitis. Anti-virale medicatie is niet effectief. De effectiviteit van corticosteroïden is omstreden. In het algemeen mag van corticosteroïden geen groot effect verwacht worden, tenzij miscchien bij de meest ernstig zieke patiënten. Ook een vaccin voor RSV is er nog niet. Een studie in de jaren '60 met een formaline-geïnactiveerd vaccin is desastreus verlopen. RSV bronchiolitis werd niet voorkomen door het vaccin en kinderen die nadien met RSV werden geinfecteerd werden ernstiger ziek met zelfs toegenomen mortaliteit als gevolg. In de afwezigheid van een vaccin zijn RSV-specifieke antistoffen (passieve immunisatie) ontwikkeld die bij risicokinderen een deel van de ziekenhuisopnames voor RSV bronchiolitis voorkomen.

Het doel van de cohortstudie beschreven in dit proefschrift was het ontwikkelen van prognostische modellen voor het acute beloop van RSV bronchiolitis en voor luchtwegmorbiditeit op de langere termijn. Dit werd gedaan door de potentiële voorspellende waarde te bepalen van klinische en immunologische factoren voor korte en lange termijn luchtwegmorbiditeit.

Hoofdstuk 2 is een literatuuroverzicht van klinische, virologische en immunologische factoren die een rol spelen bij kinderen die beademd worden in verband met RSV bron-

chiolitis. Er wordt ingegaan op de pathofysiologie van RSV infectie die leidt tot kunstmatige beademing van gezonde prematuur en à term geboren kinderen en neonaten. In het algemeen hebben zeer jonge kinderen een verlaagd nivo van cellulaire immuniteit wat samenhangt met immunologische onrijpheid. Dit maakt deze kinderen kwetsbaar voor virale infecties. Of dit ook geldt voor RSV is nog niet zeker. Naast verlaagde cellulaire immuniteit lijken lage titers van RSV-specifieke antistoffen in het bloed het risico op ernstige RSV infectie te verhogen.

Hoofdstuk 3 is een overzicht van de literatuur over de relatie tussen RSV bronchiolitis en de daaropvolgende luchtwegmorbiditeit. Ondanks verschillen in onderzoeksmethoden, tonen de meeste onderzoeken dat ongeveer de helft van de kinderen na RSV bronchiolitis recurrent episodes van piepen ontwikkelt. Deze klachten zijn geassocieerd met afwijkende longfunctie, zoals bronchiale hyperreactiviteit. De ernst van de luchtwegsymptomen neemt geleidelijk af en op de leeftijd van 6 jaar wordt piepende ademhaling niet vaker gevonden dan bij andere kinderen van dezelfde leeftijd. De mechanismen die ten grondslag liggen aan lange termijn luchtwegmorbiditeit na RSV bronchiolitis zijn onzeker. Aan de ene kant zijn er aanwijzingen dat aangeboren longfunctieafwijkingen de oorzaak zijn van zowel RSV bronchiolitis als de luchtwegmorbiditeit die daarop volgt. Aan de andere kant is het mogelijk dat RSV veranderingen in de lagere luchtwegen of het immuunsysteem induceert die resulteren in luchtwegmorbiditeit. Diermodellen suggereren dat RSV infectie de ontwikkeling van allergie kan stimuleren, maar de meeste studies bij kinderen tonen dat atopie geen belangrijke rol speelt bij de ontwikkeling van recurrent piepen volgend op RSV bronchiolitis.

Sinds de introductie van RSV profylaxe is er behoefte aan selectie van kinderen die de meeste baat hebben bij profylaxe. Om die reden onderzochten wij gedurende welke periode prematuur geboren kinderen zonder chronisch longlijden risico lopen op RSV leidend tot mechanische beademing (**Hoofdstuk 5**). Wij vonden dat na de post-conceptionele leeftijd van 44 weken het risico op deze ernstige vorm van RSV infectie relatief laag is.

Om de rol van cellulaire immuniteit tijdens RSV lagere luchtweginfectie (LLWI) te bestuderen werd de immuunrespons in het perifere bloed en in neusslijm tijdens RSV LLWI bij beademde en niet beademde kinderen vergeleken. De rol van T cel IFN-γ and monocytair IL-12 stond hierbij centraal. Bij beademde kinderen vonden wij een verlaagde lymfoproliferatieve respons en IFN-γ productie door cellen in het perifere bloed na niet-specifieke stimulatie *in vitro (***Hoofdstuk 6**). Ook *in vivo* concentraties van IFN-g in neusslijm waren lager bij beademde patiënten (**Hoofdstuk 7**). Om verder de oorzaak van verlaagde cellulaire immuniteit tijdens RSV LLWI bij beademde kinderen te onderzoeken hebben wij monocytair IL-12 productie in het bloed gemeten. IL-12 productie door monocyten was verlaagd bij beademde patienten en was bovendien omgekeerd gerelateerd met beademingsduur tijdens RSV LLWI (**Hoofdstuk 8**). Op basis van deze bevindingen hebben wij geconcludeerd dat RSV LLWI leidend tot mechanische beademing samenhangt met verminderde cellulaire immuniteit en dat cellulaire immuniteit waarschijnlijk nodig is voor herstel tijdens beademing als gevolg van RSV LLWI.

Wij onderzochten vervolgens of RSV-specifieke cellulaire immuniteit, geïnduceerd tijdens de primaire RSV LLWI, beschermt tegen reïnfectie met RSV in het volgende winterseizoen (**Hoofdstuk 9**). Wij vonden dat bij vrijwel alle kinderen RSV-specifiek geheugen werd geïnduceerd tijdens de primaire infectie, maar dat dit geen bescherming

bood tegen reïnfectie. Ook bleek reïnfectie het RSV-specifieke geheugen niet te versterken (boosting effect). Deze bevindingen zijn wellicht relevant voor de toekomstige ontwikkeling van een RSV vaccin.
In het verleden is zonder succes gezocht naar klinische parameters tijdens RSV LLWI met voorspellende waarde van het vervolgens ontwikkelen van luchtwegmorbiditeit. Geslacht, leeftijd bij opname voor RSV LLWI, ziekte-ernst waren niet gerelateerd aan luchtwegmorbiditeit volgend op RSV LLWI. Wij vonden dat de aanwezigheid van symptomen van bronchusobstructie tijdens RSV LLWI een positief voorspellende waarde had voor recurrente episodes van piepen tijdens de follow-up periode, zoals geregistreerd in dagboekjes door de ouders (**Hoofdstuk 10**). Wij vonden verder dat een positieve familieanamnese voor atopie, en ook Th2 cytokine profielen, niet geassocieerd waren met recurrent piepen volgend op RSV LLWI (**Hoofdstuk 11**). Echter, de voorspellende waarde van monocytaire IL-10 productie tijdens de herstelfase van RSV LLWI voor recurrent piepen is één van de belangrijkste resultaten uit dit proefschrift (**Hoofdstuk 11**).
Dit proefschrift laat zien dat de meerderheid van de kinderen zonder onderliggend lijden met RSV LLWI leidend tot kunstmatige beademing een post-conceptionele leeftijd van < 45 weken heeft. Het ligt daarom voor de hand dat onrijpheid van het immuunsysteem, en wellicht ook van de luchtwegen, een belangrijke rol speelt in de pathogenese van deze ernstige vorm van RSV LLWI. Zo hebben wij getoond dat RSV LLWI leidend tot kunstmatige beademing samenhangt met verlaagde cellulaire immuniteit. Dit leidt mogelijk tot verminderde of vertraagde afweer tegen het virus. Verder heeft dit proefschrift getoond dat monocytair IL-12 en IL-10 voorspellers zijn voor korte respectievelijk lange termijn luchtwegmorbiditeit. Deze bevindingen leiden tot de conclusie dat de antigeen-presenterende cel (APC) mogelijk een orkestrerende rol vervult in de immuunrespons tijdens RSV bronchiolitis. Onderzoek naar de rol van andere APC, zoals dendritische cellen, in de pathogenese van RSV bronchiolitis is daarom gerechtvaardigd.

Curriculum vitae

Louis Bont was born on March 16, 1970 in Amsterdam, The Netherlands. He graduated from Secondary School (Casimir Lyceum) in 1988. In 1989 he started his medical studies at the University of Amsterdam and obtained his Medical Degree in 1996. During his studies he performed a parasitological schistosomiasis study in Cameroon and an epidemiological malaria study in Vietnam. After graduation he worked as a resident in Pediatrics at the Victoria Hospital, Blackpool, United Kingdom and the Rijnstate Hospital, Arnhem, The Netherlands. In August 1997 he began as a PhD-student at the Wilhelmina Children's Hospital, University Medical Center Utrecht to study the relationship between cellular immune responses during respiratory syncytial virus (RSV) infection and the subsequent development of airway morbidity. He was awarded the European Society for Pediatric Infectious Diseases Fellowship Award in 2000. He received a Ter Meulen Fund stipendium from the Royal Netherlands Academy of Arts and Sciences in 2000. From June 2000 until May 2001 he studied the pathogenesis of RSV infection in mice as a Research Fellow at the University of Texas Medical Branch, Galveston, Texas, United States under Prof. R.P. Garofalo.
He is married to Corine Tiebosch. They have two children, Marin and Neil.

Publications

Bont L, Schepel N, De Vries P, Kager PA. Malaria in een veranderende gezondheidszorg in Vietnam. *Ned Tijdschr Geneeskd 1995; 139:1928-1931.*

Scholte R, Sama-Ekobo A, Slootweg R, Bont L, Polderman AM. The contribution of existing health facilities to the control of urinary schistosomiasis in northern Cameroon. *Acta Trop 1995; 60:109-117.*

Bont L, Brus F, Dijkman-Neerincx RHM, Jansen TLThA, Meyer JWR, Janssen M. The clinical spectrum of post-streptococcal syndromes with arthritis in children. *Clin Exp Rheumatol 1998; 16:750-752.*

Bont L, Bosch F, Dijkman-Neerincx RHM, Yska JPP, Bosker HA. Tosade de pointes na pipamperonintoxicatie. *Cardiologie 1998; 5:193-195.*

Bont L, Heijnen CJ, Kavelaars A, van Aalderen WMC, Brus F, Draaisma JMTh, Geelen SM, van Vught HJ, Kimpen JLL. Association of peripheral blood cytokine responses with disease severity in respiratory syncytial virus bronchiolitis. *Eur Respir J 1999; 14:144-149.*

Bont L, van Vught HJ, Kimpen JLL. Prophylaxis against respiratory syncytial virus (RSV) in premature infants. *Lancet 1999; 354(9183):1003-1004.*

Bont L, van Aalderen WMC, Kimpen JLL. Long-term consequences of respiratory syncytial virus (RSV) bronchiolitis. *Pediatr Respir Rev 2000; 1:221-227.*

Bont L, Heijnen CJ, Kavelaars A, van Aalderen WMC, Brus F, Draaisma JMTh, Geelen SM, Kimpen JL. Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one-year follow-up study. *Am J Respir Crit Care Med 2000; 161:1518-1523.*

Bont L, Kavelaars A, Heijnen CJ, van Vught AJ, Kimpen JL. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. *J Infect Dis 2000; 181:1772-1775.*

Bont L, van Aalderen WMC, Versteegh J, Brus F, Draaisma JMTh, Pekelharing-Berghuis M, van Diemen JAAM, Kimpen JLL. Airflow limitation during respiratory syncytial virus (RSV) lower respiratory tract infection predicts recurrent wheezing. *Pediatr Infect Dis J 2001; 20:277-282.*

Bont L, Heijnen CJ, Kavelaars A, van Aalderen WMC, Brus F, Draaisma JMTh, Pekelharing-Berghuis M, van Diemen JAAM, Kimpen JLL. Local interferon-γ levels during respiratory syncytial virus lower respiratory tract infection are associated with disease severity. *J Infect Dis in press.*

Dankwoord

Dit proefschrift kwam tot stand uit een samenwerking tussen het Wilhelmina Kinderziekenhuis / Utrecht Medisch Centrum, de Beatrix Kliniek / Academisch Ziekenhuis Groningen en de kinderafdelingen van het Rijnstate Ziekenhuis te Arnhem, het Elisabeth Ziekenhuis te Tilburg, het Diakonessenhuis te Utrecht en het Sint Antonius Ziekenhuis te Nieuwegein. Voordat ik de kinderen en hun ouders bedank die deelnamen aan dit onderzoek, wil ik graag een aantal andere mensen bedanken die een belangrijke bijdrage leverden aan dit proefschrift. Vooral wil ik dié mensen bedanken die mij in de gelegenheid stelden om dit onderzoek met plezier uit te voeren.

Prof.dr. J.L.L.Kimpen. Beste Jan, hartelijk dank voor het vertrouwen dat je in mij stelde door mij aan te stellen als jonge onderzoeker. Je enthousiasme is vanaf het begin een stimulans geweest. Ik herinner me nog goed dat je in de gang van het (oude) Wilhelmina Kinderziekenhuis me feliciteerde met de inclusie van de eerste patiënt in dit onderzoek. Ook je bereidheid heeft mij gemotiveerd me in te zetten voor dit project. Zo heb ik zelden langer dan een paar dagen gewacht op een reactie op mijn manuscripten-in-wording. Je deur stond altijd voor me open, zelfs toen ander werk het maximale van je vereiste. Dat je het mij tenslotte mogelijk maakte om gedurende een jaar basaal wetenschappelijke ervaring op te doen in de Verenigde Staten ben ik je dankbaar voor. Je uiterst zakelijke houding was altijd doorzichtig en daarom prettig. Het heeft wel voorkomen dat we ooit een biertje in de kroeg zijn gaan drinken (en ik heb er nog wel één of twee tegoed). Misschien dat dat er nog eens van komt. Jan, ik ben trots dat jij mijn promotor bent.

Prof.dr. C.J.Heijnen en dr. A.Kavelaars. Beste Cobi en Annemieke, ik bedank jullie samen, omdat jullie voor mij een eenheid zijn geweest. Zonder je enthousiasme, Cobi, op het voor jouw vreemde terrein van respiratoir syncytieel virus bronchiolitis en jouw uiterste scherpte, Annemieke, in mijn begeleiding was dit proefschrift niet tot stand gekomen. Jullie bijdrage gaat veel verder dan het bijbrengen van een aantal laboratoriumtechnieken. Jullie stonden aan de basis van een goed doordachte opzet om cytokineprofielen te bepalen bij de patiënten in mijn onderzoek. Het cytokine-onderzoek was haalbaar en naar mijn inzicht toch volledig. De combinatie van haalbaarheid en volledigheid was toch een van de grootste uitdagingen van het cytokine-onderzoek. Ik weet dat jullie mij graag wat meer kneepjes van de experimentele wetenschap hadden bijgebracht en het spijt me dat dat er maar zo zelden van is gekomen. Tenslotte wil ik jullie bedanken dat jullie mij in de eerste "eenzame" maanden in de groep hebben opgenomen en hebben laten meegenieten van de bourgondische avonden aan het Wilhelminapark.

De andere directe mede-onderzoekers: Prof.dr. H.A. van Vught, dr. W.M.C. van Aalderen, dr. F.Brus, dr. J.M.Th. Draaisma, dr. J.A.A.M. van Diemen-Steenvoorde, drs. M. Pekelharing-Berghuis, dr. S.M. Geelen. Beste Hans, Wim, Frank, Jos, Ronnie, Martha en Sybil, jullie maakten mijn onderzoek mogelijk. Jullie betrokken houding was stimulerend en jullie inhoudelijke bijdrage heeft het onderzoek verrijkt. Bovendien hebben jullie zorg gedragen voor de inclusie van de patiënten, waarmee de basis van het onderzoek werd gelegd.

Beste Nathalie, Boris, Erik, Jorien, Minh, Jettie, Wendy en Sandra. Als student namen jullie gedurende een aantal maanden deel aan het onderzoek. Het was een groot plezier om met jullie samen te werken. Met ongekende energie hebben jullie een groot deel van het onderzoek uitgevoerd. Afname van vragenlijsten, telefonisch enqueteren, thuisbezoeken, vergelijken van sensitiviteit/specificiteit van immuunfluorescentie en PCR voor RSV, uitvoeren van immunologische bloedkweken en longfunctieonderzoek bij kinderen op de leeftijd van 2 jaar. Ik hoop dat jullie nooit het enthousiasme zullen verliezen, dat ik heb mogen meemaken.

Beste Marijke, Rogier, Arieke, Yvet, Michiel, Jeanne-Marie en alle andere (tijdelijke) bewoners van de "flex-kamer" op de vierde verdieping van het nieuwe WKZ. Hartelijk dank voor onvergetelijke en gezellige jaren. We deelden de frustraties van patiëntgebonden onderzoek en het plezier van mooie nieuwe data. En ook koekjes, ijsjes en andere geneugten. Beste Marijke, terwijl ik dit schrijf leid jij het hele RSV project. Het gemak waarmee je in het onderzoek stapte en het geduld dat je had met mijn eigenwijsheid bewonder ik. Ik heb genoten van de samenwerking. Rogier, de vele discussies over "wel of niet Th2" en andere raakvlakken tussen RSV en koemelkeiwitallergie waren essentieel voor dit proefschrift. Ik hoop dat onze wegen nog vaak zullen kruisen.

Alle AIO's, post-docs en andere medewerkers van het Immunologie laboratorium van het WKZ. Het begrip voor mijn onervarenheid en stommiteiten op jullie lab heb ik als een steun ervaren. Ik wil jullie daarnaast ook bedanken voor alles wat ik van jullie heb geleerd. Ik had vooraf aan mijn onderzoek letterlijk nog nooit een pipet vastgehouden. Marion, Jitske en Anita, jullie wil ik in het bijzonder bedanken voor de hulp bij de bloedkweken, ELISA's en het analyseren van de snottebellen.

En dan nog een paar mensen die ik wil bedanken. Beste Corine, als poli-assistente in het Sint Elisabeth Ziekenhuis in Tilburg heb je veel werk verricht voor deze cohort-studie. Beste Cuno, bedankt voor je epidemiologische ondersteuning. Beste Carla, bedankt voor je bijdrage bij de data-beheer. De medewerkers van het seretariaat Algemene Kindergeneeskunde, beste Bea, Ellen en Marieke, jullie waardeer ik voor diverse diensten, die jullie, belangeloos, ten gunste van dit onderzoek uitvoerden. In het bijzonder wil ook alle artsen, verpleegkundigen en andere medewerkers van de Intensive Care Algemene Pediatrie van het WKZ/UMC bedanken, omdat ik me op jullie afdeling altijd zo bijzonder welkom heb gevoeld.

Beste ouders van de kinderen uit deze cohortstudie. Jullie kinderen zijn het onderzoek. Jullie waren bereid lastige vragen te beantwoorden terwijl jullie baby opgenomen was met een ernstige RSV infectie. En daarbij gaven jullie nog toestemming voor bloed-en slijmafname. En later nog meer prikken en longfunctieonderzoek. Voor mij was het meest indrukwekkend dat het jullie lukten om gedurende enkele jaren dagelijks luchtwegklachten te noteren. Jullie bereidheid mee te werken aan dit RSV onderzoek heeft mijn verwachtingen steeds opnieuw overtroffen. Bedankt!!!

Professor Garofalo. Dear Roberto, although my work in Galveston has not been part of this thesis I felt that my work at your lab was a logical consequence of my study in the Netherlands. I thank you for the opportunity to work with you and for the inspiring supervision. I learned how to perform RSV research in a mouse model. I have realized that this type of work will provide answers to important questions that can not be addressed in human studies. I also thank Antonella and you for your very warm hospitality that made our Galveston adventure a thrill.

Dr Haeberle. Dear Helene, I want to thank you for everything you have taught me. You have introduced me to the mouse work. Your patience allowed me to learn, your enthusiasm and energy have made a great environment for experiencing the fundamentals of basic research.
Lieve mama, dank zij jou is dit een prachtig boekje geworden. Terwijl ik in de Verenigde Staten verblijf, zorg jij, met oog voor alle details, voor de laatste loodjes van dit proefschrift. Lieve papa, natuurlijk bedank ik jou ook. Jij en ma hebben mij opgevoed met respect. Respect voor mij, respect voor elkaar en respect voor anderen. Maar ook respect voor nieuwsgierigheid en wetenschap. Verder wil ik jullie bedanken voor zaken waar ik geen woorden voor heb kunnen vinden. Ik hoop deze dank op een andere manier over te brengen.
En tenslotte Corine, Marin en Neil. *You have rocked my world!*

Address

Room KB.03.023.2
Wilhelmina Children's Hospital
University Medical Center Utrecht
POB 85090
NL 3508 AB Utrecht
the Netherlands
Tel: 31-30-2504194
Fax: 31-30-2505346
l.bont@wkz.azu.nl