Respiratory Syncytial Virus Infections in Infants

Determinants of Clinical Severity

Afke Hélène Brandenburg

Cover photograph: Negative constrast electron micrograph of RSV, a kind gift from dr. J.J. Habova of the department of Virology of the Erasmus Medical Center Rotterdam.

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Determinants of Clinical Severity

Respiratoir syncytieel virus infecties in jonge kinderen

Factoren van invloed op klinische ernst

PROEFSCHRIFT

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op gezag van de rector magnificus

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En volgens besluit van het College voor Promoties

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Introduction and scope of this thesis

1.1. History

In 1955 a virus was isolated by Morris et al. from a chimpanzee with an upper respiratory tract infection (80). This apparently new virus was originally called chimpanzee coryza agent. Soon afterwards, when it was isolated from children with respiratory disease, it became clear that this virus was a major human pathogen (18-20). The virus was from then onward called respiratory syncytial virus (RSV) because of its ability to cause respiratory disease and to induce large syncytia in cell culture. RSV is now known as the single most common cause of severe respiratory tract infection in childhood. In fact up to 70% of hospital admissions of infants for respiratory infections during the winter season may be caused by RSV alone (37).

Soon after RSV was found to be a significant cause of morbidity and mortality in childhood the search for a vaccine began. During the sixties a formalin inactivated RSV (FI-RSV) candidate vaccine, known as "lot100", was developed and administered to children of two to seven years old. This vaccine, in stead of protecting vaccinees against RSV infection, predisposed for more severe disease upon natural infection in the following RSV season. Hospitalization rates were as high as 80% and two of the vaccinees died (22,34,69).

At this moment, despite considerable research efforts, no licensed vaccine is available against this important pathogen. Development of a vaccine against RSV is one of the priorities of the Global Program for Vaccines of the World Health Organization (27).

1.2. Virology

RSV is a member of the family of Paramyxoviridae. It belongs to the genus Pneumovirus, together with bovine RSV – which causes a disease in calves similar to the severe manifestations of human RSV in infants – the turkey rhinotracheitisvirus and the pneumonia virus of mice (Table 1.1.). The virion contains one single-stranded negative-sense RNA genome, of 15.222 base pairs long, which contains the genetic information for the ten known RSV proteins (61). The RSV genome is enclosed within the nucleocapsid of about 13.5 nm in diameter, which is encapsulated by a lipid bilayer envelope of 120-300 nm. The members of the genus Pneumovirus differ from other paramyxovirusses by having envelope proteins without hemaglutinin or neuraminidase activity and also by having a second matrix protein (24).

RSV contains 8 structural proteins. The two main envelope glycoproteins, the fusion protein (F) and the glycoprotein (G) form the spikes which are visible as a fuzzy layer by electron microscopy (see also Figure 1.1.). The F protein of about 70kD mediates both viral penetration and cell to cell spread by fusion of membranes. This protein contains N linked oligosacharide groups and is composed of 2 disulfide-linked subunits of 47kD (F1) and 20kD (F2). The larger envelope protein G of about 90kD serves a function corresponding to

Table 1.1. Classification of Paramyxoviridae

Subfamily *genus*	virus	disease in humans
Subfamily Paramyxovirinae		
Paramyxovirus	human parainfluenzavirus type 1	common cold, croup
	human parainfluenzavirus type 3	common cold, croup, pneumonia
	sendai virus (mouse parainfluenzavirus type 1)	
	bovine parainfluenza virus type 3	
	simian parainfluenzavirus type 10	
Rubulavirus	human parainfluenzavirus type 2	common cold, croup
	human parainfluenzavirus type 4 A and B	
	mumpsvirus	mumps
	porcine rubulavirus	
	simian virus 5 (canine parainfluenzavirus type2)	
	newcastle disease virus (avian paramyxovirus 1)	
	Yucaipavirus (avian paramyxovirus 2)	
	avian paramyxovirus 3 to 9	
Morbillivirus	measlesvirus	measles
	canine distemper virus	
	rinderpest virus	
	peste-des-petit-ruminant virus	
	phocine distemper virus	
	dolphin morbillivirus	
	porpoise distempervirus	
tentative new genus	Hendra virus	respiratory symptoms
	Nipah virus	flu-like symptoms, encephalitis
	Menangle virus	
Subfamily Pneumovirinae		
Pneumovirus	human respiratory syncytial virus	common cold, bronchiolitis, pneumonia
	bovine respiratory syncytial virus	
	mouse pneumonia virus	
	turkey rhinotracheitis virus	

Data obtained from references 23,54,72,76,89

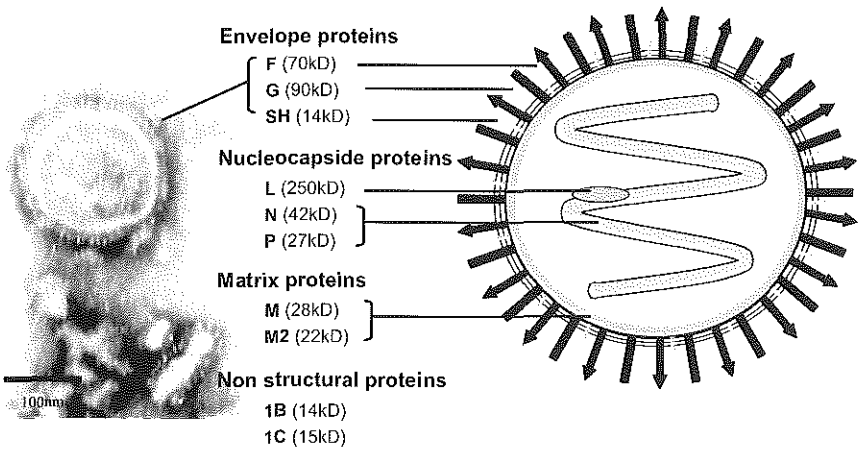

Figure 1.1. Negative contrast electron micrograph (A) and schematic diagram (B) of the RSV virion showing the different RSV proteins. (The electron micrograph was a kind gift from dr. J.J. Habova of the department of Virology of the Erasmus Medical Center Rotterdam).

the host cell attachment of the hemaglutinin of other paramyxovirusses. It is heavily glycosilated with both O- and N-linked oligosacharide groups. In fact more than 60% of the molecular weight of the G protein consists of sugar moieties. RSV contains one smaller envelope glycoprotein, the SH or 1A protein of about 14kD, of which the function is essentially unknown. There are two matrix proteins, M of about 28kD and M2 of about 22kD. RSV contains 3 nucleocapsid-associated proteins; the nucleoprotein (N) of about 42kD, the phosphoprotein (P) of about 27kD and the large protein (L) of about 250kD, which harbors the RNA polymerase function. Furthermore, RSV contains two small non-structural proteins; the 1B and 1C protein of about 14 and 15kD respectively, of which the functions are unknown.

Antigenic diversity in RSV strains is extensive, especially for the G protein, the most variable protein of the virus (66). RSV strains can be divided into two main groups, RSV-A and -B, by their reaction patterns with monoclonal antibody panels (4,81,82) and on the basis of the nucleotide sequence differences between several of their genes (13,65,101,103).
Between the two groups antigenic homology is found to be about 25%. For the F protein it is about 50%. For the G protein amino acid homology has been found to be as low as 53% and antigenic homology only 5%, between the two groups. This makes the G protein the protein most tolerable to mutations without loss off function known in nature (24,59,66). The two antigenic categories of RSV have variously been described in literature as groups, subgroups, types or subtypes. The term "group" is currently used most frequently (102).

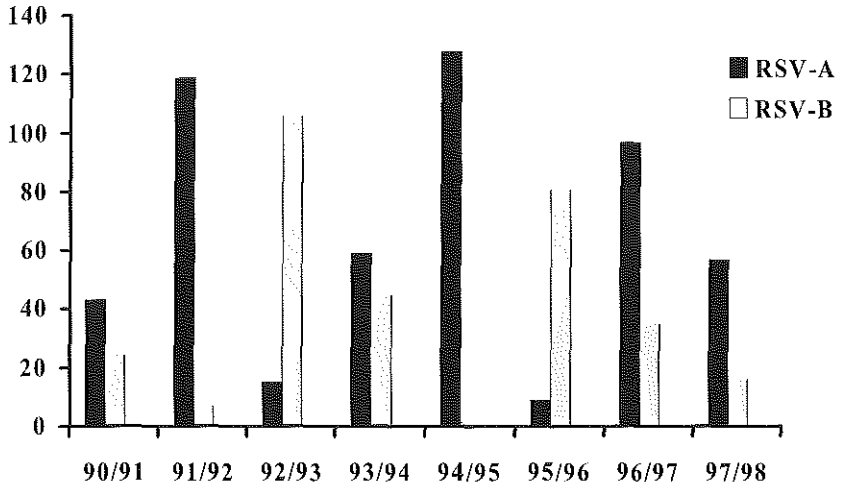

Figure 1.2. Annual incidence of RSV group A and B isolations as detected at the department of Virology of the Erasmus Medical Center Rotterdam from 1990 to 1998 (kindly provided by G. Aaron and Dr. Ph.H. Rothbarth).

The RSV groups A and B circulate independently in the population with group A being somewhat more prevalent (2,60,81,106). Figure 1.2. illustrates this by showing the number of isolates of groups A and B over eight years in the Erasmus Medical Center Rotterdam. Also within the two groups several lineages can be identified, mainly on the basis of differences in the G protein. These strains also seem to co-circulate independently in the population (10,101).

1.3. Epidemiology

RSV infections have a worldwide geographical distribution. Wherever studies have been performed, RSV was found to be the main cause of severe lower respiratory tract infection in young children (24,37,113). In temperate climate zones RSV infections occur virtually only during the winter season during yearly epidemics (24), as is illustrated in Figure 1.3. Epidemics usually last for about 5 months but 40% of infections are usually found during one peak month, being mostly December or January. In tropical climates a different pattern is found. Infections may be detected year round with peaks often found during the rainy season (113). In some years mainly group A viruses are isolated, whereas in others mainly group B viruses are found. In some years a co-circulation of both group A and B viruses is observed (see also Figure 1.2.).

RSV is spread via infected respiratory secretions. Transmission occurs mainly through

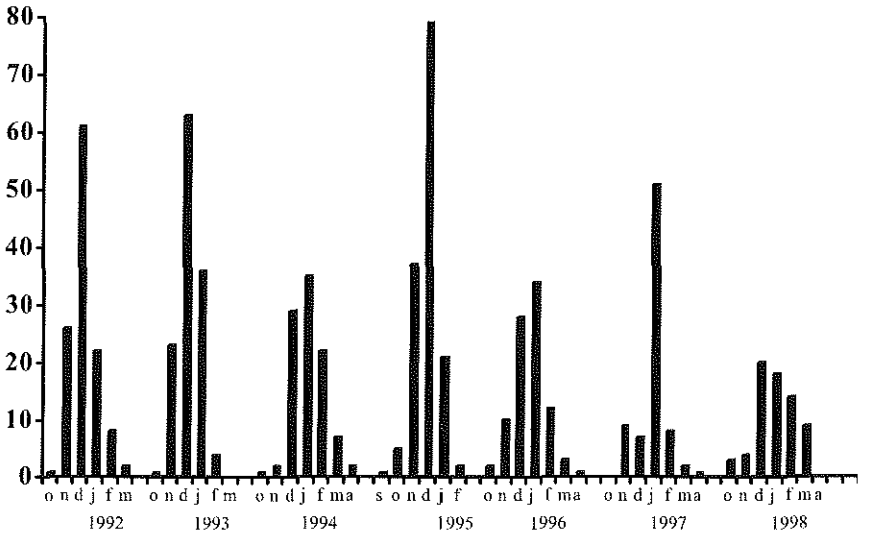

Figure 1.3. Monthly RSV isolations as detected at the department of Virology of the Erasmus Medical Center Rotterdam from 1991 to 1998 illustrating the yearly winter outbreaks of RSV in our setting (kindly provided by G. Aaron and dr. Ph.H. Rothbarth).

direct close contact with infected individuals or contact with surfaces contaminated with respiratory secretions and subsequent "self-infection" via contaminated hands to nasal and conjunctival mucosa. Transmission does not occur via small particle aerosols (46). RSV can survive on clothes for about 30 minutes and on smooth surfaces like countertops and stethoscopes for several hours (9,49).

RSV causes respiratory tract infections of patients of any age. Clinically severe RSV infections are mainly found in the very young, presumably at their first infection. The highest morbidity of RSV infections is seen in infants between six weeks and six months of age (38,40,50,88).

Reinfections with RSV are common. They tend to have a milder clinical course and usually remain limited to the upper respiratory tract. In a study of Hall et al about 25% of adult volunteers could be reinfected with RSV of the same group two months after natural infection (52). Frequent reinfections enable RSV to remain highly prevalent in the population. At least 50% of children are infected during their first winter. By two years of age almost all children will have been infected at least once and over 50% will have been infected twice (58).

1.4. Clinical manifestations of RSV infection

RSV infection usually starts with upper respiratory tract infection characterized by rhinitis, cough and a low-grade fever. Two to five days later lower respiratory tract involvement may develop characterized by tachypnea, chest retractions and sometimes wheezing. Often hypoxemia and hypercapnia is observed. Only in a minority of children fever is found at that stage. A high percentage (up to 40%) of young children infected with RSV for the first time will show signs of lower respiratory tract involvement (39,67,100), but only some will be so severely affected that hospital admission is necessary. Apnea is found in 10 – 20 % of infants hospitalized for RSV and in the very young apnea may be the only presenting symptom of RSV infection. An estimated 0.5 – 2% of all infants are hospitalized with an RSV infection in their first year of life (38,40,50,75,85) and 7% – 21% of these hospitalized infants will develop respiratory insufficiency that requires ventilatory support (11,12,105). Especially at risk for a severe clinical course of RSV infection are infants with underlying risk factors such as prematurity (85), bronchopulmonary dysplasia (41), congenital heart disease with pulmonary hypertension (74) or immune deficiency (51). However, most children admitted to hospital because of a RSV infection do not posses one or more of these risk factors.

The most common clinical presentation of RSV infection in older children and adults is a mild upper respiratory infection. Although RSV is described mainly as a cause of lower respiratory tract infection in young infants, in recent years more and more reports of severe RSV infections in adults are seen (6,32,73,114). Severe clinical RSV infection is seen in immune compromised adults, especially shortly after bone marrow transplantation, where high mortality rates are found (33,55). During outbreaks among institutionalized elderly, RSV infections are frequently complicated by pneumonia and mortality rates of 2 to 20% have been described (43,56,87,97). RSV infections have also been related to community acquired pneumonia and excacerbations of COPD in adult patients (44,98,108).

1.5. Humoral immune response

In response to RSV infection, antibodies to most RSV proteins develop (36,107,109-111). Neutralizing antibodies are mainly directed to the envelope glycoproteins and are involved in protection. Reinfection however, is readily possible (52,58) also in adults, indicating that protection is incomplete even when high levels of neutralizing antibodies are present.
Antibodies detected locally in the respiratory tract are related to protection against infection or reinfection. Presence of neutralizing antibodies in the respiratory tract was described to correlate with relative protection against experimental infection in human adults (79,84) and development of local IgA has been found to coincide with virus clearance (77).

In the past antibodies have often been incriminated as playing a role in the pathology of RSV lower respiratory tract infection mainly based on two facts. (i) Most severe infections are seen at an age when infants still have maternally derived antibodies (39,68), and (ii) the FI-RSV candidate vaccine, which predisposed for more severe disease upon subsequent infection, induced high levels of antibodies (22,83). However it has become apparent since, that RSV specific antibodies do not play a role in pathogenesis of severe lower respiratory tract disease. In fact RSV neutralizing antibodies induce protection to infection, although high titers are necessary. This protective effect of high titers of RSV neutralizing antibodies is found both in animal models (90) and in infants in which higher levels of maternally derived antibodies have been found to correlate with a relative protection to the development of lower respiratory tract infection (38). Furthermore in recent years passive administration of antibodies to high-risk children has been found to be valuable in decreasing the chance to develop severe clinical disease upon RSV infection (42,57,96). Recently RSV hyperimmuneglobulin (RespiGam®) and a humanized monoclonal antibody directed to the F protein (Paluvizumab®) have been licensed for prophylactic use in high-risk infants (1,78,94)

1.6. Cellular immune response

RSV specific cellular immune response is considered to play a major role in clearance of the virus and recovery from infection. This is illustrated by the observation that normal children stop shedding virus within 1 to 3 weeks but T cell immune compromised children can shed virus for many months (17,51). Also in mouse models, T cell depleted animals can shed RSV for prolonged periods of time, whereas the administration of RSV specific CD4+ and/or CD8+ cells can induce clearance of the virus in these animals. Interestingly the administration of these cells also tends to increase lung damage (3,16,26).
Especially RSV specific cytotoxic T cells (CTL) are found to play an important role in clearing the virus (3,15,71,104). CTL against most RSV proteins can be readily detected after infection in several animal models (3,7,14,15,71,86,104) and in humans (7,8,14,21,62). However, no CTL response to the RSV G protein has yet been demonstrated (7).

Apart from being important in protection, RSV specific T cell responses also play a role in the induction of lung pathology. In mouse models, upon challenge with live virus animals vaccinated with FI-RSV, a striking type 2 T cell response is induced which correlates with enhanced lung disease largely characterized by eosinophilia (3,25,26). This type 2 T cell response proved to correlate with the absence of a cytotoxic T cell response (95,99).
Measurement of cellular immune memory response in peripheral blood mononuclear cells (PBMC) from normal children and adults showed a type 1 like T cell response (5).

Indications for a type 2 like T cell response are also found in humans. *In vitro* stimulation of PBMC from normal humans, with live RSV, FI-RSV or RSV proteins was shown to induce a type 1 like response for live RSV and the F protein whereas FI-RSV and the G protein induced predominantly a type 2 like T cell response (63). Moreover type 2 like cytokines have been detected in supernatants of PBMC from children who experienced a RSV bronchiolitis at an early age (93), indicating a direct relationship between severe RSV lower respiratory tract infection and T cell responses.

1.7. Pathogenesis of RSV and the possible role of the immune response in pathogenesis

In experimental and natural settings the incubation period for RSV was found to be about 4 to 5 days (46,64,67,70,100). At the beginning of the infection RSV primarily replicates in the epithelial cells of the nasopharynx (53), inducing signs of upper respiratory tract infection. The mechanism by which RSV spreads to the lower respiratory tract is essentially unknown, but it is thought that spread occurs mainly by the aspiration of nasopharingeal secretions. RSV may also spread directly from cell to cell. It is however unlikely that, in the natural situation, this is an important route for viral spread to the lower respiratory tract, because in certain animal studies the tracheal epithelium shows only a patchy infection at any time (31,91). RSV infections usually stay limited to the respiratory tract and do not become systemic even in severely affected children (24,35). No culturable virus is found in blood samples of immunologically competent patients, although RSV genome may be detected in bloodsamples of infected young children (29,92). Signs of lower respiratory tract involvement, like tachypnea, usually start 1 to 3 days after the onset of disease, probably indicating viral spread to the lower respiratory tract (24).

Pathologic findings in severe RSV infection resemble those observed in severe infections by other respiratory viruses. In these cases destruction of epithelial cells is found and cell debris is released into the bronchiolar space, which is accompanied by enhanced mucus secretion. In addition a peri-bronchiolar inflammatory response is found, characterized by a cellular infiltrate mainly consisting of mononuclear cells. Also edema of the mucosa and submucosa develops. Mucosal necrosis and mucus secretion together with swelling of submucosal tissue may induce obstruction of the bronchioli, leading to hyperinflation and atelectasis of parts of the lungs, contributing to the classical signs and symptoms of RSV lower respiratory tract infection (24).

Clinical severity of lower respiratory tract involvement in RSV infection is, at least partially, determined by the direct cytopathic effect of the virus infection on lung tissue. A role for an aberrant immunological response, occurring in part of the infected children, which increases the inflammatory process, has long been suspected. These speculations were based

on several observations. (i) Most importantly, the vaccine debacle, predisposing for a more severe disease upon subsequent infection (22,34,69). (ii) Severe infections found at an age when maternal antibodies are still present. (iii) Clinical signs that have been reported to diminish while virus excretion from the nasopharynx continued (47,48). (iv) Children who experienced a clinically severe RSV infection at a young age have higher chance of developing recurrent wheezing later in childhood (30,45,112).

Enhanced disease after vaccination with FI-RSV has, in the mouse model, convincingly been related to an aberrant type 2 like T cell response leading to more severe lung disease. Newborn infants still have an immature immune response and are known to be more prone to produce a type 2 like T cell response upon infection (28), possibly leading to more severe inflammation in these children upon RSV infection. The presence of type 2 cytokines in supernatants of PBMC from children who experienced a RSV infection at an early age have been reported (93).

1.8. Scope of this thesis

Although for many years RSV has been recognized as the most prevalent cause of severe respiratory infection in young infants, the development of effective preventive and therapeutic strategies has been hampered by the lack of understanding of the pathogenesis of lower respiratory tract involvement occurring in part of the RSV infected infants.

As outlined above it has long been suspected that an immune pathologic phenomenon may be at the basis of the development of severe RSV lower respiratory tract infection. However other factors related to the virus, the patient or the environment may have an impact on the clinical outcome of naturally occurring RSV infection in young infants.

In this thesis the role of viral (chapter 2), environmental (chapter 3) and immunological factors (chapter 4 and 5) are studied in relation to the clinical severity of RSV infection. The goal of these studies was to obtain more insight in factors related to the clinical severity of RSV lower respiratory tract infection. This knowledge will be valuable for the rational development of preventive and therapeutic intervention strategies for RSV infection in young infants.

References

1. Anonymous1998. Prevention of respiratory syncytial virus infections: indications for the use of palivizumab and update on the use of RSV-IGIV. American Academy of Pediatrics Committee on Infectious Diseases and Committee of Fetus and Newborn. Pediatrics 102:1211-1216.
2. Akerlind, B. and E. Norrby. 1986. Occurrence of respiratory syncytial virus subtypes A and B strains in Sweden. J. Med. Virol. 19:241-247.
3. Alwan, W. H., F. M. Record, and P. J. M. Openshaw. 1992. CD4+ T cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T cells.

Clin. Exp. Immunol. 88:527-536.
4. Anderson, L. J., J. C. Hierholzer, C. Tsou, R. M. Hendry, B. F. Fernie, Y. Stone, and K. Mcintosh. 1985. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J. Infect. Dis. 151:626-633.
5. Anderson, L. J., C. Tsou, C. Potter, H. L. Keyserling, T. F. Smith, G. Ananaba, and C. R. M. Bangham. 1994. Cytokine response to respiratory syncytial virus stimulation of human peripheral blood mononuclear cells. J. Infect. Dis. 170:1201-1208.
6. Aylward, R. B. and D. R. Burdge. 1991. Ribavirin therapy of adult respiratory syncytial virus pneumonitis. Arch. Intern. Med. 151:2303-2304.
7. Bangham, C. R. M., P. J. M. Openshaw, L. A. Ball, A. M. Q. King, G. W. Wertz, and B. A. Askonas. 1986. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoproteins (G), expressed by vaccinia virus recombinants. J. Immunol. 137:3973-3977.
8. Belshe, R. B., L. S. Richardson, W. T. London, D. L. Sly, E. Camargo, D. A. Prevar, and R. M. Chanock. 1978. Evaluation of five temperature-sensitive mutants of respiratory syncytial virus in primates: II. Genetic analysis of virus recovered during infection. J. Med. Virol. 3:101-110.
9. Blydt-Hansen, T., K. Subbarao, P. Quennec, and J. McDonald. 1999. Recovery of respiratory syncytial virus from stethoscopes by conventional viral culture and polymerase chain reaction. Pediatr. Infect. Dis. J. 18:164-165.
10. Cane, P. A., D. A. Matthews, and C. R. Pringle. 1992. Analysis of relatedness of subgroup A respiratory syncytial viruses isolated worldwide. Virus Res. 25:15-22.
11. Cane, P. A., D. A. Matthews, and C. R. Pringle. 1993. Frequent polymerase errors observed in a restricted area of clones derived from the attachment (G) protein gene of respiratory syncytial virus. J. Virol. 67:1090-1093.
12. Cane, P. A. and C. R. Pringle. 1992. Molecular epidemiology of respiratory syncytial virus: rapid identification of subgroup A lineages. J. Virol. Methods 40:297-306.
13. Cane, P. A. and C. R. Pringle. 1995. Evolution of subgroup A respiratory syncytial virus: evidence for progressive accumulation of amino acid changes in the attachment protein. J. Virol. 69:2918-2925.
14. Cannon, M. J. and C. R. M. Bangham. 1989. Recognition of Respiratory Syncytial Virus Fusion Protein by Mouse cytotoxic T Cell Clones and a Human Cytotoxic T Cell Line. J. Gen. Virol. 70:79-87.
15. Cannon, M. J., P. J. M. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168:1163-1168.
16. Cannon, M. J., E. J. Stott, G. Taylor, and B. A. Askonas. 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. Immunology 62:133-138.
17. Chandwani, S., W. Borkowsky, K. Krasinski, R. Lawrence, and R. Welliver. 1990. Respiratory syncytial virus infection in human immunodeficiency virus- infected children. J. Pediatr. 117:251-254.
18. Chanock, R. M. and L. finberg. 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza gent (CCA). II. Epidemiological aspects of infection in infants and young children. Am. J. Hyg. 66:291-300.
19. Chanock, R. M., H. W. Kim, A. J. Vargosko, A. Deleva, K. M. Johnson, C. Cumming, and R. H. Parrott. 1961. Respiratory syncytial virus I. Virus recovery and Other observations during 1960 outbreak of bronchiolititis, pneumonia and minor respiratory disease in children. J. A. M. A. 176:647-653.
20. Chanock, R. M., B. Roizman, and R. Myers. 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent. I. Isolation properties and characterization. Am. J. Hyg. 66:281-290.
21. Chiba, Y., Y. Higashidate, K. Suga, K. Honjo, H. Tsatsumi, and P. L. Ogra. 1989. Development of cell-mediated cytotoxic immunity to respiratory syncytial virus in human infants following naturally acquired infection. J. Med. Virol. 28:133-139.
22. Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette. 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. 89:449-463.
23. Chua, K. B., W. J. Bellini, P. A. Rota, B. H. Harcourt, A. Tamin, S. K. Lam, T. G. Ksiazek, P. E. Rollin, S. R. Zaki, W. Shieh, C. S. Goldsmith, D. J. Gubler, J. T. Roehrig, B. Eaton, A. R. Gould, J. Olson, H.

Field, P. Daniels, A. E. Ling, C. J. Peters, L. J. Anderson, and B. W. Mahy. 2000. Nipah virus: a recently emergent deadly paramyxovirus. Science 2000. May. 26;288(5470):1432-5. 288:1432-1435.
24. Collins, P. L., K. Mcintosh, and R. M. Chanock. 1996. Respiratory syncytial virus, p. 1313-1351. In B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Fields virology. Lippincott Raven Publishers, Philadelphia.
25. Connors, M., N. A. Giese, A. B. Kulkarni, C.-Y. Firestone, H. C. Morse III, and B. R. Murphy. 1994. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. J. Virol. 68:5321-5325.
26. Connors, M., A. B. Kulkarni, C.-Y. Firestone, K. L. Holmes, H. C. Morse III, A. V. Sotnikov, and B. R. Murphy. 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J. Virol. 66:7444-7451.
27. Crowe, J. E., Jr. 1995. Current approaches to the development of vaccines against disease caused by respiratory syncytial virus (RSV) and parainfluenza virus (PIV). A meeting report of the WHO Programme for Vaccine Development. Vaccine 13:415-421.
28. Delespesse, G., L. P. Yang, Y. Ohshima, C. Demeure, U. Shu, D. G. Byun, and M. Sarfati. 1998. Maturation of human neonatal CD4+ and CD8+ T lymphocytes into Th1/Th2 effectors. Vaccine 16:1415-1419.
29. Domurat, F., N. J. Roberts,Jr., E. E. Walsh, and R. Dagan. 1985. Respiratory syncytial virus infection of human mononuclear leukocytes in vitro and in vivo. J. Infect. Dis. 152:895-902.
30. Duiverman, E. J., H. J. Neijens, R. van Strik, M. J. Affourtit, and K. F. Kerrebijn. 1987. Lung function and bronchial responsiveness in children who had infantile bronchiolitis. Pediatr. Pulmonol. 3:38-44.
31. Fishaut, M., J. D. Schwartzman, K. Mcintosh, and S. R. Mostow. 1978. Behavior of respiratory syncytial virus in piglet tracheal organ culture. J. Infect. Dis. 138:644-649.
32. Fleming, D. M. and K. W. Cross. 1993. Respiratory syncytial virus or influenza? Lancet 342:1507-1510.
33. Fouillard, L., L. Mouthon, J. P. Laporte, F. Isnard, J. Stachowiak, M. Aoudjhane, J. C. Lucet, M. Wolf, F. Bricourt, and L. Douay. 1992. Severe respiratory syncytial virus pneumonia after autologous bone marrow transplantation: a report of three cases and review. Bone Marrow Transplant. 9:97-100.
34. Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn. 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aquaeous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. 89:435-448.
35. Gardner, P. S., J. McQuillin, and S. D. Court. 1970. Speculation on pathogenesis in death from respiratory syncytial virus infection. Br. Med. J. 1:327-330.
36. Gimenez, H. B., H. M. Keir, and P. Cash. 1987. Immunoblot analysis of the human antibody response to respiratory syncytial virus infection. J. Gen. Virol. 68:1267-1275.
37. Glezen, P. and F. W. Denny. 1973. Epidemiology of acute lower respiratory disease in children. N. Engl. J. Med. 288:498-505.
38. Glezen, W. P., A. Paredes, J. E. Allison, L. H. Taber, and A. L. Frank. 1981. Risk of respiratory syncytial virus infection for infants from low- income families in relationship to age, sex, ethnic group, and maternal antibody level. J. Pediatr. 98:708-715.
39. Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child 140:543-546.
40. Green, M., A. F. Brayer, K. A. Schenkman, and E. R. Wald. 1989. Duration of hospitalization in previously well infants with respiratory syncytial virus infection. Pediatr. Infect. Dis. J. 8:601-605.
41. Groothuis, J. R., K. M. Gutierrez, and B. A. Lauer. 1988. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 82:199-203.
42. Groothuis, J. R., E. A. Simoes, M. J. Levin, C. B. Hall, C. E. Long, W. J. Rodriguez, J. Arrobio, H. C. Meissner, D. R. Fulton, and R. C. Welliver. 1993. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. The Respiratory Syncytial Virus Immune Globulin Study Group. N. Engl. J. Med. 329:1524-1530.
43. Gross, P. A., M. Rodstein, J. R. LaMontagne, R. A. Kaslow, A. J. Saah, S. Wallenstein, R. Neufeld, C. Denning, P. Gaerlan, and G. V. Quinnan. 1988. Epidemiology of acute respiratory illness during an

influenza outbreak in a nursing home. A prospective study. Arch. Intern. Med. 148:559-561.
44. Guidry, G. G., C. A. Black-Payne, D. K. Payne, R. M. Jamison, R. B. George, and J. A. Bocchini,Jr. 1991. Respiratory syncytial virus infection among intubated adults in a university medical intensive care unit. Chest 100:1377-1384.
45. Gurwitz, D., C. Mindorff, and H. Levison. 1981. Increased incidence of bronchial reactivity in children with a history of bronchiolitis. J. Pediatr. 98:551-555.
46. Hall, C. B. and R. G. Douglas,Jr. 1981. Modes of transmission of respiratory syncytial virus. J. Pediatr. 99:100-103.
47. Hall, C. B., R. G. Douglas,Jr., and J. M. Geiman. 1975. Quantitative shedding patterns of respiratory syncytial virus in infants. J. Infect. Dis. 132:151-156.
48. Hall, C. B., R. G. Douglas,Jr., and J. M. Geiman. 1976. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. J. Pediatr. 89:11-15.
49. Hall, C. B., R. G. Douglas,Jr., and J. M. Geiman. 1980. Possible transmission by fomites of respiratory syncytial virus. J. Infect. Dis. 141:98-102.
50. Hall, C. B., W. J. Hall, and D. M. Speers. 1979. Clinical and physiological manifestations of bronchiolitis and pneumonia. Outcome of respiratory syncytial virus. Am. J. Dis. Child 133:798-802.
51. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315:77-81.
52. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. 163:693-698.
53. Hall, W. J., C. B. Hall, and D. M. Speers. 1978. Respiratory syncytial virus infection in adults: clinical, virologic, and serial pulmonary function studies. Ann. Intern. Med. 88:203-205.
54. Harcourt, B. H., A. Tamin, T. G. Ksiazek, P. E. Rollin, L. J. Anderson, W. J. Bellini, and P. A. Rota. 2000. Molecular characterization of nipah virus, a newly emergent paramyxovirus. Virology 2000. Jun. 5;271(2):334-49. 271:334-349.
55. Harrington, R. D., T. M. Hooton, R. C. Hackman, G. A. Storch, B. Osborne, C. A. Gleaves, A. Benson, and J. D. Meyers. 1992. An outbreak of respiratory syncytial virus in a bone marrow transplant center. J. Infect. Dis. 165:987-993.
56. Hart, R. J. 1984. An outbreak of respiratory syncytial virus infection in an old people's home. J. Infect. 8:259-261.
57. Hemming, V. G., G. A. Prince, J. R. Groothuis, and G. R. Siber. 1995. Hyperimmune globulins in prevention and treatment of respiratory syncytial virus infections. Clin. Microbiol. Rev. 8:22-33.
58. Henderson, F. W., A. M. Collier, W. A. Clyde,Jr., and F. W. Denny. 1979. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N. Engl. J. Med. 300:530-534.
59. Hendry, R. M., J. C. Burns, E. E. Walsh, B. S. Graham, P. F. Wright, V. G. Hemming, W. J. Rodriguez, H. W. Kim, G. A. Prince, and K. Mcintosh. 1988. Strain-specific serum antibody responses in infants undergoing primary infection with respiratory syncytial virus. J. Infect. Dis. 157:640-647.
60. Hendry, R. M., A. L. Talis, E. Godfrey, L. J. Anderson, B. F. Fernie, and K. Mcintosh. 1986. Concurrent circulation of antigenically distinct strains of respiratory syncytial virus during community outbreaks. J. Infect. Dis. 153:291-297.
61. Huang, Y. T., P. L. Collins, and G. W. Wertz. 1985. Characterization of the 10 proteins of human respiratory syncytial virus: identification of a fourth envelope-associated protein. Virus Res. 2:157-173.
62. Isaacs, D., C. R. M. Bangham, and A. J. McMichael. 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet 2:769-771.
63. Jackson, M. and R. Scott. 1996. Different patterns of cytokine induction in cultures of respiratory syncytial (RS) virus-specific human Th cell lines following stimulation with RS virus and RS virus proteins. J. Med. Virol. 49:161-169.
64. Johnson, K. M., R. M. Chanock, D. Rifkind, H. M. Kravetz, and V. Knight. 1961. Resopiratory syncytial virus IV. Correlation of virus shedding, serologic response, and Illness in adult volunteers. J. A. M. A. 176:663-667.
65. Johnson, P. R., M. K. Spriggs, R. A. Olmsted, and P. L. Collins. 1987. The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: extensive sequence divergence between antigenically related proteins. Proc. Natl. Acad. Sci. USA 84:5625-5629.

66. Johnson, P. R., Jr., R. A. Olmsted, G. A. Prince, B. R. Murphy, D. W. Alling, E. E. Walsh, and P. L. Collins. 1987. Antigenic relatedness between glycoproteins of human respiratory syncytial virus subgroups A and B: evaluation of the contributions of F and G glycoproteins to immunity. J. Virol. 61:3163-3166.
67. Kapikian, A. Z., J. A. Bell, F. M. Mastrota, K. M. Johnson, R. J. Huebner, and R. M. Channock. 1961. An outbreak of febrile illnessand pneumonia associated with respiratory syncytial virus infection. Am. J. Hyg. 74:234-248.
68. Kim, H. W., J. O. Arrobio, C. D. Brandt, B. C. Jeffries, G. Pyles, J. L. Reid, R. M. Chanock, and R. H. Parrott. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. Am. J. Epidemiol. 98:216-225.
69. Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. E. Jensen, and R. H. Parrott. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89:422-434.
70. Kravetz, H. M., V. Knight, R. M. Chanock, J. A. Moris, K. M. Johnson, D. Rifkind, and J. P. Utz. 1961. Respiratory syncytial virus III. Production of Illness and clinical observations in adult volunteers. J. A. M. A. 176:657-663.
71. Kulkarni, A. B., M. Connors, C.-Y. Firestone, H. C. Morse III, and B. R. Murphy. 1993. The cytolytic activity of pulmonary CD8+ lymphocytes, induced by infection with a vaccinia recombinant expressing the M2 protein of respiratory syncytial virus (RSV), correlates with resistance to RSV infection in mice. J. Virol. 67:1044-1049.
72. Lamb, R. A. and D. Kolakofsky. 1996. Paramyxoviridae: the viruses and their replication, p. 1177-1204. In B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Fields Virology. Lippnicott-Raven Publishers, Philadelphia.
73. Levenson, R. M. and O. S. Kantor. 1987. Fatal pneumonia in an adult due to respiratory syncytial virus. Arch. Intern. Med. 147:791-792.
74. MacDonald, N. E., C. B. Hall, S. C. Suffin, C. Alexson, P. J. Harris, and J. A. Manning. 1982. Respiratory syncytial viral infection in infants with congenital heart disease. N. Engl. J. Med. 307:397-400.
75. Martin, A. J., P. S. Gardner, and J. McQuillin. 1978. Epidemiology of respiratory viral infection among paediatric inpatients over a six-year period in north-east England. Lancet 2:1035-1038.
76. McCormack, J. G. 2000. Hendra, Menangle and Nipah viruses. Aust. N. Z. J. Med. 2000. Feb;30(1):9-10. 30:9-10.
77. Mcintosh, K., H. B. Masters, I. Orr, R. K. Chao, and R. M. Barkin. 1978. The immunologic response to infection with respiratory syncytial virus in infants. J. Infect. Dis. 138:24-32.
78. Meissner, H. C., R. C. Welliver, S. A. Chartrand, B. J. Law, L. E. Weisman, H. L. Dorkin, and W. J. Rodriguez. 1999. Immunoprophylaxis with palivizumab, a humanized respiratory syncytial virus monoclonal antibody, for prevention of respiratory syncytial virus infection in high risk infants: a consensus opinion. Pediatr. Infect. Dis. J. 18:223-231.
79. Mills, J., 5th, J. E. Van Kirk, P. F. Wright, and R. M. Chanock. 1971. Experimental respiratory syncytial virus infection of adults. Possible mechanisms of resistance to infection and illness. J. Immunol. 107:123-130.
80. Morris, J. A., R. E. Blount,Jr., and R. E. Savage. 1956. Recovery of Cytopathogenic Agent from Chimpanzees with Coryza. Proc. Soc. Exper. Biol. & Med. 92:544-549.
81. Mufson, M. A., R. B. Belshe, C. Orvell, and E. Norrby. 1988. Respiratory syncytial virus epidemics: variable dominance of subgroups A and B strains among children, 1981-1986. J. Infect. Dis. 157:143-148.
82. Mufson, M. A., C. Orvell, B. Rafnar, and E. Norrby. 1985. Two distinct subtypes of human respiratory syncytial virus. J. Gen. Virol. 66:2111-2124.
83. Murphy, B. R., G. A. Prince, E. E. Walsh, H. W. Kim, R. H. Parrott, V. G. Hemming, W. J. Rodriguez, and R. M. Chanock. 1986. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J. Clin. Microbiol. 24:197-202.
84. Nadal, D. and P. L. Ogra. 1990. Development of local immunity: role in mechanisms of protection against or pathogenesis of respiratory syncytial viral infections. Lung 168 Suppl:379-87:379-387.

85. Navas, L., E. Wang, V. de Carvalho, and J. Robinson. 1992. Improved outcome of respiratory syncytial virus infection in a high- risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J. Pediatr. 121:348-354.
86. Openshaw, P. J. M., K. Anderson, G. W. Wertz, and B. A. Askonas. 1990. The 22,000-Kilodalton Protein of Respiratory Syncytial Virus Is a Major Target for Kd-Restricted Cytotoxic T Lymphocytes from Mice Primed by Infection. J. Virol. 64:1683-1689.
87. Osterweil, D. and D. Norman. 1990. An outbreak of an influenza-like illness in a nursing home. J. Am. Geriatr. Soc. 38:659-662.
88. Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98:289-300.
89. Paton, N. I., Y. S. Leo, S. R. Zaki, A. P. Auchus, K. E. Lee, A. E. Ling, S. K. Chew, B. Ang, P. E. Rollin, T. Umapathi, I. Sng, C. C. Lee, E. Lim, and T. G. Ksiazek. 1999. Outbreak of Nipah-virus infection among abattoir workers in Singapore. Lancet 354:1253-1256.
90. Prince, G. A., R. L. Horswood, and R. M. Chanock. 1985. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J. Virol. 55:517-520.
91. Richardson, L. S., R. B. Belshe, D. L. Sly, W. T. London, D. A. Prevar, E. Camargo, and R. M. Chanock. 1978. Experimental respiratory syncytial virus pneumonia in cebus monkeys. J. Med. Virol. 2:45-59.
92. Rohwedder, A., O. Keminer, J. Forster, K. Schneider, E. Schneider, and H. Werchau. 1998. Detection of respiratory syncytial virus RNA in blood of neonates by polymerase chain reaction. J. Med. Virol. 54:320-327.
93. Roman, M., W. J. Calhoun, K. L. Hinton, L. F. Avendano, V. Simon, A. M. Escobar, A. Gaggero, and P. V. Diaz. 1997. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am. J. Respir. Crit. Care Med. 156:190-195.
94. Saez-Llorens, X., E. Castano, D. Null, J. Steichen, P. J. Sanchez, O. Ramilo, F. H. Top,Jr., and E. Connor. 1998. Safety and pharmacokinetics of an intramuscular humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. The MEDI-493 Study Group. Pediatr. Infect. Dis. J. 17:787-791.
95. Schwarze, J., G. Cieslewicz, A. Joetham, T. Ikemura, E. Hamelmann, and E. W. Gelfand. 1999. CD8 T cells are essential in the development of respiratory syncytial virus-induced lung eosinophilia and airway hyperresponsiveness. J. Immunol. 162:4207-4211.
96. Siber, G. R., J. Leszcynski, V. Pena-Cruz, C. Ferren-Gardner, R. Anderson, V. G. Hemming, E. E. Walsh, J. Burns, K. Mcintosh, and R. Gonin. 1992. Protective activity of a human respiratory syncytial virus immune globulin prepared from donors screened by microneutralization assay. J. Infect. Dis. 165:456-463.
97. Sorvillo, F. J., S. F. Huie, M. A. Strassburg, A. Butsumyo, W. X. Shandera, and S. L. Fannin. 1984. An outbreak of respiratory syncytial virus pneumonia in a nursing home for the elderly. J. Infect. 9:252-256.
98. Spelman, D. W. and P. A. Stanley. 1983. Respiratory syncytial virus pneumonitis in adults. Med. J. Aust. 1:430-431.
99. Srikiatkhachorn, A. and T. J. Braciale. 1997. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. J. Exp. Med. 186:421-432.
100. Sterner, G., S. Wolontis, B. Bloth, and G. DeHevesy. 1966. Respiratory syncytial virus: an outbreak of acute respiratory illnesses in a home forc infants. Acta Paediatr. Scan. 55:273-279.
101. Storch, G. A., L. J. Anderson, C. S. Park, C. Tsou, and D. E. Dohner. 1991. Antigenic and genomic diversity within group A respiratory syncytial virus. J. Infect. Dis. 163:858-861.
102. Sullender, W. M. 2000. Respiratory Syncytial Virus Genetic and Antigenic Diversity. Clin. Microbiol. Rev. 2000. Jan;13(1):1-15. 13:1-15.
103. Sullender, W. M., M. A. Mufson, L. J. Anderson, and G. W. Wertz. 1991. Genetic diversity of the attachment protein of subgroup B respiratory syncytial viruses. J. Virol. 65:5425-5434.
104. Taylor, G., L. H. Thomas, S. G. Wyld, J. Furze, P. Sopp, and C. J. Howard. 1995. Role of T-lymphocyte subsets in recovery from respiratory syncytial virus infection in calves. J. Virol. 69:6658-6664.

105. Tissing, W. J. E., H. A. Van Steensel-Moll, and M. Offringa. 1993. Risk factors for mechanical ventilation in respiratory syncytial virus infection. Eur. J. pediatr. 152:125-127.
106. Tsutsumi, H., M. Onuma, K. Suga, T. Honjo, Y. Chiba, S. Chiba, and P. L. Ogra. 1988. Occurrence of respiratory syncytial virus subgroup A and B strains in Japan, 1980 to 1987. J. Clin. Microbiol. 26:1171-1174.
107. Vainionpaa, R., O. Meurman, and H. Sarkkinen. 1985. Antibody response to respiratory syncytial virus structural proteins in children with acute respiratory syncytial virus infection. J. Virol. 53:976-979.
108. Vikerfors, T., M. Grandien, and P. Olcen. 1987. Respiratory syncytial virus infections in adults. Am. Rev. Respir. Dis. 136:561-564.
109. Wagner, D. K., B. S. Graham, P. F. Wright, E. E. Walsh, H. W. Kim, C. B. Reimer, D. L. Nelson, R. M. Chanock, and B. R. Murphy. 1986. Serum immunoglobulin G antibody subclass responses to respiratory syncytial virus F and G glycoproteins after primary infection. J. Clin. Microbiol. 24:304-306.
110. Wagner, R. K., P. Muelenaer, F. W. Henderson, M. H. Snyder, C. B. Reimer, E. E. Walsh, L. J. Anderson, D. L. Nelson, and B. R. Murphy. 1989. Serum immunoglobulin G antibody subclass response to respiratory syncytial virus F and G glycoproteins after first, second and third infections. J. Clin. Microbiol. 27:589-592.
111. Ward, K. A., P. R. Lambden, M. M. Ogilvie, and P. J. Watt. 1983. Antibodies to respiratory syncytial virus polypeptides and their significance in human infection. J. Gen. Virol. 64:1867-1876.
112. Webb, M. S., R. L. Henry, A. D. Milner, G. M. Stokes, and A. S. Swarbrick. 1985. Continuing respiratory problems three and a half years after acute viral bronchiolitis. Arch. Dis. Child 60:1064-1067.
113. Weber, M. W., E. K. Mulholland, and B. M. Greenwood. 1998. Respiratory syncytial virus infection in tropical and developing countries. Trop. Med. Int. Health 3:268-280.
114. Whimbey, E., R. B. Couch, J. A. Englund, M. Andreeff, J. M. Goodrich, I. I. Raad, V. Lewis, N. Mirza, M. A. Luna, and B. Baxter. 1995. Respiratory syncytial virus pneumonia in hospitalized adult patients with leukemia. Clin. Infect. Dis. 21:376-379.

Chapter 2.
Strain variation and clinical severity

2.1 Relationship between clinical severity of respiratory syncytial virus (RSV) infection and subtype.

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Summary

We investigated whether a relationship between clinical severity of RSV infection and distribution of subtype A or B could be demonstrated. The data of 232 children, who were admitted to or diagnosed with RSV infection in the outpatient department of the Sophia Children's Hospital, Rotterdam between 1992 and 1995, were studied. The diagnosis of RSV was confirmed by a direct immunofluorescence assay. Subtyping was performed by an indirect immunofluorescence assay using specific monoclonal antibodies. Gender, age at diagnosis, gestational age and birthweight, the presence of underlying diseases, impaired feeding, the presence of wheezing and retractions, respiratory rate, temperature, clinical diagnosis at presentation, SaO_2, pCO_2 and pH, characteristics of hospitalisation and the need for mechanical ventilation were observed. Analysis was performed on data from all patients diagnosed with RSV infection in the period between 1992 and 1995 spanning three RSV seasons, and seperately on the RSV season 1993 – 1994. The outcome of the three year analysis (150 (64.7%) subtype A vs. 82 (35.3%) subtype B) was compared with the outcome of the season 1993 – 1994, a mixed epidemic with 37 (60.7%) subtype A and 24 (39.3%) subtype B isolates. None of the variables observed in the season 1993 – 1994 differed significantly between RSV-subtype A and B. Similar results were obtained from the analysis in the period 1992 until 1995, with the exception of pCO_2 (a more high pCO_2 was found in subtype A, $p < 0.001$) and retractions (more retractions were noted in patients with subtype A, $p = 0.03$). After correcting for possible confounders using regression analysis, these differences were not significant anymore.

Our data indicate that there is no relationship between clinical severity of RSV infection and subtype.

Introduction

Respiratory syncytial virus (RSV) is the most common cause of respiratory tract infections in infants and young children. The clinical presentation can vary from a mild upper respiratory tract infection to severe bronchiolitis. The incidence of RSV bronchiolitis reaches a maximum at the age of two months and declines within the first year of life (13). The majority of infections have a mild clinical course. A minority of infants, 0.5 to 2%, has to be admitted to the hospital. Between 7% and 21% of these patients will need mechanical ventilation (4,5).

Severe infections may occur in premature infants or patients suffering from an underlying disease, such as congenital heart disease (CHD), bronchopulmonary dysplasia (BPD) or T-cell-immunodeficiency (3). The mortality of infants hospitalised with RSV is approximately 0.5% to 1.5%. In infants with an underlying disease higher mortality rates are found (7,8).

Two major antigenic groups of RSV, subtype A and subtype B, can be identified by specific monoclonal antibodies (1). The antigenic diversity between these two subtypes is determined mainly by G-proteins, which are responsible for attachment of the virus and to a lesser extent by the fusion proteins, the F-proteins. The two subtypes can circulate independently from each other, but they may also cocirculate during one epidemic (2). The identification of the two subtypes has led to the speculation that there might be a relationship between clinical severity of infection and RSV subtype. A limited number of studies were carried out to establish such a relationship (6,9,10,14). These studies provide conflicting results.

The objective of our study was to investigate whether a relationship between clinical severity of RSV infection and subtype could be demonstrated in children younger than 12 months.

Patients and methods

Patients younger than 12 months who either were admitted to the Sophia Children's Hospital or visited the outpatient department due to RSV infection in the period between 1992 and 1995, were included in this study. The Sophia Children's Hospital is a combined secondary-tertiary care university hospital.

RSV infection was confirmed by a positive direct immunofluorescent assay (IFA) using FITC labeled monoclonal antibodies against RSV (DAKO, Ely, UK) performed on cells of nasopharyngeal washing and/or a positive viral culture on HEp-2 cells (11). In 97.4% (n = 226) both DIFA and viral culture were positive, in 1.3% (n = 3) only the DIFA was positive and in 1.3% (n = 3) only the viral culture was positive. RSV subtyping was performed using RSV subtype specific monoclonal antibodies (MAB) in an immunofluorescence assay on cells of nasopharyngeal washings as described by Taylor et. al. (15). MABs used were 92-

Table 2.1.1. Patient characteristics

	subtype A (n = 150) (%)	subtype B (n = 82) (%)
Boys	63.3	67.1
Girls	36.7	32.9
Mean age (months)	3.5 ± 3.0	3.6 ± 2.9
Patients < 3 months	48.0	47.6
3 – 6 months	26.7	26.8
> 6 months	25.3	25.6
Mean gestational age (weeks)	37.3 ± 3.8	37.8 ± 3.3
Gestational age < 35 weeks	18.4	12.7
35 – 37 weeks	10.3	12.7
> 37 weeks	71.3	74.6
Mean birthweight (g)	2936 ± 875	3023 ± 804
Underlying disease state	16.3	11.0
Prematurity and BPD (n)	10	4
CHD (n)	14	3
BPD (n)	0	2

No significant differences between RSV subtype A and B were found.
Data expressed as percentages unless stated otherwise.
BPD : bronchopulmonary dysplasia; CHD : congenital heart disease

11C for subtype A and 102-10B for subtype B (Chemicon, Temecula USA) (1). Briefly, cells of nasopharyngeal washings were fixed on glass slides in acetone. RSV subtype specific MAB's were incubated for 30 minutes at 37° C. After washing three times in PBS the slides were incubated with the conjugate anti-mouse FITC (Dako, Ely,UK). When the IFA was negative and viral culture was positive, the subtype specific immunofluorescence was performed on RSV infected HEp-2 cells.

Epidemiological and clinical data were retrospectively obtained from the medical charts. Patient characteristics include gender, age at diagnosis, birthweight, gestational age and the presence of underlying disease (congenital heart disease with hemodynamic consequences i.e. left/right shunt, bronchopulmonary disease and T-cell immune deficiency). Clinical observations at presentation include impaired feeding (defined as normal feeding, slow feeding with normal volume, decreased volume and no oral feeding), the presence of wheezing and retractions, and temperature. The use of mechanical ventilation was registered during admission. The decision for ICU admission and mechanical ventilation was made before subtype was known. Other clinical parameters include characteristics of hospitalisation:

Table 2.1.2. Clinical parameters at presentation

	subtype A (n = 150) (%)	subtype B (n = 82) (%)
Impaired feeding	71.6	64.6
Wheezing	36.0	34.1
Retractions	58.0	41.8
Mean respiratory rate (min^{-1})	52 ± 18	51 ± 13
Mean temperature (°C)	37.8 ± 1.0	38.0 ± 0.8
Mean SaO_2 (%)	90.3 ± 11.3	90.4 ± 9.2
Mean pCO_2	6.8 ± 2.2	5.8 ± 1.0
Mean pH	7.35 ± 0.1	7.37 ± 0.

No significant differences between RSV subtype A and B were found, except for the presence of retractions ($p = 0.03$) and pCO_2 ($p < 0.001$).
Data expressed as epercentages unless stated otherwise.

number of admissions and length of stay in hospital and number of intensive care unit (ICU) admissions and length of stay in ICU.

Laboratory parameters measured at presentation included measurement of oxygen saturation (SaO_2), pCO_2 and pH. SaO_2 was measured transcutaneously with the use of a pulse oximeter, pCO_2 was measured on capillary blood samples. All parameters were measured irrespective of severity of disease.

Three diagnostic categories were defined: bronchiolitis/pneumonia (lower respiratory tract infection, LRTI), upper respiratory tract infection (URTI) and apnoea. The diagnosis bronchiolitis was based on clinical features and hypertranslucency, atelectasis or bronchial thickening on chest radiograph. URTI was defined as coughing and/or rhinitis, with no abnormalities on chest radiograph. Apnoea at presentation was defined as a cessation of respiration for a period over 15 seconds and/or bradycardia with accompanying cyanosis. The diagnosis pneumonia was based on clinical features and the presence of an infiltrate on chest radiograph.

Statistical analysis was performed using the χ^2 test, the student's t-test and the Mann-Whitney U test. P-value < 0.05 was considered significant. In order to examine the indepent effect of virus subtype on the severity of disease we adjusted for gender, age in months, prematurity (gestational age ≤ 37 weeks), the presence of underlying disease and year of diagnosis with logistic and linear regression analysis. Linear regression was used to investigate if there were differences between the two virus subtypes in SaO_2 or pCO_2. Logistic regression was performed with dichotomous outcome variables such as impaired feeding, the presence of wheezing and retractions, the clinical diagnosis (bronchiolitis/pneumonia or

Table 2.1.3. Clinical diagnosis at presentation

	subtype A (n = 150) (%)	subtype B (n = 82) (%)
Apnoea ± URTI	5.3	3.7
URTI	24.7	28.0
Bronchiolitis/pneumonia	3.3	2.4
with URTI	52.0	56.1
with apnoea and URTI	14.7	9.8

No significant differences between subtype A and B were found.
URTI : upper respiratory tract infection

upper respiratory tract infection), need of mechanical ventilation and ICU admission.

Results

In the period between 1992 and 1995 covering three RSV seasons, 232 children with RSV infection visited our hospital. In 1992 – 1993 a predominance of subtype B was found. Ten out of 68 children were infected with subtype A (14.7%) versus 58 children with subtype B (85.3%). The season 1993 – 1994 showed a mixed epidemic: 37 children were infected with subtype A (60.7%) and 24 children were infected with subtype B (39.3%). The season 1994 – 1995 showed a epidemic in which all 103 children were infected with subtype A.

Based upon the subtype distribution, two analysis were perfomed: one on the the entire period from 1992 until 1995, covering three RSV seasons and one on the mixed A/B season 1993 – 1994. The results of the outcome of the analysis of the period 1992 – 1995 were compared with the outcome of the analysis of the season 1993 – 1994.

Analysis of the data from the period 1992 until 1995

This period included 232 children: 150 (64.7%) were infected with subtype A, 82 (35.3%) with subtype B. The male/female ratio was 1.8 (150/82). Table 2.1.1. shows the population characteristics.

Clinical parameters are shown in Table 2.1.2. No significant differences were found regarding impaired feeding, the presence of wheezing, respiratory rate, temperature, SaO_2 and pH. Significant differences were found regarding pCO_2 and the presence of retractions. The average pCO_2 for subtype A is 6.8 kPa against 5.8 kPa for subtype B ($p < 0.001$). Retractions were noted in 84 childrens infected with subtype A and in 28 children infected with subtype B ($p = 0.03$). No significant differences were found regarding underlying disease. Table 2.1.3. shows the clinical diagnosis at presentation. In Table 2.1.4. the characteristics of hospitalisation are shown.

The pCO_2 was measured in 213 children: 136 (90.1%) samples were collected in patients with subtype A and 77 (94.0%) for subtype B. We noted in the season 1994 – 1995 more children with a high pCO_2 (above 10.0 kPa) than in the other two seasons. We also found in the season 1994 – 1995 a significant difference in the number of children < 2 months of age (in the first two seasons 19.4% against 34.0% of the children in the season 1994 – 1995, p = 0.01). Furthermore we found a significantly higher pCO_2 in children < 2 months of age (7.5 kPa vs. 6.0 kPa for children ≥ 2 months of age, p < 0.001). This also applies to all three seasons separately.

Twenty-eight (12.1%) children required mechanical ventilation. Twenty-one of these were infected with subtype A, 7 with subtype B (p = 0.22). Two children died. One of them was infected with subtype A, the other child was infected with subtype B.

Table 2.1.4. Characteristics of hospitalisation

	subtype A (n = 150) (%)	subtype B (n = 82) (%)
Number of admissions	76.7	85.4
Mean length of stay (days)	10.4(6.2)	9.3(4.8)
Number of ICU admissions	34.0	23.2
Mean length of stay in ICU (days)	6.2(8.3)	5.9(5.0)
Mechanical ventilation	14.0	8.5

No significant differences between RSV subtype A and B were found.
Data expressed as percentages unless stated otherwise.
ICU: intensive care unit

Adjustment for confounders with regression analysis did not reveil a significant difference between subtype A and B for any of the disease outcome parameters such as impaired feeding, presence of wheezing, presence of retractions, SaO_2 and pCO_2, lower respiratory tract infection, ICU-admission and mechanical ventilation.

Analysis of the season 1993 – 1994 and comparison of both analysis

This period included 61 patients. The male/female ratio was 1.3 (35/26). We did not find a significant difference in the studied population characteristics between subtype A and subtype B. No significant differences were found between subtype A and subtype B for all the observed variables.

The outcome of the analysis of the data from the season 1993 – 1994 was equal to the outcome of the analysis of the data from the period 1992 – 1995, covering three seperate RSV seasons, except for pCO_2 and the presence of retractions.

Discussion

We investigated whether a relationship between clinical severity of RSV infection and subtype A or B could be demonstrated. The results of our study indicate that such a relationship does not exist.

Two analysis were performed: one on data from the whole period between 1992 and 1995, covering three RSV seasons, and one on data of the season 1993 – 1994. By comparing the outcome of one season with the outcome of three seasons, the possible effect of confounding variables from a particular season was ruled out.

As strong indicators for severity of infection in this study were used oxygen saturation (SaO_2), pCO_2, clinical diagnosis (upper respiratory tract infection versus lower respiratory tract infection), ICU-admission and the need for mechanical ventilation. These indicators are strongly related to pulmonary dysfunction (6,16).

None of the variables observed in the season 1993 – 1994 were significantly different between patients infected with subtype A and subtype B. This was also found in the analysis of the period 1992 – 1995, except for the presence of retractions and pCO_2. More retractions were noted in patients with subtype A than in patients with subtype B. However, the association did not hold after correcting for confounders using logistic regression analysis.

The other variable that showed a significant difference is pCO_2. This is due to a relationship between young age and high pCO_2, as has been demonstrated by Mulholland et al.(12). In the season 1994 – 1995 (a season with only subtype A infections) significantly more children younger than two months of age were admitted with RSV than in the two preceding epidemics. The mean pCO_2 of these children was significantly higher than the mean pCO_2 of children $\geq$ 2 months. This influences the mean pCO_2 for subtype A in the whole period covering the three seperate RSV-seasons. After correcting for age, the association between high pCO_2 and subtype A did not hold.

Several studies investigated the possible relationship between clinical severity of RSV infection and subtype. McConnochie et al. conclude that subtype A is related to a more severe disease (9). They analysed data from 157 patients with known subtype, using arbitrary cut-off values at which a variable was to be considered severe. Significant differences were observed in $pCO_2 > 45$ mm Hg, $SaO_2 < 87\%$ and respiratory rate > 72 min^{-1}. A significant difference was also observed between subtype A and subtype B regarding mechanical ventilation. Straliotto et al. concluded that subtype B is related to a more severe disease, but their population was rather small: 29 patients (14). The results of both these studies are conflicting with those from our study. McIntosh et al. concluded that there is no difference in severity between subtype A and subtype B. They analysed their data using a severity index consisting of three groups: severe, moderate and mild (10).

In conclusion, the outcome of the analysis of the data obtained in our study indicate that

there is no relationship between clinical severity of RSV infection and subtype A or B. Hence it is not necessary to determine subtype at presentation, because there are no consequences for clinical management. The outcome of our study also implies that the development of a vaccine has to be aimed in the direction of a vaccine which protects for subtype A as well as subtype B.

References

1. Anderson, L. J., J. C. Hierholzer, C. Tsou, R. M. Hendry, B. F. Fernie, Y. Stone, and K. Mcintosh. 1985. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J. Infect. Dis. 151:626-633.
2. Belshe, R. B. and M. A. Mufson. 1991. Respiratory syncitial virus. p. 388-402. In R. B. Belshe (ed.), Textbook of Human Virology.
3. Brunell, P. A., R. S. Daum, C. B. Hall, M. L. Lepow, and G. H. McCracken Jr. 1987. (Committee on infectious diseases 1986 - 1987). Ribavirin therapy of respiratory syncitial virus. Paediatrics 79:475-476.
4. Everard, M. L. and A. D. Milner. 1992. The respiratory syncitial virus and its role in acute bronchiolitis. Eur. J. pediatr. 151:638-651.
5. Frankel, L. R., N. J. Lewiston, D. W. Smith, and D. K. Stevenson. 1986. Clinical observations on mechanical ventilation for respiratory failure in bronchiolitis. Pediatr. Pulmonol. 2:307-311.
6. Hall, C. B., E. E. Walsh, K. C. Schnabel, C. E. Long, K. M. McConnochie, S. W. Hildreth, and L. J. Anderson. 1990. 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. 162:1283-1290.
7. La Via, W. V., S. W. Grant, H. R. Stutman, and M. I. Marks. 1993. Clinical profile of pediatric patients hospitalized with respiratory syncytial virus infection. Clin. Pediatr. 32:450-454.
8. La Via, W. V., M. I. Marks, and H. R. Stutman. 1992. Respiratory syncytial virus puzzle: clinical features, pathophysiology, treatment, and prevention. J. Pediatr. 121:503-510.
9. McConnochie, K. M., C. B. Hall, E. E. Walsh, and K. J. Roghmann. 1990. Variation in severity of respiratory syncytial virus infections with subtype. J. Pediatr. 117:52-62.
10. McIntosh, E. D., L. M. De Silva, and R. K. Oates. 1993. Clinical severity of respiratory syncytial virus group A and B infection in Sydney, Australia. Pediatr. Infect. Dis. J. 12:815-819.
11. Minnich, L. L. and C. G. Ray. 1982. Comparison of direct and indirect immunofluorescence staining of clinical specimens for detection of respiratory syncytial virus antigen. J. Clin. Microbiol. 15:969-970.
12. Mulholland, E. K., A. Olinsky, and F. A. Shann. 1990. Clinical findings and severity of acute bronchiolitis. Lancet 335:1259-1261.
13. Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98:289-300.
14. Straliotto, S. M., B. Roitman, J. B. Lima, G. B. Fischer, and M. M. Siqueira. 1994. Respiratory syncytial virus (RSV) bronchiolitis: comparative study of RSV groups A and B infected children. Rev. Soc. Bras. Med. Trop. 27:1-4.
15. Taylor, C. E., S. Morrow, M. Scott, B. Young, and G. L. Toms. 1989. Comparative virulence of respiratory syncytial virus subgroups A and B. Lancet 1:777-778.
16. Van Steensel-Moll, H. A., E. Van der Voort, A. P. Bos, P. H. Rothbarth, and H. J. Neijens. 1989. Respiratory syncytial virus infections in children admitted to the intensive care unit. Pediatrie. 44:583-588.

2.2 G protein variation in respiratory syncytial virus group A does not correlate with clinical severity

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Summary

Respiratory syncytial virus group A strain variations of 28 isolates from The Netherlands collected during three consecutive seasons were studied by analyzing G protein sequences. Several lineages circulated repeatedly and simultaneously during the respective seasons. No relationships were found between lineages on the one hand and clinical severity or age on the other.

Respiratory syncytial virus (RSV) can be divided into two groups, A and B, on the basis of the reaction with monoclonal antibodies directed against the F and G protein (1,23) and nucleotide sequence differences of several genes (5,16,30,31). These two groups circulate independently in the human population, with group A being the most prevalent (14,23).
Also, within the two groups substantial strain differences have been described, mainly associated with the divergence in the gene encoding the G protein (17), which is the most variable protein of the virus. Several lineages within groups A and B also seem to co-circulate simultaneously in the population (3,30). Studies on RSV strains show an accumulation of amino acid changes over the years, suggesting antigenic drift-based immunity-mediated selection (4,5,8,15,27).
One of the most interesting features of RSV is its ability to cause repeated infections throughout life (9,11). This enables RSV to remain present at high levels in the population, and it has been estimated that at least 50% of children encounter their first RSV infection during their first winter season. Strain variation is thought to contribute to its ability to cause frequent reinfections (4,8,32).
The clinical severity of RSV infection is associated with epidemiological and host factors, which include socioeconomic status (26), age (26), prematurity (25), and underlying heart and/or lung disease (10,19). Several studies have evaluated differences in clinical severity between groups A and B. In about half of these studies no differences in clinical severity were detected between groups involved (14,18,21,22,28,34,37) and in the other studies group A seemed to be associated with more severe clinical disease (12,13,20,23,29,33,36). It has been suggested that virus variants within group A are responsible for this discrepancy (7,12,36).
To further address this issue, we selected group A strains from three consecutive winter seasons and subjected isolates of these strains to sequence analyses of part of the G protein. The strains were isolated from children for whom standardized clinical data were available from our previous study concerning RSV-A versus RSV-B and clinical severity (18).

RSV isolates (n = 293) found in routine diagnostics during three consecutive winter seasons were typed by performing direct immune fluorescence on cells from nasopharyngeal washings using specific monoclonal antibodies MAB 92-11C for group A and MAB 109-10B for group B (Chemicon, Temecula, CA) as previously described (2). Twenty-eight RSV group A isolates were selected for sequence analysis.
All five group A strains available from the first season (1992-93) were included. Eleven from the second season (1993-94) and twelve from the third season (1994-95) were selected from children who had experienced either a mild infection (not admitted) or a severe infection as determined by clinical parameters upon admission (see below).

Demographic and clinical data on the children during the acute phase and at the time of the control visit were collected in a previous study (18). Briefly, the data included gender, age, duration of pregnancy, underlying disease, feeding difficulties, history of apnea, the presence of retractions, respiratory rate, oxygen saturation (SaO_2) in room air, partial CO_2 pressure (pCO_2), pH, abnormalities on X ray, admission to an intensive care unit, and the need for artificial ventilation. Severe RSV infection was defined as meeting one or more of the following criteria: $pCO_2 > 6.6$ kPa $SaO_2 < 90\%$, and/or the need for artificial ventilation. Viral RNA extraction and amplification of the viral RNA by reverse transcriptase PCR was carried out as described previously (35). Briefly, RNA was extracted from 100 µl of culture supernatant using a guanidinium isothiocyanate solution and was collected by precipitation with isopropanol. The viral RNA was then amplified by reverse transcriptase PCR using oligonucleotide primers G(A)-173s (GGCAATGATAATCTCAACTTC) and G(A)-525as (TGAATATGCTGCAGGGTACT), which resulted in an amplified fragment of 392 bp spanning the first hypervariable region of the G protein (AA 100-132). The amplified products were subjected to nucleotide sequence analysis by cycle sequencing using an ABI dye terminator sequencing system and analysis on an ABI Prism 377 DNA sequencer (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Alignment of the nucleotide sequences of the G protein gene of the RSV isolates was carried out using the GCG pack-

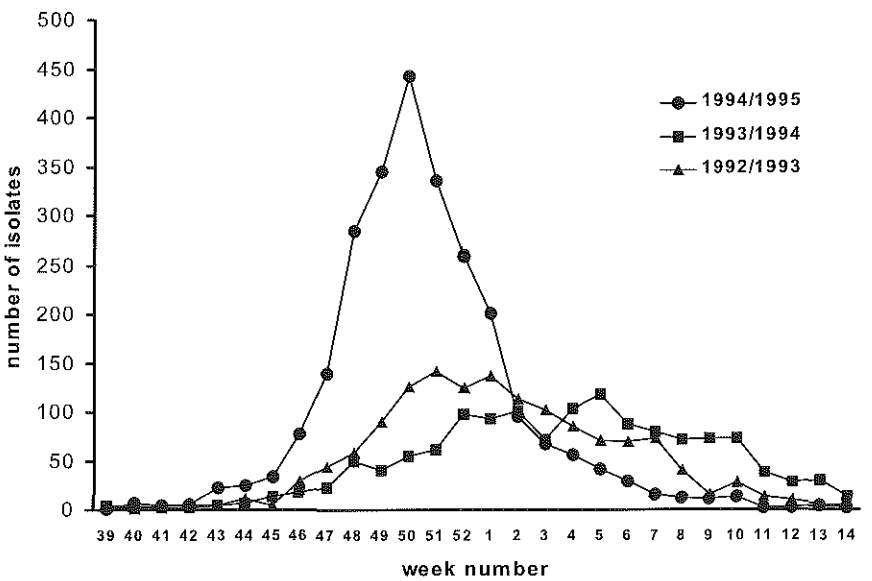

Figure 2.2.1. Number of RSV isolates per week during the three seasons studied as recorded by the combined Dutch Virology Laboratories. (Published with permission of the Dutch Working Group on Clinical Virology.).

age (Madison, Wiscon.). Multiple sequence files were analysed by DNAPARS in the PHYLIP package (6). Subsequently, phenograms were generated using the DRAWGRAM program.
Clinical data of patients from the respective seasons were compared in a χ^2 test, Fisher's exact, or Mann-Whitney U test when applicable.

During the three winter seasons 232 children younger than 12 months of age were diagnosed with a RSV infection by direct immune fluorescence and/or virus isolation. In 1992-93 a predominance of group B viruses was found, season 1993-94 showed a mixed epidemic, and in season 1994-95 all children were infected with group A viruses (18). Figure 2.2.1. shows the numbers of RSV isolates in The Netherlands per week during the three seasons. In the 1994-95 season, a short steep peak in the first weeks of December was observed. During this third season, more children younger than 1 month of age were admitted. Children in the third season had a higher mean pCO_2 and lower pH (Table 2.2.1.) than children in the first two seasons. No other differences in parameters known to correlate with clinical severity could be objectively measured.
G protein amplicons of 28 RSV group A isolates divided over the three seasons were studied by sequence analysis and a phenogram was generated (Figure 2.2.2.). Season of infec-

Table 2.2.1.. Clinical parameters of RSV-infected patients during three consecutive seasons

Patient variable	1992-1993 and 1993-1994	1994-1995	P[a]
No. of children	130	102	NS
No. (%) <37 gestation	37 (28.5)	21 (20.5)	NS
No. (%) <1 month	9 (6.9)	17 (16.7)	0.035
Mean (SD) respiratory rate	51.9 (13.4)	51,6 (20.3)	NS
No. (%) with hstory of apnea	23 (17.7)	23 (22.5)	NS
No. (%) wheezing	47 (36.1)	34 (33.2)	NS
Mean (SD) pCO_2	6.1 (1.4)	6.93 (2.35)	0.027
Mean (SD) pH	7.36 (0.07)	7.33 (0.11)	0.040
Mean (SD) SaO_2	90.7 (8.7)	89.9 (12.2)	NS
No. (%) on artificial ventilation	13 (10.0)	15 (14.7)	NS
No. (%) with severe RSV[b]	51 (39.2)	46 (45.1)	NS

a. Clinical data of patients were compared in a χ^2 test, Fisher's exact test, or Mann-Whitney U test when applicable. NS = no statistical difference.
b. Severe RSV infection was defined as meeting one or more of the following criteria: pCO_2 > 6.6 kPa SaO_2 < 90% and/or on artificial ventilation.

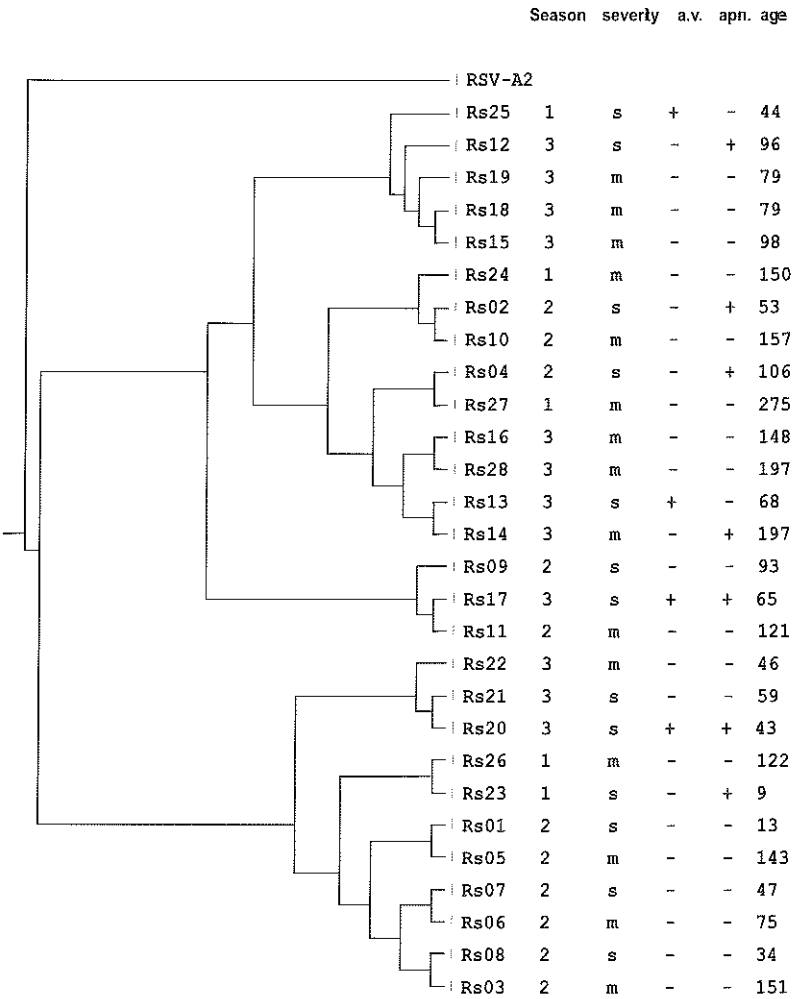

Figure 2.2.2. Phylogenetic dendrogram showing relatedness of group A isolates determined by sequence analyses of the first hypervariable region of the G protein. Isolates were selected from three consecutive seasons in the Sophia Children's Hospital Rotterdam. Seasons are indicated as follows: 1; 1992-1993; 2, 1993-1994; 3, 1994-1995. For each isolate the following patient characteristics are indicated. Severity of RSV infection: s, severe, m, mild. Severe was defined as meeting one or more of the following criteria: pCO_2 > 6.6 kPa, SaO_2 < 90%, and for artificial ventilation a.v., the need for artificial ventilation. apn., a history of apneas; age, the age in days upon admission.

tion, age upon diagnosis, and clinical parameters – severity score, artificial ventilation, and apnea – are indicated in the phenogram.

Several lineages of RSV were found to be present during the three seasons studied, and sev-

eral lineages could be identified during all three seasons. Closely related strains were also found to occur in subsequent seasons. The observed clustering of the RSV isolates proved to be independent of season or patient related parameters (Figure 2.2.2.).

Thus, several lineages of RSV-A cocirculated during the three seasons studied, and clinically severe as well as milder cases were evenly distributed over the different lineages found.

RSV infections are usually found during several months in the winter season. In the 1994-95 season, a relatively high incidence of RSV infections during a relatively short period was found. In the 1994-95 season, more children from the very young age group were admitted. The only clinical parameters objectively found to be more severe in the 1994-95 season were the pCO_2 and the pH. These parameters may be directly related to the younger age of the children involved, since a significant relation between pCO_2 and age has been previously described (24).

The RSV-G protein is the most variable of the RSV proteins; therefore, we choose to sequence a variable part of the RSV-G protein to study strain variation within subgroup A. However, it is not known where on the RSV genome putative virulence factors would be located. Since we sequenced only a small part of the genome, it cannot be fully excluded that mutations important for virulence elsewhere on the RSV genome were missed.

The isolates from the 1994-95 season were all of group A. We investigated whether this peak represented a single, possibly more virulent, strain of RSV-A. Despite the limited number of strains that were sequenced, it was clear that in the 1994-95 season, as well as in the other two seasons, several different strains cocirculated, and severe infections or younger age proved not to be related to one particular strain. In addition, closely related strains were found during different seasons as has been described previously (3,8,30).

Collectively, our data show that during a winter season when relatively many children are admitted during a relatively short period, several strains may cocirculate in the population. In addition, it was shown that clinically more severe cases were found spread over the branches of the phylogenetic tree. Therefore, severity of infection could not be attributed to particular lineages of RSV.

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References

1. Anderson, L. J., J. C. Hierholzer, C. Tsou, R. M. Hendry, B. F. Fernie, Y. Stone, and K. Mcintosh. 1985. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J. Infect. Dis. 151:626-633.
2 Brandenburg, A. H., J. Groen, H. A. Van Steensel-Moll, E. J. C. Claas, P. H. Rothbarth, H. J. Neijens,

and A. D. M. E. Osterhaus. 1997. Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. J. Med. Virol. 52:97-104.
3. Cane, P. A., D. A. Matthews, and C. R. Pringle. 1992. Analysis of relatedness of subgroup A respiratory syncytial viruses isolated worldwide. Virus Res. 25:15-22.
4. Cane, P. A., D. A. Matthews, and C. R. Pringle. 1994. Analysis of respiratory syncytial virus strain variation in successive epidemics in one city. J. Clin. Microbiol. 32:1-4.
5. Cane, P. A. and C. R. Pringle. 1995. Evolution of subgroup A respiratory syncytial virus: evidence for progressive accumulation of amino acid changes in the attachment protein. J. Virol. 69:2918-2925.
6. Felsenstein J. 1989. Phylip-Phylogeny Interference Package. Cladistics 5:164-166.
7. Fletcher, J. N., R. L. Smyth, H. M. Thomas, D. Ashby, and C. A. Hart. 1997. Respiratory syncytial virus genotypes and disease severity among children in hospital. Arch. Dis. Child 77:508-511.
8. Garcia, O., M. Martin, J. Dopazo, J. Arbiza, S. Frabasile, J. Russi, M. Hortal, P. Perez-Brena, I. Martinez, and B. Garcia-Barreno. 1994. Evolutionary pattern of human respiratory syncytial virus (subgroup A): cocirculating lineages and correlation of genetic and antigenic changes in the G glycoprotein. J. Virol. 68:5448-5459.
9. Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child 140:543-546.
10. Groothuis, J. R., K. M. Gutierrez, and B. A. Lauer. 1988. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 82:199-203.
11. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis 163:693-698.
12. Hall, C. B., E. E. Walsh, K. C. Schnabel, C. E. Long, K. M. McConnochie, S. W. Hildreth, and L. J. Anderson. 1990. 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. 162:1283-1290.
13. Heikkinen, T., M. Waris, O. Ruuskanen, A. Putto-Laurila, and J. Mertsola. 1995. Incidence of acute otitis media associated with group A and B respiratory syncytial virus infections. Acta Paediatr. 84:419-423.
14. Hendry, R. M., A. L. Talis, E. Godfrey, L. J. Anderson, B. F. Fernie, and K. Mcintosh. 1986. Concurrent circulation of antigenically distinct strains of respiratory syncytial virus during community outbreaks. J. Infect. Dis. 153:291-297.
15. Johansen, J., L. S. Christensen, A. Hornsleth, B. Klug, K. S. Hansen, and M. Nir. 1997. Restriction pattern variability of respiratory syncytial virus during three consecutive epidemics in Denmark. APMIS 105:303-308.
16. Johnson, P. R., M. K. Spriggs, R. A. Olmsted, and P. L. Collins. 1987. The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: extensive sequence divergence between antigenically related proteins. Proc. Natl. Acad. Sci. USA 84:5625-5629.
17. Johnson, P. R., Jr., R. A. Olmsted, G. A. Prince, B. R. Murphy, D. W. Alling, E. E. Walsh, and P. L. Collins. 1987. Antigenic relatedness between glycoproteins of human respiratory syncytial virus subgroups A and B: evaluation of the contributions of F and G glycoproteins to immunity. J. Virol. 61:3163-3166.
18. Kneyber, M. C., A. H. Brandenburg, P. H. Rothbarth, R. de Groot, A. Ott, and H. A. Van Steensel-Moll. 1996. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch. Dis. Child 75:137-140.
19. MacDonald, N. E., C. B. Hall, S. C. Suffin, C. Alexson, P. J. Harris, and J. A. Manning. 1982. Respiratory syncytial viral infection in infants with congenital heart disease. N. Engl. J. Med. 307:397-400.
20. McConnochie, K. M., C. B. Hall, E. E. Walsh, and K. J. Roghmann. 1990. Variation in severity of respiratory syncytial virus infections with subtype. J. Pediatr. 117:52-62.
21. McIntosh, E. D., L. M. De Silva, and R. K. Oates. 1993. Clinical severity of respiratory syncytial virus group A and B infection in Sydney, Australia. Pediatr. Infect. Dis. J. 12:815-819.
22. Monto, A. S. and S. Ohmit. 1990. Respiratory syncytial virus in a community population: circulation of subgroups A and B since 1965. J. Infect. Dis. 161:781-783.
23. Mufson, M. A., R. B. Belshe, C. Orvell, and E. Norrby. 1988. Respiratory syncytial virus epidemics: variable dominance of subgroups A and B strains among children, 1981-1986. J. Infect. Dis. 157:143-

148.
24. Mulholland, E. K., A. Olinsky, and F. A. Shann. 1990. Clinical findings and severity of acute bronchiolitis. Lancet 335:1259-1261.
25. Navas, L., E. Wang, V. de Carvalho, and J. Robinson. 1992. Improved outcome of respiratory syncytial virus infection in a high- risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J. Pediatr. 121:348-354.
26. Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98:289-300.
27. Peret, T. C., C. B. Hall, K. C. Schnabel, J. A. Golub, and L. J. Anderson. 1998. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J. Gen. Virol. 79:2221-2229.
28. Russi, J. C., H. Chiparelli, A. Montano, P. Etorena, and M. Hortal. 1989. Respiratory syncytial virus subgroups and pneumonia in children (letter). Lancet 2:1039-1040.
29. Salomon, H. E., M. M. Avila, M. C. Cerqueiro, C. Orvell, and M. Weissenbacher. 1991. Clinical and epidemiologic aspects of respiratory syncytial virus antigenic variants in Argentinian children (letter). J. Infect. Dis. 163:1167
30. Storch, G. A., L. J. Anderson, C. S. Park, C. Tsou, and D. E. Dohner. 1991. Antigenic and genomic diversity within group A respiratory syncytial virus. J. Infect. Dis. 163:858-861.
31. Sullender, W. M., M. A. Mufson, L. J. Anderson, and G. W. Wertz. 1991. Genetic diversity of the attachment protein of subgroup B respiratory syncytial viruses. J. Virol. 65:5425-5434.
32. Sullender, W. M., M. A. Mufson, G. A. Prince, L. J. Anderson, and G. W. Wertz. 1998. Antigenic and genetic diversity among the attachment proteins of group A respiratory syncytial viruses that have caused repeat infections in children. J. Infect. Dis. 178:925-932.
33. Taylor, C. E., S. Morrow, M. Scott, B. Young, and G. L. Toms. 1989. Comparative virulence of respiratory syncytial virus subgroups A and B (letter). Lancet 1:777-778.
34. Tsutsumi, H., M. Onuma, K. Nagai, H. Yamazaki, and S. Chiba. 1991. Clinical characteristics of respiratory syncytial virus (RSV) subgroup infections in Japan. Scand. J. Infect. Dis. 23:671-674.
35. van Milaan, A. J., M. J. Sprenger, P. H. Rothbarth, A. H. Brandenburg, N. Masurel, and E. C. Claas. 1994. Detection of respiratory syncytial virus by RNA-polymerase chain reaction and differentiation of subgroups with oligonucleotide probes. J. Med. Virol. 44:80-87.
36. Walsh, E. E., K. M. McConnochie, C. E. Long, and C. B. Hall. 1997. Severity of respiratory syncytial virus infection is related to virus strain. J. Infect. Dis. 175:814-820.
37. Wang, E. E., B. J. Law, and D. Stephens. 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.

Chapter 3
Local variation and clinical severity

3.1 Local variability in respiratory syncytial virus disease severity

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Summary

Respiratory syncytial virus (RSV) lower respiratory tract infections are considered to be a serious disease in centres such as the Sophia Children's Hospital (Rotterdam, the Netherlands), but as more benign infections in others such as the Geneva Children's Hospital (Switzerland). To assess the clinical severity of RSV infections at the two sites, 151 infants primarily admitted with a virologically confirmed RSV infection were studied prospectively (1994-5) and retrospectively (1993-4) (55 infants in Geneva and 96 in Rotterdam). Parameters of RSV morbidity which were more severe in Rotterdam during the two winter seasons were apnoea (1.8 v 23.9%), the rate of admission to the intensive care unit (3.6 v 28.1%), mechanical ventilation (0 v 7.3%), and length of stay in hospital (6.8 v 9.1 days). In Geneva higher respiratory rates (59.2 v 51.2), more wheezing (65.5 v 28.8%), and more retractions (81.8 v 63.3%) were recorded. Fewer infants younger than 4 months (54.9 v 68.7%), but more breast fed infants (94.1 v 38.5%), were admitted in Geneva, although the morbidity parameters remained different after correction for these two variables in multivariate analyses. Thus unidentified local factors influence the pattern and severity of RSV infection and may affect the results of multicentre prophylactic and therapeutic studies.

Introduction

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection (for example, bronchiolitis, pneumonia) in young children. RSV infections occur in yearly winter epidemics and most children are infected before the age of 2 years (5,13). The highest morbidity of RSV disease is seen in infants aged less than 6 months (4,6,8,23) and in children with risk factors such as prematurity (22), bronchopulmonary dysplasia (7), congenital heart disease with pulmonary hypertension (17), or immune deficiency (9). An estimated 0.5-2% of all infants with RSV infection are admitted to hospital (4,6,8,18,22) and 7-21% of these infants will develop respiratory insufficiency and require respiratory support (2,3,29). The proportion of infants eventually dying from RSV infection has been estimated at 0.5-1.5% of all infants admitted to hospital, and higher mortality is seen in infants with underlying disease (15-17).

This classically described RSV morbidity, however, does not reflect the RSV morbidity observed in the Geneva Children's Hospital (Switzerland), where RSV bronchiolitis is considered a common, but relatively benign, disease, in spite of an annual birth cohort including all defined risk groups. Over the past 10 years few infants admitted to hospital in Geneva with an RSV infection developed respiratory insufficiency requiring respiratory support, and no death directly attributable to RSV was reported. In contrast, RSV disease is considered a serious, sometimes life threatening, disease at the Sophia Children's Hospital (Rotterdam, the Netherlands).

In the present study we compared the clinical characteristics and outcome of disease in infants admitted to hospital with RSV infections in these two centres (a) to objectively assess the morbidity of RSV infections at each site and thus the local potential for preventive or therapeutic measures and (b) to evaluate whether known parameters of disease severity explain the local variability of clinical characteristics and outcome of RSV disease in infants.

Patients and methods

All children less than 12 months of age admitted to the Geneva Children's Hospital or the Sophia Children's Hospital, Rotterdam with a virologically confirmed RSV infection in the winter seasons 1993-4 and 1994-5 were included in the study. The study was approved by institutional ethical committees from the two hospitals. The RSV infection was defined as a positive result in direct immune fluorescence assay performed on cells from nasopharyngeal washings using fluorescein isothiocyanate labelled RSV specific monoclonal antibodies (DAKO, Ely) or detection of the viral antigen by indirect ELISA (24) and subsequent con-

firmation by viral culture. Duplicate samples collected in Geneva were frozen and sent to the department of virology of Erasmus University for confirmation analyses. Children referred by other hospitals and nosocomially infected children were excluded from the analyses to minimise the potential influence of different referral systems.

The Geneva Children's Hospital is a university hospital providing primary, secondary, and tertiary care for a population of approximately 500 000 inhabitants with an annual cohort of 5800 births in 1994. As it is the only children's hospital it admits all infants from this defined area, including all prematurely born infants. The Sophia Children's Hospital, Rotterdam is a university hospital with a combined secondary-tertiary care function. As one of several hospitals in the area it admits only some of the children from the Rotterdam area requiring admission to hospital. Most patients seen in the emergency care outpatient clinic of the Sophia Children's Hospital receive basic paediatric care (90%) and only 17% come from outside the Rotterdam area (30).

Epidemiological and clinical variables were prospectively obtained on admission and discharge or at a control visit for the season 1994-5, and retrospectively from the patient charts for the season 1993-4. Demographic and clinical data were recorded on a standardised form with common definitions for all items. The demographic variables included gender, age, duration of pregnancy, existence of underlying disease (defined as congenital heart disease, bronchopulmonary dysplasia, or T cell immune deficiency), breast feeding, a positive family history of asthma or eczema, number of children in the household, day care attendance, and smoking in the household. The clinical data included the number of days with breathing problems before admission, feeding difficulties (defined as an increase of time required for feeding or a decrease in feeding volume), a positive history of apnoea (defined as either a history of respiratory arrest with cyanosis or an observation of respiratory arrest for a period of more than 20 seconds and/or bradycardia with accompanying cyanosis in the paediatric emergency room or during hospital admission), respiratory rate, the presence of wheezing (scored positive if wheezing could be heard without using a stethoscope) and retractions, fever (defined as a rectal temperature higher than 38.5°C), oxygen saturation (SaO_2) in room air, carbon dioxide tension (PCO_2), pH, and abnormalities on a radiograph (hyperinflation, consolidation, or atelectasis) as described by the radiologist. SaO_2 was measured transcutaneously with the use of a pulse oximeter (Hewlett Packard Neonatal (Rotterdam), Nellcor N-180 (Geneva)). Intubation was indicated in both centres in the case of (a) respiratory insufficiency with hypercapnia (PCO_2 > 8 kPa and pH < 7.2), (b) hypoxia (SaO_2 <85% with a fractional inspired oxygen >60%), (c) prolonged episodes of apnoea leading to severe bradycardia requiring stimulation or hand bag ventilation, or (d) sudden clinical

deterioration. Discharge from either hospital required an adequate fluid intake for age, correction of tachypnea, and no oxygen requirement.

Data collected on the course of the disease and treatment included the occurrence of additional apnoea during the hospital stay, the maximum respiratory rate, the length of stay in hospital, admission to and length of stay in the intensive care unit, use of mechanical ventilation, administration of oxygen, bronchodilators, ribavirine and/or antibiotics, and number of deaths.

The clinical data from the Geneva and Rotterdam patients were compared in a χ^2 test, Fisher's exact test, or Mann-Whitney U test when applicable. Differences in the clinical manifestations between Geneva and Rotterdam were tested again with multiple regression analysis, adjusting for possible confounders. Linear regression was used for continuous variables and logistic regression for dichotomous variables. The clinical parameters were entered in the regression model as dependent variables, and the confounders and location were entered as independent variables. Statistical significance was accepted at $p < 0.05$. To check for seasonal differences in clinical severity the analyses were also performed separately for the two winter seasons.

Results

Rates of admission to hospital in Geneva

We calculated the rates of admission to hospital for RSV infection in the Geneva Children's Hospital for both term (n = 5800) and preterm (gestation <37 weeks, n = 312) infants younger than 12 months of age. The rate of admission to hospital was 5.3/1000 for term and 22/1000 for preterm infants for the 1994-5 winter season. The rate of admission to hospital in Rotterdam could not be reliably calculated as the size of the attachment population for the Sophia Children's Hospital cannot be precisely defined.

Demographics

A total of 208 children younger than 12 months of age (61 in Geneva, 147 in Rotterdam) were admitted to hospital with a diagnosis of RSV infection. A predominance of RSV subgroup A was found in nasopharyngeal specimens in the two centres (Rotterdam, 1993-4 60% subgroup A, 1994-5 100% subgroup A; Geneva, 1993-4, data not available, 1994-5 75% subgroup A). Six infants in Geneva (one secondary referral and five nosocomial infections) and 51 in Rotterdam (27 secondary referrals and 24 nosocomial infections) were excluded from the analysis.

Table 3.1.1. Demographic characteristics of patients

	Geneva (n=55)	Rotterdam (n=96)	P-value
Gender (boys : girls)	34 : 21	55 : 41	0.71
Age in days upon admission (median/range)	108/11–341	79/9–256	0.17
Gestational age (weeks)± SD	38.2 ± 3.3	38.0 ±3.0	0.79
Risk factors :			
Gestation < 37 weeks	10 (18.2)	23 (24.0)	0.53
Age < 6 weeks	9 (16.4)	23 (24.0)	0.37
Cardiac malformation	2 (3.6)	3 (3.1)	
Bronchopulmonary dysplasia	2 (3.6)	4 (4.2)	
T cell immune deficiency	0	0	
No risk factors	34 (61.8)	53 (55.2)	0.53
Breast feeding > 1 month	35 (70.0)	23 (29.1)	0.0001
Breast feeding upon admission	25 (50.0)	24 (31.2)	0.051
Asthma in family *	8 (25.8)	14 (26.4)	1.00
Eczema in family *	2 (6.5)	10 (18.5)	0.20
N. of children in the household* (median/range)	2/1 – 4	2/1 – 8	1.00
Daycare attendance *	3 (9.7)	3 (5.7)	0.67
Smoking in the household	12 (40.0)	23 (46.9)	0.64

* Data from the prospectively collected cohort (season 1994/95) only.
Percentages are shown in brackets.
Differences between Geneva and Rotterdam cohorts were tested by the χ^2 test or Fisher's exact test for dichotomous variables and by Mann-Whitney U test for continuous variables.

Table 3.1.1. summarises the demographic data on the 151 children included in the study. Significant differences between centres were only noted for breast feeding (Table 3.1.1.) and age at admission (Figure 3.1.1.): 55% of children admitted in Geneva and 69% of those admitted in Rotterdam were younger than 4 months (χ^2 test; p = 0.03).

Disease severity at admission

Several disease severity parameters were reported differently by the two centres (Table 3.1.2.). Rotterdam infants were more often admitted with a history of apnoea and had a lower SaO_2 and a higher PCO_2 at the time of admission than the subset of Geneva infants for whom PCO_2 values were available. Geneva infants, in contrast, had a higher mean res-

piratory rate and more often presented with wheezing and chest retractions. Feeding difficulties and chest retractions were the most common presenting symptoms in the two centres.

Course of disease and treatment

Treatment in the two centres included supplementary oxygen, empirical bronchodilator administration, and the use of antibiotics but no corticosteroids (Table 3.1.3.). Ribavirin was only used for a subset of infants in Rotterdam. Additional episodes of apnoea (two infants) were only reported in Rotterdam, as well as the death of a 4 week old infant with no known RSV risk factors who had been admitted with cardiorespiratory arrest after recurrent apnoea. The total length of stay in hospital was shorter in Geneva than in Rotterdam, where more children were admitted to the intensive care unit and required mechanical ventilation.

Separate analysis of seasons

To evaluate the influence of seasonal variability on the clinical severity of RSV, statistical analyses were performed independently for the prospectively studied 1994-5 winter season and the retrospective 1993-4 season. For the 1994-5 season, no difference in pre-existing risk factors was observed between Geneva (n = 31) and Rotterdam (n = 59) infants. Significant differences for Geneva infants were higher rates of breast feeding >1 month

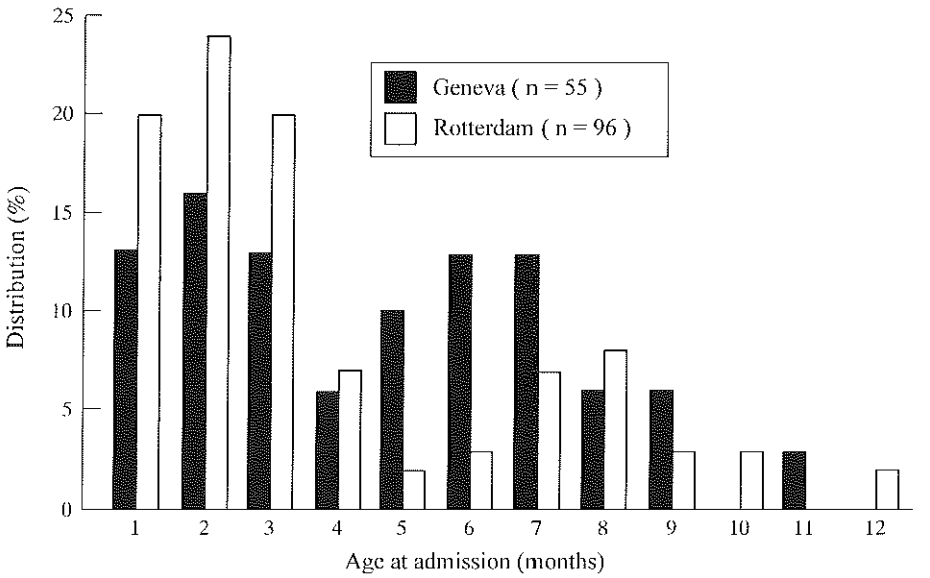

Table 3.1.2. Clinical parameters at admission.

	Geneva (n=55)	Rotterdam (n=96)	P-value
Fever	15 (27.2)	22 (23.4)	0.74
Days with breathing problems before admission* (median/range)	3/0 – 13	2/0 – 9	0.09
Feeding problems	42 (76.4)	82 (92.1)	0.17
Retractions	45 (81.8)	57 (63.3)	0.029
wheezing*	19 (65.5)	17 (28.8)	0.001
Respiratory rate/min ± SD	59.7 ± 12.2	51.2 ± 19	0.0002
Apnea	1 (1.8)	23 (23.9)	0.0008
O_2 saturation (%) ± SD	92.5 ± 8.2	90.8 ± 9.0	0.0039
pCO_2 (kPa) ± SD	5.8 ± 1.3 (n=15)	6.7 ± 1.9 (n=94)	0.032
Abnormality on X-ray	40 (74.0)	56(59.6)	0.10

* Data from the prospectively collected cohort (season 1994/95) only.
Percentages are shown in brackets.
Differences between Geneva and Rotterdam cohorts were tested by the χ^2 test or Fisher's exact test for dichotomous variables and by Mann-Whitney U test for continuous variables.

(74.2 v 30.6%, $p < 0.001$), higher respiratory rates (60.3 v 51.3, $p = 0.019$), a lower frequency of apnoea (3.2 v 23.7%, $p = 0.013$), lower PCO_2 (5.65 v 6.91, $p = 0.019$), a shorter length of hospital stay (6.0 v 8.4 days, $p = 0.01$), and a lower rate of admission to the intensive care unit (6.5 v 32.2%, $p < 0.001$). In this small sample of 90 infants differences in the frequency of chest retractions, mean SaO_2, and requirement for respiratory support did not reach statistical significance. The same trends were found for the 1993-4 season (24 infants in Geneva and 37 in Rotterdam). Significant differences for the Geneva infants were a higher rate of breast feeding >1 month (73.4 v 21.8%, $p = 0.002$), a higher mean respiratory rate (59.5 v 51.1, $p = 0.03$), a lower frequency of apnoea (0 v 24.3%, $p = 0.009$), a shorter length of hospital stay (7.3 v 9.9 days, $p = 0.02$), and a lower rate of admission to the intensive care unit (0 v 21.6%, $p = 0.02$).

Multivariate analysis

Clinical parameters which differed significantly between Geneva and Rotterdam were again compared by multivariate analysis, adjusting for the identified epidemiological factors that differed between the two centresnamely, age and breast feeding. After correction for these potential confounders a higher percentage of apnoeas, a higher rate of admission to the intensive care unit, and a longer duration of hospital stay were still observed in Rotterdam,

Table 3.1.3. Course of disease and treatment

	Geneva (n=55)	Rotterdam (n=96)	P-value
Oxygen administration	43 (78.8)	59 (68.6)	0.30
Bronchodilators	49 (89.1)	79 (84.0)	0.54
Ribavirine	0	16 (17.2)	
Antibiotics	32 (58.2)	34 (38.2)	0.03
Maximal Respiratory rate ± SD	63.3±12.3	58.7±15.4	0.026
Additional children with apnea	0	2 (2.1)	
Patients in ICU	2 (3.6)	27 (28.1)	0.0005
Patients requiring mechanical ventilation	0	7 (7.3)	0.048
Deaths	0	1 (1.0)	
Hospital stay (days) (median/range)	6.5 / 1–19	9 / 1–29	0.0011

Percentages are shown in brackets.
Differences between Geneva and Rotterdam cohorts were tested by the χ^2 test or Fisher's exact test for dichotomous variables and by Mann-Whitney U test for continuous variables.

whereas a higher respiratory rate and higher percentage of wheezing on admission in Geneva remained significant.

Discussion

In this study we confirmed that the course of RSV infections is significantly more benign in Geneva than Rotterdam. Infants admitted to hospital in Geneva less often presented with apnoea or respiratory insufficiency and thus less often required admission to the intensive care unit or respiratory support than infants admitted in Rotterdam. Their more benign status was also reflected by a significantly shorter length of stay in hospital. This demonstration of a local variability of RSV disease severity is not restricted to the two centres studied. It is in accordance with at least two previous published observations. A striking difference in RSV morbidity was first reported in 1961 in two nearby nursery groups (12). More recently, a significant association between the hospital centre and parameters of clinical severity was reported in a multicentre study of RSV outcome in Canada (31). Furthermore, paediatricians from various European centres have subjectively recognised either of the two distinct clinical patterns of RSV disease presented here as representative of the situation prevailing in their area (personal communications to C.A. Siegrist at ESPID meeting, June 1996). Importantly, this study also shows (as 35 years ago (12)) that a detailed comparison of all the parameters previously reported as affecting disease severity does not identify the

factors responsible for the observed differences in RSV disease severity.

The more benign course of RSV infection in Geneva does not appear to depend on a lower incidence of RSV infections in the first year of life. The rate of admission to hospital for an RSV infection during the first winter season at 5.3/1000 lies within the previously reported rates of admission to hospital of 1-20/1000 children (4,27). This rate of admission to hospital in Geneva is little affected by the variable severity of the RSV winter epidemic. Comparison of the two consecutive seasons of children admitted to hospital confirmed that the disease pattern and severity also remain constant. As rates of admission to hospital have been reported to be influenced by socioeconomic status, influencing the age at exposure and access to medical care (11,27,28), the potentially higher socioeconomic status of parents in Geneva would be expected to result in a reduction of rates of admission to hospital rather than of RSV disease severity. Thus the more benign course of RSV disease in Geneva than in Rotterdam essentially reflects a reduced severity of disease in the most severely sick infants who require admission to hospital.

Disease severity and the outcome of infants admitted to hospital is related to their pre-existing status such as prematurity, age less than 6 weeks, congenital heart disease, bronchopulmonary dysplasia, or immune deficiency (9,17,22). The lower severity of RSV infections in Geneva than Rotterdam is, however, not explained by a lower number of infants presenting with these underlying risk factors. Two factors found to differ between the two cohorts were a smaller percentage of children aged less than 4 months at admission and a higher percentage of breast feeding in Geneva. Interestingly, the percentage of breast fed infants in the Rotterdam cohort was also significantly lower than the overall rate of breast feeding in the Netherlands (65% at 1 month and 55% at 3 months of age (1)). As minimal or no breast feeding has been reported to increase the risk of admission to hospital for respiratory infections (27), mucosal protection could participate in the observed reduction of disease severity. Correcting for breast feeding and age in multivariate analyses did not correct the differences in disease severity, however.

Differences in subtype virulence have also been suggested to explain the yearly variation of disease severity (10,19), although no relation between clinical severity and RSV subtypes was found in a study in Rotterdam (14). In the present study a predominance of subtype A was observed in Geneva and in Rotterdam. Although virulence could still differ within strains of the same subtype, strain virulence differences are unlikely to result in a higher morbidity in the same centre over two consecutive winter seasons.

Other epidemiological factors that could explain the reported variation in disease severity (see under methods) were carefully compared and found to be similar in the two cohorts. Differences in referral systems were minimised in our study by only including primarily referred infants to either centre, but differences in hospital policies still affect rates of admission to the intensive care unit. In Rotterdam all RSV infected children less than 2 months of age or born prematurely are initially monitored in the intensive care unit, whereas admission to the Geneva intensive care unit depends exclusively on the clinical status. These hospital policies cannot, however, explain the differences in clinical parameters at admission or indication for mechanical ventilation.

Unexpectedly, we recognised two different disease patterns in our two cohorts: respiratory rate and frequency of wheezing and chest retractions were significantly higher in the Geneva infants. In contrast, respiratory insufficiency was more common in Rotterdam, although the duration of reported respiratory symptoms before admission was shorter. We postulate that the efficacy of compensatory hyperventilation in response to lung disease could be a critical factor distinguishing the two cohorts. This dissociation between an increased respiratory effort (previously described as a poor predictor of clinical severity (21)) and the clinical outcome suggests that environmental factors such as air quality may exert an influence on RSV morbidity by modulating the infant's capacity to respond to pulmonary disease by compensatory hyperventilation. Parental smoking (similar in the two cohorts) and the use of wood burning stoves (20,28,32) have been shown to increase the risk and severity of RSV infections. It is important to define the role of air humidity or temperature, either indoors or outdoors, or of industrial air pollution, which is responsible for an excess of cardiovascular deaths among adult or elderly patients (26) and which could also affect the capacity of young infants to cope with respiratory infections (25). A relatively preserved air quality in medium sized cities such as Geneva compared with large industrialised urban agglomerations such as Rotterdam could well contribute to a lower morbidity of infant respiratory diseases. Air quality could thus contribute to the high RSV morbidity reported by large American or European centres, mostly located in dense urban environments.

In conclusion, parameters to be collected in multicentre studies assessing RSV disease severity have not yet all been identified. Whether air quality affects RSV disease in infants and elderly patients should be specifically addressed through prospective studies collecting air samples. Until these additional factors responsible for the geographical variations of RSV morbidity are identified, the many prophylactic or therapeutic strategies planned for the next decade should probably take into careful account the existence of different local

disease patterns.

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References

1. Anonymous1994. Vademecum health statistics. Statistics Netherlands & Ministery of welfare, Health and Cultural Affairs,
2. Everard, M. L. and A. D. Milner. 1992. The respiratory syncitial virus and its role in acute bronchiolitis. Eur. J. pediatr. 151:638-651.
3. Frankel, L. R., N. J. Lewiston, D. W. Smith, and D. K. Stevenson. 1986. Clinical observations on mechanical ventilation for respiratory failure in bronchiolitis. Pediatr. Pulmonol. 2:307-311.
4. Glezen, W. P., A. Paredes, J. E. Allison, L. H. Taber, and A. L. Frank. 1981. Risk of respiratory syncytial virus infection for infants from low- income families in relationship to age, sex, ethnic group, and maternal antibody level. J. Pediatr. 98:708-715.
5. Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child 140:543-546.
6. Green, M., A. F. Brayer, K. A. Schenkman, and E. R. Wald. 1989. Duration of hospitalization in previously well infants with respiratory syncytial virus infection. Pediatr. Infect. Dis. J. 8:601-605.
7. Groothuis, J. R., K. M. Gutierrez, and B. A. Lauer. 1988. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 82:199-203.
8. Hall, C. B., W. J. Hall, and D. M. Speers. 1979. Clinical and physiological manifestations of bronchiolitis and pneumonia. Outcome of respiratory syncytial virus. Am. J. Dis. Child 133:798-802.
9. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315:77-81.
10. Hall, C. B., E. E. Walsh, K. C. Schnabel, C. E. Long, K. M. McConnochie, S. W. Hildreth, and L. J. Anderson. 1990. 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. 162:1283-1290.
11. Holberg, C. J., A. L. Wright, F. D. Martinez, C. G. Ray, L. M. Taussig, and M. D. Lebowitz. 1991. Risk factors for respiratory syncytial virus-associated lower respiratory illnesses in the first year of life. Am. J. Epidemiol. 133:1135-1151.
12. Kapikian, A. Z., J. A. Bell, F. M. Mastrota, K. M. Johnson, R. J. Huebner, and R. M. Channock. 1961. An outbreak of febrile illnessand pneumonia associated with respiratory syncytial virus infection. Am. J. Hyg. 74:234-248.
13. Kim, H. W., J. O. Arrobio, C. D. Brandt, B. C. Jeffries, G. Pyles, J. L. Reid, R. M. Chanock, and R. H. Parrott. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. Am. J. Epidemiol. 98:216-225.
14. Kneyber, M. C., A. H. Brandenburg, P. H. Rothbarth, R. de Groot, A. Ott, and H. A. Van Steensel-Moll. 1996. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch. Dis Child 75:137-140.
15. La Via, W. V., S. W. Grant, H. R. Stutman, and M. I. Marks. 1993. Clinical profile of pediatric patients hospitalized with respiratory syncytial virus infection. Clin. Pediatr. 32:450-454.
16. La Via, W. V., M. I. Marks, and H. R. Stutman. 1992. Respiratory syncytial virus puzzle: clinical features, pathophysiology, treatment, and prevention. J. Pediatr. 121:503-510.

17. MacDonald, N. E., C. B. Hall, S. C. Suffin, C. Alexson, P. J. Harris, and J. A. Manning. 1982. Respiratory syncytial viral infection in infants with congenital heart disease. N. Engl. J. Med. 307:397-400.
18. Martin, A. J., P. S. Gardner, and J. McQuillin. 1978. Epidemiology of respiratory viral infection among paediatric inpatients over a six-year period in north-east England. Lancet 2:1035-1038.
19. McConnochie, K. M., C. B. Hall, E. E. Walsh, and K. J. Roghmann. 1990. Variation in severity of respiratory syncytial virus infections with subtype. J. Pediatr. 117:52-62.
20. McConnochie, K. M. and K. J. Roghmann. 1986. Parental smoking, presence of older siblings, and family history of asthma increase risk of bronchiolitis. Am. J. Dis. Child 140:806-812.
21. Mulholland, E. K., A. Olinsky, and F. A. Shann. 1990. Clinical findings and severity of acute bronchiolitis. Lancet 335:1259-1261.
22. Navas, L., E. Wang, V. de Carvalho, and J. Robinson. 1992. Improved outcome of respiratory syncytial virus infection in a high- risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J. Pediatr. 121:348-354.
23. Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98:289-300.
24. Sarkkinen, H. K., P. E. Halonen, and A. A. Salmi. 1981. Detection of influenza A virus by radioimmunoassay and enzyme-immunoassay from nasopharyngeal specimens. J. Med. Virol. 7:213-220.
25. Schmitzberger, R., K. Rhomberg, H. Buchele, R. Puchegger, D. Schmitzberger-Natzmer, G. Kemmler, and B. Panosch. 1993. Effects of air pollution on the respiratory tract of children. Pediatr. Pulmonol. 15:68-74.
26. Seaton, A., W. MacNee, K. Donaldson, and D. Godden. 1995. Particulate air pollution and acute health effects. Lancet 345:176-178.
27. Sims, D. G., M. A. Downham, J. McQuillin, and P. S. Gardner. 1976. Respiratory syncytial virus infection in north-east England. Br. Med. J. 2:1095-1098.
28. Singleton, R. J., K. M. Petersen, J. E. Berner, E. Schulte, K. Chiu, C. M. Lilly, E. A. Hughes, L. R. Bulkow, and T. L. Nix. 1995. Hospitalizations for respiratory syncytial virus infection in Alaska Native children. Pediatr. Infect. Dis. J. 14:26-30.
29. Tissing, W. J. E., H. A. Van Steensel-Moll, and M. Offringa. 1993. Risk factors for mechanical ventilation in respiratory syncytial virus infection. Eur. J. pediatr. 152:125-127.
30. Van Steensel-Moll, H. A., C. J. Jongkind, R. S. R. Aarsen, A. de Goede-Bolder, A. Dekker, L. W. A. van Suijlekom-Smit, S. Kraaycnoord, and G. Derksen-Lubsen. 1996. A problem oriented patient classification system in general pediatrics. Tijdsch Kindergeneeskd 64:99-104.
31. Wang, E. E., B. J. Law, and D. Stephens. 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.
32. Wright, A. L., C. Holberg, F. D. Martinez, and L. M. Taussig. 1991. Relationship of parental smoking to wheezing and nonwheezing lower respiratory tract illnesses in infancy. Group Health Medical Associates. J. Pediatr. 118:207-214.

Chapter 4
Humoral immune response against RSV

4.1 Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection

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Summary

The decline of maternal respiratory syncytial virus (RSV) specific serum antibodies was studied in 45 children during the first six months of life, using a virus neutralization assay and competition ELISAs measuring fusion protein and glycoprotein specific antibodies. In all children RSV neutralizing antibodies could be demonstrated at birth, with titers ranging from 33 to 1382. The calculated mean half life of these antibodies was 26 days. Furthermore, in a group of 38 children with suspected RSV infection, all younger than six months of age upon admission, the diagnostic value of serological assays was evaluated. In 32 children RSV infection was confirmed by virus isolation, direct immune fluorescence and RT-PCR analyses. In seven individuals of this group a significant titer rise in virus neutralization assay could be demonstrated. Six additional RSV infected children could be identified, by showing the presence of RSV-specific IgM or IgA serum antibodies or by showing an increase in fusion protein or glycoprotein specific antibodies. All serological tests together identified 13 (41%) of the 32 RSV infected patients.

It was concluded that in children in this age group, which represent the majority of patients hospitalized with RSV infections, serological assays not only have a limited diagnostic value, but are also of limited value for sero-epidemiological studies.

Introduction

In developed countries respiratory syncytial virus (RSV) is the most important cause of severe respiratory tract infections in children under two years of age (4,9). Severe RSV infection related disease is predominantly seen in children between six weeks and six months of age, when most infants still have RSV specific maternal serum antibodies. Vaccination with a formalin inactivated candidate RSV vaccine, inducing high titers of RSV specific antibodies, gave rise to more severe clinical disease upon subsequent exposure to the virus (3,15). Therefore antibodies have been incriminated as a factor in more severe clinical outcome of the infection. Especially the presence of RSV specific IgE has been suggested to be a contributing factor in RSV bronchiolitis (2,23). On the other hand there is evidence from both human and laboratory animal studies that high titered virus neutralizing (VN) antibodies may be protective against RSV infection (7,10,17,20). Here we present data on the decline of RSV specific maternal serum antibodies in 45 children, and the development of specific serum antibodies upon infection with RSV in 32 children, all younger than six months of age. In this study, existing and newly developed assays were used to measure RSV specific antibodies of different classes, isotypes and protein specificities.

Patients and methods

Patients and Sera

Group I: Serum samples collected during the years 1989-1991, from 45 healthy children participating in a hepatitis B vaccination trial were used to study the decline of maternal RSV specific antibodies. Sera were taken at birth, at 3 months and at 6 months. Children born in the months March until June were selected to minimize the chance of RSV infection in these first 6 months.

Group II: Serum samples from 38 children, all younger than six months of age, seen at the Sophia's children hospital during the period November 1993 to April 1994, with a respiratory infection suspected of RSV, were collected in the acute stage of the disease and three to four weeks later. Demographic and clinical data of these children were collected on admission and at control visit. They included gender, age, duration of pregnancy, presence of underlying disease (defined as congenital heart disease, bronchopulmonary dysplasia or immune deficiency), feeding difficulties (defined as an increase of time required for feeding or a decrease of feeding volume), a positive history for apnea (defined as either a history of respiratory arrest with cyanosis or an observation of respiratory arrest for a period of more than 20 seconds and/or bradycardia with accompanying cyanosis in the pediatric emergency room or during hospital admission), the presence of retractions, respiratory rate, oxygen saturation (SaO_2) in room air, pCO_2, pH and abnormalities on X-ray (hyperinflation,

consolidation or atelectasis) as described by a radiologist, admission to an intensive care unit and the need for artificial ventilation.

Virus preparation

The A2 strain of human RSV (ATCC VR1302) was used in serological tests. The virus was passaged in HEp-2 cells and cultured until the majority of the monolayer exhibited cytopathic changes. The supernatant was cleared of cell debris by centrifugation for 10 minutes at 1000 G and subsequently aliquoted and frozen at -70°C until use in virus neutralization assays (VN). For all virus neutralization tests the same stock of RSV was used.

Virus for competition ELISAs and IgM and IgA capture ELISAs was partially purified by polyethylene glycol precipitation of the supernatant of infected cells and subsequently centrifuged for 2 hours at 25000 rpm through a discontinuous 30-60% (w/w) sucrose gradient in a Beckman SW28 rotor. The resulting opaque virus containing band was collected, aliquoted and frozen at -70°C until use.

Virus isolation(VI) and direct immune fluorescence assay (DIFA)

Nasopharyngeal washings were taken from the children of group II. The samples were diluted with 5 ml DMEM, homogenated, and centrifuged for 10 minutes at 1000 rpm. The supernatant was used for DIFA. The pellet was pipetted onto multispot slides, dried at room temperature and fixed with acetone. These slides were used for DIFA as previously described (19), using fluorescein isothiocyanate (FITC) labeled RSV specific monoclonal antibodies (DAKO, Ely, UK). For RSV subtyping, monoclonal antibodies against subtype A (MAB 92-11c, Chemicon, Temecula, USA) or subtype B (MAB 109-10B, Chemicon, Temecula, USA) were pipetted on the slides and incubated for 30 minutes at 37°C. After washing three times with phosphate buffered saline pH 7.2 (PBS) a FITC labeled anti mouse conjugate (DAKO, Ely, UK) was applied and incubated for 30 minutes at 37°C. After washing three times with PBS and once in distilled water the slides were examined under a epifluorescence microscope using a 620 nm filter. Slides were scored positive when a typical granular fluorescence was observed in the cytoplasm of cells.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed on nasopharyngeal washings of the children as previously described (22). In short, total RNA of nasopharyngeal washings was extracted using guanidinium isothiocyanaat and phenol/chloroform. RNA PCR was performed in two steps. For cDNA synthesis, 10 µl of the RNA template and 1 µl (containing 10 pmol) cDNA primer were heated to 80°C for 2 minutes and put on ice. Then 14 µl of a cDNA reaction mixture was added, which made a solution containing 50 mM Tris-HCL pH 8.3, 37.5 mM KCl 3

mM $MgCl_2$, 10 mM DTT, 0.5mM of each of the dNTP's (dATP, dCTP, dGTP and dTTP), 1.6 U RNAsin (Promega, Leiden, The Netherlands) and 8 U molony murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL, Breda, the Netherlands). The mixture was incubated at 42°C for 45 minutes, heated at 95°C and put on ice. Then 75 µl of the PCR mix was added resulting in a 100 µl PCR solution containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton-X-100, 0.2 mM of each of the dNTP's, 20 pmol cDNA primer, 20 pmol reverse primer and 1 U of Taq polymerase (Promega, Leiden, The Netherlands). A PCR program containing 36 cycles of 3 steps (1 min at 95°C, 1 minute at 52°C, 1 minute at 74°C) was used.

For analysis of the amplified products 25µl of the PCR products were electrophoresed in a 1.5% agarose gel. The gel was then denatured for 10 minutes in 0.4 N NaOH and the amplified products were transferred to nylon membrane using a semidry blotter (Biorad, California USA) at 3 mA/cm^2, for southern blot analysis. Southern blots were hybridized overnight using subtype A and B specific 32P labeled oligonucleotides.

Virus neutralization (VN) assay

50µl serially diluted sera were incubated with 50µl DMEM containing 100 TCID50 of RSV-A2 for one hour at 37°C in 96 well tissue culture microtiter plates (Costar plastics, Badhoevedorp, the Netherlands). Control wells contained no serum (positive or virus growth control) or only DMEM (negative or cell control). Then HEp-2 cells were added to all wells and incubated for three days at 37°C 5% CO_2. Subsequently the cells were washed once carefully with PBS and fixed in ethanol at -70°C for 30 minutes.

The expression of viral antigen on the HEp-2 cells was detected in an ELISA system; the plates were blocked with 100 µl 1% gelatin in PBS for 30 minutes and washed five times with PBS 0.05% tween 20 (PBST). 50 µl of an RSV fusion protein specific mouse monoclonal (MAB 92-11C, Chemicon, Temecula USA) was added to the cells and incubated for 2 hours at 37°C. After washing five times, HRPO labeled rabbit anti-mouse (DAKO, Ely, UK) was used as conjugate, incubated for one hour at 37°C, washed five times and shaken dry. Then the substrate, tetramethylbenzidine/H_2O_2 was added. The reaction was stopped after 10 minutes with 2 M H_2SO_4. The optical density (OD) was read at 450 nm. The percentage virus neutralization was calculated by the following formula:

$$\frac{\text{experimental OD} - \text{cell control OD}}{\text{virus control OD} - \text{cell control OD}} \times 100\%$$

The titer of the serum was defined as the reciprocal of the dilution which gave 50 % virus neutralization. A significant rise in titer to confirm infection was arbitrarily defined as a

greater than a threefold titer rise.

Competition ELISA

To measure antibodies against separate RSV membrane proteins, competition ELISAs for RSV fusion protein (comp-F ELISA) and glycoprotein (comp-G ELISA) were carried out essentially according to methods which we have previously described for Hantavirus serology (5). Briefly, antigen was coated (2 ng/well) to 96 well ELISA plates (Costar plastics, Badhoevedorp, The Netherlands) overnight at 4°C. 100 µl of a tenfold serum dilution in ELISA buffer (PBS with 3% NaCl, 0.1% BSA, 0.1% milk powder, 5% normal rabbit serum and 1% fetal calf serum) were pipetted into the wells. For the negative control (0% inhibition) ELISA buffer containing no serum was used. For the positive control (100% inhibition) ELISA buffer containing no serum was used, and ELISA buffer containing no monoclonal antibody was used in the second step. After incubation for 2 hours at room temperature half (50 µl) of the serum dilution was removed and replaced by 50 µl of a mouse monoclonal anti-fusion protein (133/1H, Chemicon MAB 858-1, Temecula, CA) or anti- glycoprotein (131/2G, Chemicon MAB 858-2, Temecula, CA) RSV dilution in ELISA buffer and incubated for one hour at 37°C. Specificity of these monoclonals has been described previously (1). Subsequently the plates were washed 3 times in PBST. HRPO labeled rabbit anti-mouse (DAKO, Ely, UK) was used as the conjugate and incubated for one hour at 37°C, washed three times and shaken dry. Then 100 µl tetramethylbenzidine/ H_2O_2 substrate was added. The reaction was stopped after 10 minutes with 2 M H_2SO_4. The OD was spectrophotometrically read at 450 nm. Percentage inhibition was calculated by the formula:

$$\frac{\text{experimental OD} - \text{OD}_{100\%\text{inhibition}}}{\text{OD}_{0\%\text{inhibition}} - \text{OD}_{100\%\text{inhibition}}} = \%\text{inhibition}$$

A significant rise in inhibition to confirm infection was arbitrarily defined as a greater than threefold rise in inhibition percentage.

Sera proved negative tested in a 1:10 dilution in this assay never reached inhibition levels above 10%.

RSV specific IgA and IgM detection in serum

RSV specific antibodies of the IgA and IgM class were detected in sera by capture ELISA essentially as previously described for hantavirus serology (5). Positive results were confirmed with an indirect immune fluorescence assay as previously described (6).

For capture ELISA, goat anti-human IgA or IgM was coated to 96 well ELISA plates overnight at 4°C. 100 µl of a hundredfold serum dilution, in ELISA buffer, was pipetted in the wells and incubated for one hour at 37°C. Subsequently the plates were washed three times in PBST. The optimal dilution of RSV antigen was pipetted in the wells and incubated for one hour at 37°C and the plates were washed three times in PBST. Subsequently 50 µl of biotinilated polyclonal goat anti-RSV (Chemicon, Temecula, CA) was added to the cells, incubated for one hour at 37°C, washed three times in PBST and shaken dry. Streptavidine-HRPO (Amersham, Little Chalfont, UK) was used as conjugate, incubated for 30 minutes at room temperature, washed three times and shaken dry. Then 100µl tetramethylbenzidine/H_2O_2 substrate was added. The reaction was stopped after 10 minutes with 2 M H_2SO_4. OD was measured at 450 nm. An OD exceeding more than 2 times the OD of the negative control was considered positive.

For confirmation of positive results in an indirect immune fluorescense assay, cells infected with RSV were mixed with uninfected HEp-2 cells and spotted on 12 well multispot slides. The slides were dried at room temperature and the cells were fixed with ethanol at -70°C. Sera were pre-incubated with sheep anti-human Fc gamma globulin (RIVM, Bilthoven, The Netherlands). Pre-incubated sera (1:16 end dilution) were pipetted on the wells with RSV infected cells and incubated 2 hours at 37°C. Subsequently the slides were washed three times for five minutes in PBS and 1 minute in distilled water. Then FITC labeled anti-human IgM or IgA (DAKO, Ely, UK) was pipetted on the slides and incubated for 30 minutes at 37°C. After washing, the slides were examined in an epifluorescence microscope using a 620 nm filter. Results were considered positive when a characteristic granular fluorescence pattern in the cytoplasm of the cells was observed.

Complement fixation assay (CF)

The CF assay was carried out as previously described (8). Briefly, serum was inactivated at 56°C for 30 minutes. A 2 log titration of the serum starting at 1:7 was made. The working dilution of first RSV antigen (Bio-Withakker, Walkersville Maryland) and then complement (Bio-Withakker, Walkersville Maryland), were added to each serum dilution. After incubation of 18 hours at 4°C, sheep red blood cells, sensitized with hemolysin (Bio-Withakker, Walkersville MD), were added and incubated for one hour at 37°C. After settling of the red blood cells titers were read at 50% end point.

Statistical analyses

For statistical analyses, geometric mean titers (GMT) were calculated for the VN assay. For the competition ELISAs, mean inhibition percentages were calculated. For comparison of mean rise in antibodies in infected and non-infected children a 2-tailed student's t-test for

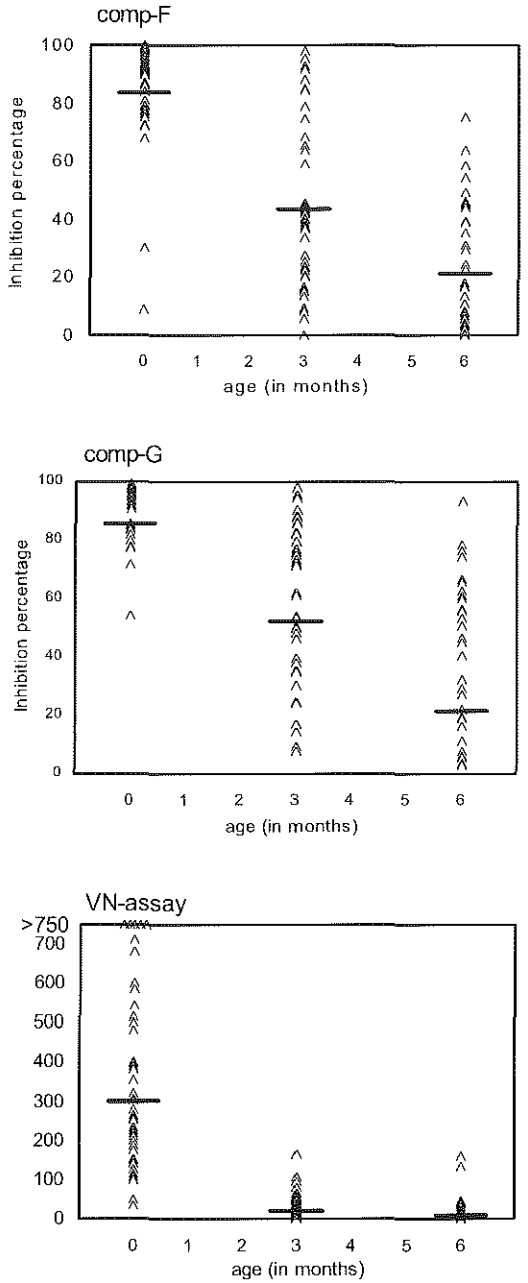

Figure 4.1.1. Decline of maternal RSV specific serum antibodies in 45 children (Group I). Sera were taken at birth, three months and six months of age. Individual and mean test results are indicated.
Mean inhibition percentages for comp-F and comp-G ELISAs and geometric mean titers for the VN assay are indicated with horizontal bars.

paired samples was used. For comparing RSV specific titers upon admission with parameters of clinical severity a 2-tailed student's t-test for independent samples was used.

Results

Decline of maternal RSV specific antibodies

The decline of RSV specific maternal antibodies during the first six months of life was monitored in 45 children (Group I) using the VN assay and competition ELISAs (Figure 4.1.1.). At birth VN antibodies were present in the sera of all 45 children with titers ranging from 33 to 1382 and a geometric mean titer of 301. Geometric mean titers at three and six months were 24 and 10 respectively. In the majority of sera taken at six months after birth, no VN antibodies could be demonstrated. Comparison of mean inhibition percentages measured in comp-F ELISA and comp-G ELISA at birth, at three and at six months showed a linear decline 86%, 43% and 21% in the comp-F ELISA and 91%, 58% and 21% in the comp G-ELISA respectively. Two of the 45 children tested showed a significant titer rise in VN between three and six months after birth (from < 10 to 48 and from 22 to 132 respectively), indicating that these infants had been infected with RSV during this period. In these paired sera a rise in inhibition percentage was found from 8.4% to 38.9% and 43.0% to 63.7% respectively in the comp F-ELISA. However, no rise was observed in the comp-G ELISA. From the comparison of the VN serum antibody titers at birth with those found three months later, a mean half-live of maternally derived serum antibodies of 26 days was calculated.

Kinetics of RSV specific antibodies after infection

Of the 38 infants with a suspected RSV infection (group II), 32 indeed proved to be infected with RSV. This was shown by VI, DIFA and RT-PCR analyses. No other viral infections were found in any of the 38 infants. Individual titers and GMT of RSV infected and non-infected children are shown in Figure 4.1.2. Comparing the serum antibody titers measured in the VN assay upon admission and three to four weeks later showed a significant mean antibody titer rise measured in children with a confirmed infection (GMT1 = 51; GMT2 = 75; p=0.01) and a decline in antibody titer in uninfected children (GMT1 = 48, GMT2 = 30; p=0.06). The mean titer rise in infected children was significantly different from the mean titer decline of noninfected children (ΔT-infected: 30, ΔT-noninfected = -44; p=0.04). No significant changes in mean inhibition levels were found between infected and uninfected children when using the comp-F and comp-G ELISA.

Comparison of diagnostic test results of individual patients with suspected RSV infection

The results of all diagnostic tests carried out with specimens of the 38 patients with sus-

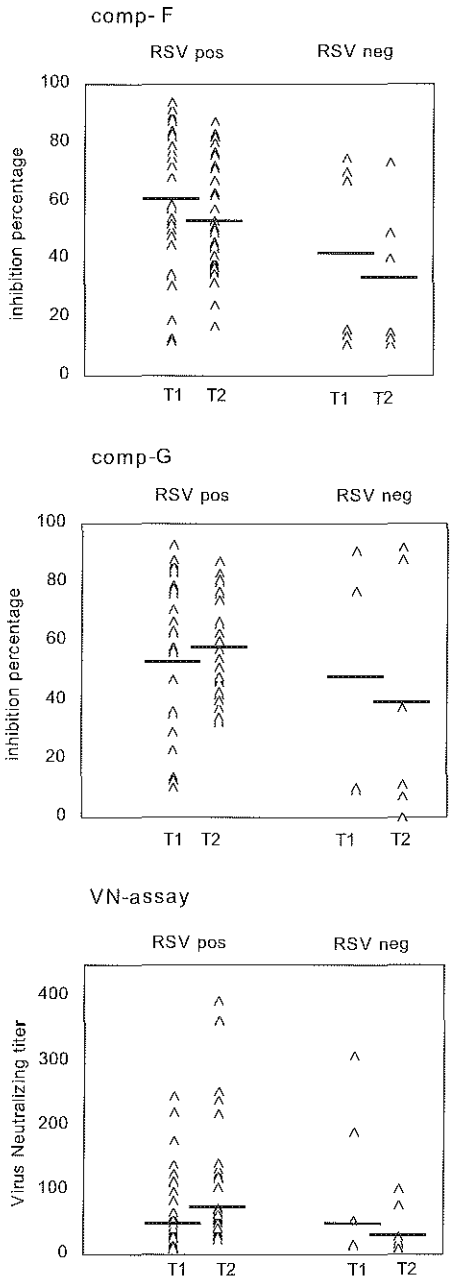

Figure 4.1.2. Development of serum antibody titers in 38 children with respiratory disease suspect for RSV infection (group II). Individual and mean test results are indicated.
Inhibition percentages (comp-F and comp-G ELISAs) and VN titers of children with confirmed RSV infection (left) and no RSV infection (right) upon admission (T1) and three to four weeks later (T2) are indicated. Mean inhibition percentages (ELISAs) and geometric mean titers (VN assay) are indicated with horizontal bars.

Table 4.1.1. Summary of diagnostic analyses in 38 children, < 6 months of age, clinically suspected of RSV infection

patient	VI	DIFA subtype	RT-PCR subtype	VN*	competition ELISA*		capture ELISA		CF*
					anti-F	anti-G	IgM	IgA	
1	+	A	A	+	+	+	–	–	–
2	+	A	A	+	–	+	–	–	–
3	+	A	A	+	–	+	–	–	–
4	+	A	A	+	–	+	–	–	–
5	+	B	B	+	–	–	–	+	–
6	+	B	B	+	–	+	–	–	–
7	+	A	A	+	–	–	–	–	–
8	+	A	A	–	–	+	–	+	–
9	+	A	A	–	–	+	–	–	–
10	+	A	A	–	–	–	+	–	–
11	+	A	A	–	–	–	–	+	–
12	+	A	A	–	–	–	–	+	–
13	+	A	A	–	–	–	–	+	–
14–19 (n=6)	+	A	A	–	–	–	–	–	–
20–32 (n=13)	+	B	B	–	–	–	–	–	–
33–38 (n=6)	–	–	–	–	–	–	–	–	–

VI = virus isolation, DIFA = direct immune fluorescence on cells of nasopharingeal washings using subtype A and B specific monoclonal antibodies, RT-PCR = Reversed transcriptase polymerase chain reaction using subtype A and B specific probes. VN = virus neutralization assay, anti-F = anti fusion protein antibodies, anti-G = anti glycoprotein antibodies. CF = complement fixation test.

* A three fold rise in antibody level was considered positive in VN, CF, and anti F and G competition ELISA.

pected RSV infection are presented in Table 4.1.1. The results of VI, DIFA and RT-PCR assays were in complete agreement: in 32 patients an RSV infection was identified, of which 17 were of subtype A and 15 of subtype B as shown with DIFA and RT-PCR. Analyses of paired sera, in the VN assay allowed the identification of seven (22%) RSV infected infants by showing a greater than threefold titer rise. With the comp-F and comp-G ELISA one (3%) and seven (22%) RSV infected infants could be identified respectively. Detection of RSV specific IgM and IgA in single serum samples identified one (3%) and five (16%) RSV infected infants respectively. With a CF assay no RSV infected infants were detected. All serological tests together only identified 13 (41%) of the 32 RSV infected patients.

As shown in Figure 4.1.3., a greater than threefold rise in RSV specific serum antibody rise

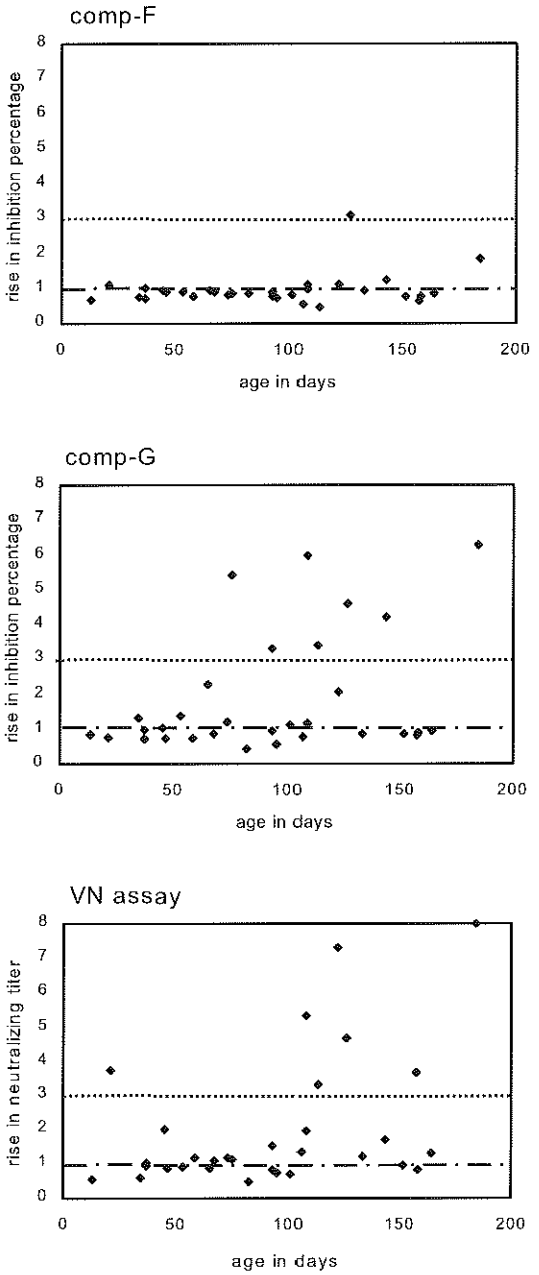

Figure 4.1.3. Rise in antibody levels in 32 children with a confirmed RSV infection plotted against the age in days upon admission. Rise in antibody levels is expressed as the quotient of the inhibition percentage upon admission and the inhibition percentage in the convalescent phase for the competition ELISAs or as the quotient of the VN titer upon admission and the VN titer in the convalescent phase for individual children.

was predominantly found in RSV infected children older than three months.

Comparison of VN titer upon admission with disease severity

In order to evaluate whether a relationship exists between the presence of RSV specific maternal antibodies on one hand and severity of RSV related disease on the other, antibody titers measured in the VN assay and inhibition percentages in the comp-F and comp-G ELISA were related to parameters of clinical severity (pCO_2, SaO_2, ICU admission, artificial ventilation).

Children with a higher pCO_2 upon admission had significantly higher titers in the VN assay ($p = 0.05$). A higher pCO_2 also correlated strongly with a younger age upon admission. No correlation could be found between SaO_2, ICU admission, or artificial ventilation and VN titers or percentage inhibition in the competition ELISAs (data not shown).

Discussion

In the present paper we have shown that in children under six months of age the diagnostic value of RSV serology is limited and by far inferior to the direct detection methods for RSV antigen or viral RNA. This may, at least in part, be caused by the relative inability of the young infants to mount a specific antibody response upon infection. This is best illustrated by the virtual absence of RSV specific IgM, which is not vertically transmitted via the placenta. Furthermore preexisting maternal antibodies may interfere with the antibody response upon infection and may also hinder the interpretation of serological results.

The detection of IgM, IgG, and IgA antibodies for the serodiagnosis of RSV in young infants is known to be relatively insensitive as a diagnostic tool. However, we investigated whether antibody recognition of different structural RSV proteins, would be a useful parameter for the diagnosis of RSV infection. This is a well established approach for other virus infections, like those with HIV (16) and hantavirus (5).

All children in group I had detectable maternal antibodies at birth, which declined with a half-life of 26 days in the first months of life. This value is in agreement with normal half-live values of passively acquired antibodies, which is estimated to be three to four weeks (13). The presence of RSV specific antibodies has been shown to correlate with protection against severe RSV infection in mice (17) and in children (7), although relatively high titers seemed to be required for protection. Studies in children have shown that the administration of high titered anti RSV immune globulines may protect young children from developing a severe RSV infection with the involvement of the lower respiratory tract (7,11). This allows speculation about the protective value of maternal antibodies, which may provide sufficient protection against severe disease development after birth. However, with a half-life of 26 days antibody levels may be expected to drop relatively fast to unprotective levels.

With the exception of pCO_2 levels, which correlated with VN titers upon admission, non of the parameters of clinical severity correlated with antibody levels upon admission in group II. However, the correlation of pCO_2 levels with VN titers may probably be explained by the higher pCO_2 usually found in younger infants with RSV infection (14), at which age higher maternal antibody levels are also present. Thus no causal relationship between RSV specific antibody titers and severity of infection was detected in this study.

The discrepancy between the data generated in the VN assay and the competition ELISAs may be explained by the fact that the inhibition percentages of maternal antibodies found in noninfected children are relatively high, as compared to VN antibodies. Therefore maternal antibodies should be expected to cause more interference in competition ELISAs than in the VN assay. This would result in the absence of a demonstrable rise in inhibition percentages upon infection.

We used a 1:10 dilution of the serum sample in our competition ELISA although we realized that such a low dilution might cause nonspecific binding. When the RSV competition ELISA was established we tested several negative serum samples at a 1:10 dilution. None of these sera caused nonspecific reduction of the absorbance. Furthermore the use of the principle of a competition ELISA with 1:10 diluted serum samples has been investigated extensively by us in several other systems, including infections with hantavirus (5), rabiesvirus (21) and Borrelia burgdorferi (18).

At different intervals after infection or reinfection, serum antibodies have different affinities for the respective epitopes. Especially in the acute phase of RSV infection, antibodies with low affinity may be present (12). In the competition ELISA the monoclonal antibodies which have a relative high affinity may interfere with the binding of the serum antibodies, especially when the whole sample is removed. We therefore removed half of the sample only and replaced it with 50 µl of the respective monoclonal antibody preparation.

Although the children with a proved subtype A infection showed more often a serological response in the assays used than children with a subtype B infection, the number of individuals studied is too small to conclude that this may have been due to the use of a subtype A strain in the assays.

Taken together, the data presented in this study show that VI, DIFA, and RT-PCR are more reliable tools for the diagnosis of RSV infections than serological methods. For practical reasons, the use of DIFA, followed by confirmatory VI, is probably the best option at present for the rapid and accurate diagnosis of RSV infection.

The overall poor performance of the serological assays used, indicates the limited diagnostic value of serology in these young children. Furthermore it shows that serology cannot be used for sero-epidemiological studies, at least in the age group of children younger than six

months, which comprises more than 50% of all patients hospitalized for RSV infection.

References

1. Anderson, L. J., J. C. Hierholzer, C. Tsou, R. M. Hendry, B. F. Fernie, Y. Stone, and K. Mcintosh. 1985. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J. Infect. Dis. 151:626-633.
2. Bui, R. H., G. A. Molinaro, J. D. Kettering, D. C. Heiner, D. T. Imagawa, and J. W. St.Geme,Jr. 1987. Virus-specific IgE and IgG4 antibodies in serum of children infected with respiratory syncytial virus. J. Pediatr. 110:87-90.
3. Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette. 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. 89:449-463.
4. Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child 140:543-546.
5. Groen, J., J. Dalrymple, S. Fisher-Hoch, J. G. Jordans, J. P. Clement, and A. D. Osterhaus. 1992. Serum antibodies to structural proteins of Hantavirus arise at different times after infection. J. Med. Virol. 37:283-287.
6. Groen, J., G. van der Groen, G. Hoofd, and A. Osterhaus. 1989. Comparison of immunofluorescence and enzyme-linked immunosorbent assays for the serology of Hantaan virus infections. J. Virol. Methods 23:195-203.
7. Groothuis, J. R., E. A. F. Simoes, M. J. Levin, C. B. Hall, C. E. Long, W. J. Rodriguez, J. Arrobio, H. C. Meissner, D. R. Fulton, R. C. Welliver, D. A. Tristram, G. R. Siber, G. A. Prince, M. Van Raden, V. G. Hemming, and The Respiratory Syncitial Virus Immune Globulin Study Group. 1993. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. New England J. Med. 329:1524-1530.
8. Hawkes, R. A. 1979. General principles underlying laboratory diagnosis of viral infections. p. 3-48. In E. H. Lenette and N. J. Smidt (eds.), Diagnostic procedures for Viral, Rickettsial and Chlamydial Infections. American Public Health Association, Washington DC.
9. Kim, H. W., J. O. Arrobio, C. D. Brandt, B. C. Jeffries, G. Pyles, J. L. Reid, R. M. Chanock, and R. H. Parrott. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. Am. J. Epidemiol. 98:216-225.
10. Mcintosh, K. 1993. Respiratory syncytial virus--successful immunoprophylaxis at last. N. Engl. J. Med. 329:1572-1574.
11. Meissner, H. C., D. R. Fulton, J. R. Groothuis, R. L. Geggel, G. R. Marx, V. G. Hemming, T. Hougen, and D. R. Snydman. 1993. Controlled trial to evaluate protection of high-risk infants against respiratory syncytial virus disease by using standard intravenous immune globulin. Antimicrob. Agents Chemother. 37:1655-1658.
12. Meurman, O., M. Waris, and K. Hedman. 1992. Immunoglobulin G antibody avidity in patients with respiratory syncytial virus infection. J. Clin. Microbiol. 30:1479-1484.
13. Morell, A., W. D. Terry, and T. A. Waldmann. 1970. Metabolic properties of IgG subclasses in man. J. Clin. Invest. 49:673-680.
14. Mulholland, E. K., A. Olinsky, and F. A. Shann. 1990. Clinical findings and severity of acute bronchiolitis. Lancet 335:1259-1261.
15. Murphy, B. R., G. A. Prince, E. E. Walsh, H. W. Kim, R. H. Parrott, V. G. Hemming, W. J. Rodriguez, and R. M. Chanock. 1986. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J. Clin. Microbiol. 24:197-202.
16. Portera, M., F. Vitale, R. La Licata, D. R. Alesi, G. Lupo, F. Bonura, N. Romano, and G. Di Cuonzo. 1990. Free and antibody-complexed antigen and antibody profile in apparently healthy HIV seropositive individuals and in AIDS patients. J. Med. Virol. 30:30-35.
17. Prince, G. A., V. G. Hemming, R. L. Horswood, and R. M. Chanock. 1985. Immunoprophylaxis and immunotherapy of respiratory syncytial virus infection in the cotton rat. Virus Res. 3:193-206.

18. Rijpkema, S., J. Groen, M. Molkenboer, P. Herbrink, A. Osterhaus, and J. Schellekens. 1994. Serum antibodies to the flagellum of Borrelia burgdorferi measured with an inhibition enzyme linked immuno sorbent assay are diagnostic for lyme boreliosis. Serodiagn Immunother Infect Disease 6:61-67.
19. Rothbarth, P. H., J. J. Habova, and N. Masurel. 1988. Rapid diagnosis of infections caused by respiratory syncytial virus. Infection 16:252
20. Siber, G. R., J. Leszcynski, V. Pena-Cruz, C. Ferren-Gardner, R. Anderson, V. G. Hemming, E. E. Walsh, J. Burns, K. Mcintosh, and R. Gonin. 1992. Protective activity of a human respiratory syncytial virus immune globulin prepared from donors screened by microneutralization assay. J. Infect. Dis. 165:456-463.
21. van der Heijden, R. W., J. P. Langedijk, J. Groen, F. G. UytdeHaag, R. H. Meloen, and A. D. Osterhaus. 1993. Structural and functional studies on a unique linear neutralizing antigenic site (G5) of the rabies virus glycoprotein. J. Gen. Virol. 74:1539-1545.
22. van Milaan, A. J., M. J. Sprenger, P. H. Rothbarth, A. H. Brandenburg, N. Masurel, and E. C. Claas. 1994. Detection of respiratory syncytial virus by RNA-polymerase chain reaction and differentiation of subgroups with oligonucleotide probes. J. Med. Virol. 44:80-87.
23. Welliver, R. C., M. Sun, D. Rinaldo, and P. L. Ogra. 1985. Respiratory syncytial virus-specific IgE responses following infection: evidence for a predominantly mucosal response. Pediatr. Res. 19:420-424.

4.2 A subtype-specific peptide-based enzyme immunoassay for detection of antibodies to the G protein of human respiratory syncytial virus is more sensitive than routine serological tests

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Summary

Peptides deduced from the central conserved region (residues 158 to 189) of protein G of human respiratory syncytial virus (HRSV) subtypes A and B were used as antigens in subtype-specific enzyme-linked immunosorbent assays (G-peptide ELISAs). These G-peptide ELISAs were compared with seven other serological assays to detect HRSV infection: ELISAs based on complete protein G, on fusion protein F, and on nucleoprotein N; a complement fixation assay; a virus neutralization test; and ELISAs for the detection of immunoglobulin A (IgA) or IgM antibodies specific for HRSV. In paired serum samples from patients with HRSV infection, more infections were diagnosed by the G-peptide ELISA (67%) than by all other serological tests combined (48%). Furthermore, for 16 of 18 patients (89%), the G-peptide ELISAs were able to differentiate between antibodies against HRSV subtypes A and B. This study shows that peptides corresponding to the central conserved region of the attachment protein G of HRSV can successfully be used as antigens in immunoassays. The G-peptide ELISA appeared to be more sensitive than conventional tests for the detection of HRSV antibody titer rises.

Introduction

Human respiratory syncytial virus (HRSV) is the most important causative agent of bronchiolitis and pneumonia in young children. The virus is classified within the Pneumovirus genus of the Paramyxoviridae. Efficacious vaccines against respiratory syncytial virus (RSV) are not available. Because different antigenic subtypes are described for HRSV (6), it is important for epidemiological studies and vaccine developments to monitor the prevailing subtypes in a population. However, the available immunoassays (11) are based on whole virus or complete proteins that do not discriminate between subtypes of HRSV or between different RSV types.

The highly variable attachment protein G has limited homology between HRSV subtypes (53% amino acid homology) (6). However, within the subtypes the amino acid homology is much larger: >80% within HRSV subtype A (HRSV-A) strains (3) and >90% within HRSV-B strains (10). Therefore, protein G is a good candidate antigen for a discriminatory assay. We proposed that the ectodomain of protein G contains a central, conserved, relatively hydrophobic region bounded by two hydrophilic, polymeric mucin-like regions (8). The central conserved region of HRSV, bovine RSV, and ovine RSV is a major antigenic site, and peptides corresponding to this region can be used as antigens in immunoassays (1,7-9). In a previous study, it was shown that conventional serology does not provide an adequate diagnostic tool for RSV infection in children younger than 6 months of age (2). In this study, the data obtained by these conventional serological tests were compared with those obtained by enzyme-linked immunosorbent assays (ELISAs) based on peptides corresponding to the central conserved region of HRSV-A and HRSV-B. Furthermore, we demonstrated the applicability of the peptide-based ELISA for subtype-specific diagnosis.

Materials and methods

Peptide synthesis.

Peptides corresponding to the central conserved regions of protein G (residues 158 to 189)

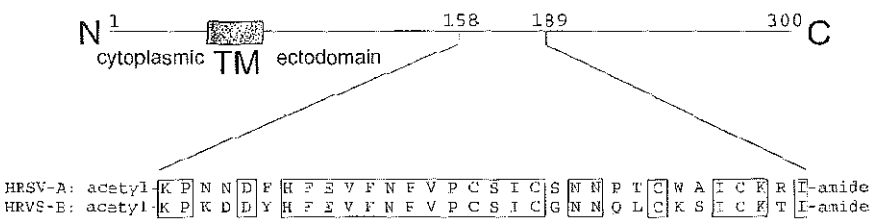

Figure 4.2.1. Schematic representation of primary structure of HRSV-G. Shaded box, transmembrane region (TM). The primary structure of the synthesized peptides corresponding to the central conserved region of the ectodomain is shown.

of HRSV-A (12) and HRSV-B (6) were synthesized (Figure 4.2.1.). Peptide synthesis has been described previously (7).

Serum samples and specimens.

Paired serum specimens from 33 different children (age, 0 to 6 months) with respiratory tract disease and suspected of having RSV infection were taken in the acute phase and 3 to 4 weeks later. Twenty-seven of these patients were confirmed to have an RSV infection by direct immunofluorescence of cells from nasopharyngeal washings, virus isolation on HEp-2 cells, and reverse transcription PCR (2). The RSV subtype was identified by using RSV subtype-specific monoclonal antibodies (MAbs; MAb 92-11C for HRSV-A and MAb 109-10B for HRSV-B; Chemicon) in an immunofluorescence assay with infected HEp-2 cells (2). Paired serum specimens from the 27 patients confirmed to be RSV positive were used to distinguish between antibody reactivity against the HRSV-A G peptide or the HRSV-B G peptide. Paired serum specimens from 14 mothers of the 14 HRSV-A-infected children described above were taken at the same time that specimens were taken from their children. These sera were tested in the HRSV-A G-peptide ELISA to measure maternal antibody titers.

As a negative control, paired serum samples from six additional patients with acute infections caused by influenza virus type A, Chlamydia psittaci, and Mycoplasma pneumoniae, but not HRSV, were tested in the G-peptide ELISAs. Two serum samples (samples 2369 and 2219) from two individuals (ages 2 and 3 years, respectively) positive for HRSV-specific antibodies collected during the 1993 to 1994 RSV epidemic were a kind gift of J. A. Melero, National Centre for Microbiology (Madrid, Spain). These two serum specimens were tested by Pepscan analysis.

Pepscan analysis.

Peptides were synthesized on functionalized polyethylene rods and were tested for their reactivity with polyclonal antisera in an ELISA by established procedures (4). Forty-seven overlapping dodecapeptides of the ectodomain of the G protein of HRSV-A (12) between amino acids 153 and 211 were synthesized. This set of peptides includes all peptides corresponding to the central conserved region.

Neutralization test.

Twofold dilutions (starting at 1:10) of test sera were incubated with 100 50% tissue culture infective doses of HRSV-A2 for 1 h at 37°C in 96-well tissue culture microtiter plates. HEp-2 cells were added to all wells, and the plates were incubated for 3 days at 37°C in 5% CO_2. The expression of viral antigen on the HEp-2 cells was detected by ELISA with an F-pro-

tein-specific MAb (MAb 92-11C; Chemicon) and antimouse horseradish peroxidase (HRPO; Dako, Glostrup, Denmark). Both incubations were for 1 h at 37°C.
Tetramethylbenzidine-H_2O_2 was used as the substrate. The reaction was stopped after 10 min with 0.2 M H_2SO_4. Absorption was read at 450 nm. The percent virus neutralization was calculated by the following formula: (experimental OD – cell control OD)/(virus control OD – cell control OD) × 100, where OD is optical density. The titer of the serum was defined as the dilution which gave 50% virus neutralization. Threefold titer rises were considered indicative of RSV infection.

Competition ELISAs.
Competition ELISAs for RSV fusion protein F, attachment protein G, and nucleoprotein N were performed as described previously (5). Partially purified RSV-A2 was coated (2 μg/well) onto 96-well ELISA plates (Costar) overnight at 4°C. Dilutions of 100 μl of test sera (diluted 1:10 and 1:100) were incubated in the wells for 2 h at room temperature. A highly positive serum sample was used as the positive control (100% inhibition), and ELISA buffer (phosphate-buffered saline, 3% extra NaCl, 0.1% bovine serum albumin, 0.1% milk powder, 5% normal rabbit serum, 1% fetal calf serum) was used as a negative control (0% inhibition). Subsequently, 50 μl of the serum dilution was discarded and 50 μl of anti-F (133/1H; Chemicon MAb 858-1), anti-G (131/2G; Chemicon MAb 858-2), or anti-N (23A3; Biosoft MAb 213-88) was added and the mixture was incubated for 1 h at 37°C. Next, the wells were incubated with HRPO-conjugated rabbit anti-mouse (Dako) for 1 h at 37°C. All dilutions were made in ELISA buffer. Tetramethylbenzidine-H_2O_2 was used as the substrate. The reaction was stopped after 10 min with 0.2 M H_2SO_4. Absorption was read at 450 nm. Percent inhibition was calculated by the following formula: (experimental OD – OD for 100% inhibition)/(OD for 0% inhibition – OD for 100% inhibition) × 100. Threefold rises in the percentage of inhibition were considered indicative of RSV infection.

IgA and IgM capture ELISA.
Anti-human immunoglobulin A (IgA) or IgM was coated onto the wells of 96-well ELISA plates (Costar) overnight at 4°C. Test sera (1:100), partially purified RSV, 50 μl of rabbit polyclonal anti-RSV serum (Dako), and HRPO-conjugated goat anti-rabbit (Dako) were each subsequently incubated for 1 h at 37°C. Tetramethylbenzidine-H_2O_2 was used as the substrate. The reaction was stopped after 10 min with 0.2 M H_2SO_4. Absorption was read at 450 nm. The cutoff value for positivity was more than two times the OD of a negative sample. A positive reaction was confirmed by immunofluorescence test.

Table 4.2.1. Antibody titers in paired serum specimens from patients with a respiratory infection determined by both G-peptide ELISAs

		Titer by the following G-peptide ELISA[a]:	
Patient no.	RSV type	Peptide A	Peptide B
2	A	**<10, 40**	<10, <10
6	A	**10, 80**	<10, <10
9	A	**20, 160**	<10, <10
11	A	80, 40	<10, <10
17	A	**10, 160**	<20, <10
18	A	10, 10	<10, <10
19	A	10, 20	<10, <10
26	A	**<10, 40**	<10, <10
27	A	10, 10	<10, <10
28	A	**<10, 40**	<10, <10
30	A	**<10, 20**	<10, <10
36	A	**<10, 640**	<10, <10
37	A	**<10, 320**	<10, <10
41	A	40, 80	<10, <10
7	B	**<10, 20**	10, <10
8	B	10, <10	**<10, 20**
10	B	40, <10	**<10, 40**
13	B	20, <10	**<10, 20**
15	B	<10, 10	<10, <10
21	B	10, <10	**<10, 40**
23	B	10, 10	**<10, 80**
24	B	10, 20	<10, 10
25	B	10, <10	<10, <10
35	B	10, <10	**<10, 40**
38	B	**10, 40**	<10, 10
39	B	10, 10	<10, <10
40	B	<10, <10	**<10, 20**
1		<10, <10	<10, <10
4		**<10, 40**	<10, <10
5		<10, <10	<10, <10
12		<10, <10	<10, <10
14		<10, <10	<10, <10
29		<10, <10	<10, <10

a The pairs of values in each column are for the paired sera from each patient. Titers in boldface represent defined titer rises.

Table 4.2.2. Antibody titers in paired serum specimens from patients with a non-RSV infection determined by both G-peptide ELISAs[a]

Patient	Non-HRSV infection	Non-HRSV titer rise	Titer by the following G-peptide ELISA: Peptide A	Peptide B
WA	Influenza virus type A	7, 32	320, 160	80, 80
B	*C. psittaci*	<16, >64	80, 80	<10, <10
G	Influenza virus type A	7, >96	40, 20	10, <10
WB	Influenza virus type A	12, 80	80, 80	10, 10
H	*M. pneumoniae*	<6, 32	20, 10	10, <10
V	*M. pneumoniae*	<6, 64	640, 320	20, 20

a The pairs of values in the last three columns are for the paired sera from each patient.

Indirect G-peptide ELISA procedure.

The G-peptide ELISA was performed as described previously (7), but with a modification in the coating procedure. One hundred fifty nanograms of crude oxidized peptide in 50 µl of carbonate buffer (0.05 M; pH 9.6) was coated per well (high-binding-capacity flat-bottom microplate; Greiner) at 37°C overnight until all water was evaporated. Twofold dilutions of test sera (starting at 1:10) and anti-human HRPO (1:1,000; Dako) were incubated for 1 h at 37°C in ELISA buffer (8.1 mM Na_2HPO_4, 2.79 mM KH_2PO_4, 0.5 M NaCl, 2.68 mM KCl, 1 mM disodium EDTA, 0.05% [vol/vol] Tween 80 [pH 7.2]) containing 4% horse serum. The substrate chromogen solution consisted of 10 mM sodium phosphate buffer (pH 6.8), 0.1 mM EDTA, 0.1% (wt/vol) 5-aminosalicylic acid, and freshly added 0.005% (vol/vol) H_2O_2. Incubation with substrate solution was performed overnight at 4°C. Color development was measured at 450 nm (Titertek Multiscan). Absorbance values higher than two times the average background value of test serum in control wells without peptide were considered positive. Fourfold titer rises or seroconversion (<10 to >=20) in paired serum specimens are designated defined titer rises and were considered indicative of RSV infection.

Results

The reactivity of a panel of paired serum specimens was tested in the HRSV-A and HRSV-B peptide ELISAs described in Materials and Methods (Table 4.2.1.). To evaluate the HRSV specificity of the ELISAs, the reactivities of paired serum specimens, which showed antibody titer rises against other agents, were tested in both peptide ELISAs (Table 4.2.2.). No titer rises were observed for the negative controls.

Table 4.2.3. Antibody titers in paired serum specimens from mothers of patients with a respiratory infection by HRSV-A G-peptide ELISA

Mother	Titer by G-peptide ELISA for peptide A[a]
2	10, 20
6	10, 80
9	160, 160
11[b]	160, 40
17	20, 20
18	20, 20
19	40, 40
26	40, 40
27	20, 80
28	160, 160
30	–[c], 20
36	10, 10
37	10, 10
41	40, 40

a The pairs of values are for the paired sera from each mother.
b Numbers in boldface represent mothers of children with a high acute-phase serum titer.
c serum was not available.

In Table 4.2.1., titer rises may be missed because of the high titer of the acute-phase serum. Because a high titer in the acute-phase serum may be due to maternal antibodies, the paired serum specimens from some of the mothers were tested in the HRSV-A peptide ELISA (Table 4.2.3.). No significant association was found for the maternal antibody titer and the corresponding titer in the acute-phase serum from the child ($p=0.13$, according to Fisher's exact test for two-way tables).

Discussion

Detection of RSV infection in young children by conventional serological tests as described in a previous study (2) was compared with detection of RSV infection with the aid of novel G-peptide ELISAs (Table 4.2.4.). In summary, 18 of 27, 6 of 27, 0 of 27, 0 of 27, 6 of 27, 5 of 27, 0 of 27, and 0 of 27 specimens scored positive in the G-peptide ELISA, protein-G ELISA, protein-F ELISA, protein-N ELISA, neutralization test, IgA ELISA, IgM ELISA, and complement fixation test, respectively (Table 4.2.4.). The G-peptide ELISAs performed even better (18 of 27 specimens) than the combination of all other serological tests (12 of 27 specimens) (Table 4.2.4.). Statistical analyses of the sensitivity of different assays are presented in Table 4.2.5. In 6 of the 33 patients, no virus or antigen could be detected (see

Table 4.2.4. Detection of HRSV infection by several serological tests

Patient no.	RSV type	Peptide-A ELISA	Peptide-B ELISA	Protein-G ELISA[a]	Protein-F ELISA[a]	Protein-N ELISA[a]	Neutralization test[a]	IgA ELISA[a]	IgM ELISA[a]	Complement fixation test[a]
2	A	+[b]	–[c]	+	–	–	+	–	–	–
6	A	+	–	–	–	–	–	–	–	–
9	A	+	–	+	–	–	+	–	–	–
11	A	–	–	–	–	–	–	–	–	–
17	A	+	–	–	–	–	–	+	–	–
18	A	–	–	–	–	–	–	+	–	–
19	A	–	–	–	–	–	–	–	–	–
26	A	+	–	–	–	–	–	–	–	–
27	A	–	–	–	–	–	–	+	–	–
28	A	+	–	+	–	–	–	–	–	–
30	A	+	–	+	–	–	–	+	–	–
36	A	+	–	+	–	–	+	–	–	–
37	A	+	–	–	–	–	+	–	–	–
41	A	–	–	–	–	–	–	–	–	–
7	B	+	–	–	–	–	–	–	–	–
8	B	–	+	–	–	–	–	–	–	–
10	B	–	+	+	–	–	–	–	–	–
13	B	–	+	–	–	–	–	–	–	–
15	B	–	–	–	–	–	–	–	–	–
21	B	–	+	–	–	–	+	+	–	–
23	B	–	+	–	–	–	–	–	–	–
24	B	–	–	–	–	–	+	–	–	–
25	B	–	–	–	–	–	–	–	–	–
35	B	–	+	–	–	–	–	–	–	–
38	B	+	–	–	–	–	–	–	–	–
39	B	–	–	–	–	–	–	–	–	–
40	B	–	+	–	–	–	–	–	–	–
1[d]		–	–	–	–	–	+	–	–	–
4[d]		+	–	–	–	–	–	–	–	–
5[d]		–	–	–	–	–	–	–	–	–
12[d]		–	–	–	–	–	–	–	–	–
14[d]		–	–	–	–	–	–	–	–	–
29[d]		–	–	–	–	–	–	–	–	–

a Data obtained from Brandenburg et al. (2).
b +, defined antibody titer rise indicative of HRSV infection.
c –, no defined antibody titer rise.
d Paired sera from patients in which no virus or antigen could be detected.

Materials and Methods) (2). In paired serum specimens from these six patients not confirmed to be infected with RSV, one RSV-specific antibody titer rise was detected in the neutralization test (Table 4.2.4.). In another patient not confirmed to be infected with RSV, a titer rise was observed in the HRSV-A G-peptide ELISA. The latter two findings may suggest that these sera reacted aspecifically. However, it may also be possible that the virus or antigen detection test was false negative or that the virus was already cleared from the body before the patients entered the hospital. No titer rises were detected in paired serum specimens from patients infected with other agents (Table 4.2.2.).

The HRSV-A and HRSV-B G-peptide ELISAs are selective for the subtype-specific detection of HRSV infection. Patients infected with HRSV-A were only diagnosed positive by the HRSV-A G-peptide ELISA. Patients infected with HRSV-B were diagnosed positive seven times by the HRSV-B G-peptide ELISA and twice by the HRSV-A G-peptide ELISA. The reactivities of two paired serum specimens from supposedly HRSV-B-infected children in the HRSV-A peptide ELISA is puzzling. Several explanations can be conceived for this observation. First, the MAb-based subtype was wrong. This explanation seems unlikely because the MAb-based subtype was confirmed by subtype-specific PCR. Second, the child was infected with both virus types, and an antibody response was developed only against subtype A. Third, the cross-reactive sera are directed against an epitope conserved in HRSV subtypes A and B, and this epitope is better exposed in the HRSV-A peptide. We do have some examples of sera that bind to a conserved epitope in the N-terminal part of the HRSV-A peptide (Figure 4.2.2.). Pepscan analysis showed two examples of human sera that bound to five successive overlapping peptides which shared the amino acid sequence DFHFEVFN (amino acids 162 to 169). This sequence is conserved in HRSV-B except for the mutation of F to Y at position 163 (Figure 4.2.1.). It is possible that this relatively conserved epitope is responsible for cross-reactivity in the peptide ELISA. Therefore, a smaller peptide, which

Table 4.2.5. Two-sided 95% confidence intervals for different assay

Serological test	Interval (%)
G-peptide ELISA	$46 < P < 83$
Protein-G ELISA	$9 < P < 41$
Protein-F ELISA	$0 < P < 13$
Protein-N ELISA	$0 < P < 13$
Neutralization test	$9 < P < 41$
IgA ELISA	$6 < P < 38$
IgM ELISA	$0 < P < 13$
Complement fixation test	$0 < P < 13$

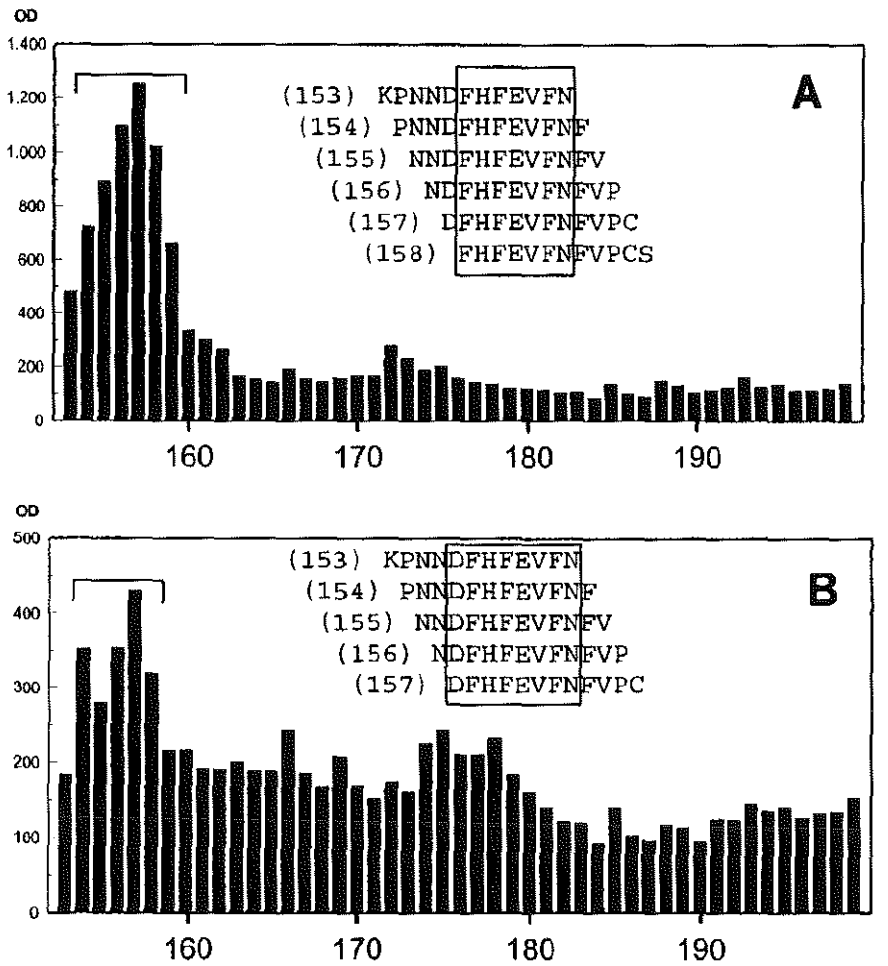

Figure 4.2.2. Reactivities of two positive control serum samples from infected children with overlapping peptides of the ectodomain of HRSV-G (12) between amino acids 153 and 211. (a) reactivity of serum sample 2369 (dilution, 1:100). (b) Reactivity of serum sample 2219 (dilution, 1:100). Numbers on the horizontal axis correspond to the N-terminal amino acid of the 12-residue peptide. Absorbances at 405 nm obtained with each peptide in an ELISA are plotted vertically. Reactive peptides are listed in the graph, and the shared amino acids are boxed.

only contains the more variable C-terminal part of the central conserved region, would seem more useful for subtype-specific diagnosis. However, a 16-residue peptide ELISA based on the shorter C-terminal peptide (residues 174 to 189) without the conserved part was significantly less sensitive than the 32-residue peptide used in the assays (7). Perhaps investigators will be able to find new immunodominant peptides, which can be combined with the 16-residue peptide, to develop an ELISA that is 100% subtype specific. However, such peptides, derived from the highly variable mucin-like regions, are probably strain specific and not completely subtype specific.

In the sera of nine patients we did not detect a defined titer rise in the HRSV-A or the HRSV-B peptide ELISA. In the case of patients 11 and 41, this may be due to the high titer in the first serum sample (Table 4.2.1.). Perhaps the first sampling date was too late for these two patients. We presumed that the high titer of the acute-phase serum was probably not caused by maternal antibodies since no evident association between the antibody titer in the mother's serum and the titer in the acutephase serum from the child was observed. The inability to diagnose an RSV infection in the seven other patients may be explained by a low or no antibody response in these children. The patients were young or prematurely born children and therefore may not have been fully immunocompetent. Two of the five serum samples that did not react in the peptide ELISA gave a positive response in the IgA ELISA only. Therefore, the sensitivity of the peptide ELISA may be increased when the reactivities of IgA antibodies are also tested.

The complete G-protein ELISA uses HRSV-A protein G as the antigen. Fewer infections were detected by the ELISA based on the complete HRSV-A protein G than by the ELISA based on the HRSV-A G peptide. The higher sensitivity of the HRSV-A G-peptide ELISA compared with that of the complete protein-G ELISA may be explained partly by the different format of the assays and the definition of a defined titer rise. Important advantages of a peptide-based ELISA are the low cost, the ease of production and the unvarying quality of the peptide, and the coated microtiter plates. Furthermore, the peptide-based RSV ELISA described in this study performs better than conventional serological assays with sera from these young children. Compared with other immunoassays, the peptide-based ELISA is a sensitive immunoassay for the detection of RSV infections. Despite considerable amino acid sequence homology between the central conserved regions of HRSV-A and HRSV-B proteins G, the peptide ELISA is specific for RSV subtype A and selective for subtype B-specific antibody detection.

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References

1. Akerlind-Stopner, B., G. Utter, M. A. Mufson, C. Orvell, R. A. Lerner, and E. Norrby. 1990. A subgroup-specific antigenic site in the G protein of respiratory syncytial virus forms a disulfide-bonded loop. J. Virol. 64:5143-5148.
2. Brandenburg, A. H., J. Groen, H. A. Van Steensel-Moll, E. J. C. Claas, P. H. Rothbarth, H. J. Neijens, and A. D. M. E. Osterhaus. 1997. Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. J. Med. Virol. 52:97-104.
3. Cane, P. A., D. A. Matthews, and C. R. Pringle. 1991. Identification of variable domains of the attachment (G) protein of subgroup A respiratory syncytial viruses. J. Gen. Virol. 72:2091-2096.
4. Geysen, H. M., R. H. Meloen, and S. J. Barteling. 1984. Use of peptide synthesis to probe viral anti-

gens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. U. S. A. 81:3998-4002.
5. Groen, J., G. van der Groen, G. Hoofd, and A. Osterhaus. 1989. Comparison of immunofluorescence and enzyme-linked immunosorbent assays for the serology of Hantaan virus infections. J. Virol. Methods 23:195-203.
6. Johnson, P. R., M. K. Spriggs, R. A. Olmsted, and P. L. Collins. 1987. The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: extensive sequence divergence between antigenically related proteins. Proc. Natl. Acad. Sci. USA 84:5625-5629.
7. Langedijk, J. P., W. G. Middel, W. M. Schaaper, R. H. Meloen, J. A. Kramps, A. H. Brandenburg, and J. T. Van Oirschot. 1996. Type-specific serologic diagnosis of respiratory syncytial virus infection, based on a synthetic peptide of the attachment protein. J. Immunol. Methods 193:157-166.
8. Langedijk, J. P. M., W. M. M. Schaaper, R. H. Meloen, and J. T. Van Oirschot. 1996. Proposed three-dimensional model for the attachment protein G of respiratory syncytial virus. J. Gen. Virol. 77:1249-1257.
9. Norrby, E., M. A. Mufson, H. Alexander, R. A. Houghten, and R. A. Lerner. 1987. Site-directed serology with synthetic peptides representing the large glycoprotein G of respiratory syncytial virus. Proc. Natl. Acad. Sci. U. S. A. 84:6572-6576.
10. Sullender, W. M., M. A. Mufson, L. J. Anderson, and G. W. Wertz. 1991. Genetic diversity of the attachment protein of subgroup B respiratory syncytial viruses. J. Virol. 65:5425-5434.
11. Welliver, R. C. 1988. Detection, pathogenesis, and therapy of respiratory syncytial virus infections. Clin. Microbiol. Rev. 1:27-39.
12. Wertz, G. W., P. L. Collins, Y. Huang, C. Gruber, S. Levine, and L. A. Ball. 1985. Nucleotide sequence of the G protein gene of human respiratory syncytial virus reveals an unusual type of viral membrane protein. Proc. Natl. Acad. Sci. U. S. A. 82:4075-4079.

Chapter 5
Cellular immune response against RSV

5.1 A type 1 like immune response is found in children with RSV infection regardless of clinical severity

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Summary

The immunological response of infants younger than six months to infection with respiratory syncytial virus (RSV) was studied in relation to clinical severity. IL-6 and IL-8 were found more frequently and at higher levels in the plasma samples of more severely ill patients and no significant differences were found in the levels of cytokines differentiating between Type 1 and Type 2 responses. Cellular infiltrates in nasopharyngeal washings consisted mainly of polymorphonuclear granulocytes and monocytes. Eosinophils, IgE positive cells and tryptase positive cells were found sporadically.

Analyses of RSV stimulated T cell cultures established from peripheral blood mononuclear cells, for intracellular and secreted cytokines showed that, irrespective of clinical severity, the responses were dominated by the production of IFN-γ, and that only low levels of IL-4 and IL-10 were detectable. Collectively these data do not indicate an association between clinical severity and a Type 2-like T cell response.

Introduction

Respiratory syncytial virus (RSV) infections are the most common cause of severe respiratory disease in young children, leading to the hospitalisation of up to 2% of children in their first year of life (16,17,19,29). The clinical presentation may vary from a mild upper respiratory tract infection to severe bronchiolitis or pneumonia. The highest morbidity of RSV disease is seen below the age of 6 months (16,17,19,32). Although there are several known clinical risk factors for developing severe RSV infection such as prematurity (30), bronchopulmonary dysplasia (18), congenital heart disease with pulmonary hypertension (27) and T cell immune deficiency (20), most children admitted with a severe RSV bronchiolitis do not have any of these risk factors.

It has been hypothesised that an aberrant virus specific immune response in these children may play a role in the pathogenesis of bronchiolitis. This was partly based on the observation that children vaccinated with an experimental formaldehyde inactivated RSV vaccine (FI-RSV) developed enhanced clinical disease upon subsequent infection (8,14,24,25). The hospitalisation rate for lower respiratory tract infection in vaccinees was up to 80% in one study, and two of the vaccinated children died (8). In rodent models vaccination with formaldehyde inactivated RSV preparations is associated with enhanced lung pathology, characterised by a striking eosinophilic infiltrate correlating with a Type 2 T cell response (31,43).

Whether a similar immune pathological mechanism plays a role in naturally occurring RSV bronchiolitis in infants remains elusive. To address this question inflammatory responses in infants younger than six months of age, with RSV infections of different clinical severities were studied. Inflammatory mediators in plasma samples, inflammatory cells in nasal washings and virus specific responses in T cell cultures established from peripheral blood mononuclear cells (PBMCs) of these children were identified.

Materials and methods

Patients and samples

One hundred and eleven children, younger than six months of age, seen at the Sophia's children hospital during the winter seasons 1993/94 and 1994/95, with a respiratory infection suspected of RSV, were included in the study. The study was approved by the ethical committee of the hospital. After informed consent was obtained from the parents, clinical data and blood samples were collected in the acute and the convalescent phase (3 to 4 weeks later) of the disease. Plasma samples were stored at -70°C until use. PBMCs were isolated by Lymphoprep (Nycomed®) gradient centrifugation and frozen at -135°C until use as previously described (40). Nasopharyngeal washings taken in the acute phase for diagnostic

purposes were used to study cellular infiltrates.

Demographic and clinical data collected included: gender, age, duration of pregnancy, presence of underlying disease (defined as congenital heart disease, bronchopulmonary dysplasia or immune deficiency), a history for apnoea (defined as either a history of respiratory arrest with cyanosis or an observation of respiratory arrest for a period of more than 20 seconds and/or bradycardia with accompanying cyanosis in the paediatric emergency room or during hospital admission), the presence of wheezing (scored positive if wheezing could be heard without using a stethoscope), the presence of retractions, respiratory rate, oxygen saturation (SaO_2) in room air, pCO_2, pH and abnormalities on X-ray (hyperinflation, consolidation or atelectasis) as described by a radiologist, admission to an intensive care unit and artificial ventilation.

Severe RSV infection was defined as meeting one or more of the following criteria: pCO_2 > 6,6kPa, SaO_2 < 90% or artificial ventilation.

RSV diagnosis

Nasopharyngeal washings (± 1 ml) were diluted with 5 ml DMEM, homogenated, and centrifuged for 10 minutes at 1000 rpm. The pellet was spotted onto multispot slides, dried at room temperature and fixed with acetone. The supernatant was used for virus isolation. The pellet was pipetted onto multispot slides, dried at room temperature and fixed with acetone. These slides were used for direkt antigen detection as described previously (35), using fluorescein isothiocyanate (FITC) labelled RSV-specific monoclonal antibodies (DAKO, Ely, UK).

Cytokine assays

Cytokine levels were measured in plasma samples and culture supernatants using commercially available ELISA systems. The following cytokine levels were determined; IL-2 (Pharmingen, San Diego, CA; detection limit 300 pg/ml), IL-4 (Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross, Amsterdam, The Netherlands; detection limit 7 pg/ml), IL-5 (Pharmingen; detection limit 20 pg/ml), IL-6 (Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross; detection limit 10 pg/ml), IL-8 (Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross; detection limit: 22 pg/ml), TNF-α (Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross; detection limit: 24 pg/ml), IFN-γ (Medgenix Diagnostics, Fleurs, Belgium; detection limit 25 pg/ml), IL-10 (Pharmingen, San Diego, CA; detection limit 20 pg/ml).

Ninety-six well ELISA plates (Costar plastics, Badhoevedorp, The Netherlands) were coated with capture antibody overnight at 4°C and blocked 30 minutes with PBS 5% milk powder. Plasma samples were diluted 1:2 in CLB High Performance buffer (Central Laboratory

of the Blood Transfusion Service of the Dutch Red Cross). 100 µl of the diluted plasma was added to the wells and incubated for 1 hour at room temperature. After washing biotin labelled detection antibody was added to the wells and the plates were incubated 1 hour at room temperature. After washing streptavidine HRPO was added to the wells and incubated 1 hour at room temperature. Then the substrate, tetramethylbenzidine/H_2O_2 was added. The reaction was stopped after 10 minutes with 2 M H_2SO_4. The optical density (OD) was read at 450 nm.

Concentrations of cytokines were calculated from the standard graph of duplicate standards included in the runs.

Immune histochemistry staining procedure

Staining of slides from nasopharyngeal washings was carried out by means of the long-chain biotin streptavidine-alkaline phosphate method (supersensitive; BioGenex, Klinipath, Duiven, The Netherlands).

Nasopharyngeal washings (± 1 ml) were diluted with 5 ml DMEM, homogenated, and centrifuged for 10 minutes at 1000 rpm. The pellet was spotted onto multispot slides, dried at room temperature and fixed with acetone. Slides were stored at -20°C until use.

The specimens were brought to room temperature, dried and fixed in PBS buffered formalin 4% for 15 minutes at 4°C and rinsed in PBS pH 7.8. The slides were incubated for 10 minutes with 0.5%-1% bovine serum album (BSA; Sigma, Bornem, Belgium) in PBS and subsequently incubated with normal goat serum (Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross) for 10 minutes. After this the following monoclonal antibodies were spotted onto the respective wells of the multispot slides and incubated for 60 minutes: Anti-CD3+ (UCHT1, 1:100; DAKO, Glostrup, Denmark), anti-CD8+ (leu2, 1:100; BD, Dorset, UK), anti-CD14+ (mon/1, 1:500; Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross), anti-CD15 (80H5, 1:50; Coulter Immunotech, Marseille, France), anti-MBP (BMK13, 1:50; Sanbio, Uden, The Netherlands), anti-IgE (HM25M, 1:750; Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross), anti-tryptase (G3, 1:250; Chemicon, Temecula, CA), anti-eosinophilic cathionic protein (EG-2, 1:50; Kabi Pharmacia, Woerden, The Netherlands). Subsequently, the slides were rinsed with PBS for 5 minutes and incubated with Link (goat anti-mouse long-chain biotinilated supersensitive AP, BioGenex AZ000UM; Klinipath) for 30 minutes, rinsed with PBS for 5 minutes, incubated with streptavidine-alkaline phosphatase (BioGenex, AZ000UM, Klinipath) for 30 minutes. They were then rinsed once more in PBS for 5 minutes and TRIS buffer (0.1 M pH 8.5) for 5 minutes and incubated for 30 minutes with a New Fuchsin substrate (Chroma, Kongen, Germany). Finally, the sections were rinsed in distilled water, counterstained with Gills haematoxylin and mounted in glycerin-gelatin.

For all spots 1000 cells were counted and the number of positive cells/1000 cells was scored.

Establishment and RSV infection of B-LCL

B lymphoblastic cell lines (B-LCL) were generated from PBMCs of patients by EBV transformation essentially as described previously (40) using 2×10^5 PBMCs.

B-LCL cultures were infected with the RSV-A2 strain (ATCC-VR1322) with a multiplicity of infection of 100, in most cases leading to a persistent infection within two to six weeks (4). These persistently infected B-LCL were used as antigen presenting cells in T cell studies. Infection of B-LCL with RSV was confirmed by immune fluorescence. To this end a sample of the cells was spotted on slides, dried at room temperature and fixed in acetone. After staining with FITC labelled RSV-specific monoclonal antibodies (DAKO, Ely, UK) fluorescent cells were scored with an epifluorescence microscope using a 620-nm filter. RSV infected cells were used as antigen presenting cells when at least 30% of the cells were found positive. Before use in T cell stimulation experiments RSV infected and control B-LCL were UV irradiated essentially as described previously for measles infected B-LCL (302 nm, 2.5×10^{-2} $\mu W/mm^2$) (41).

Bulk stimulation of PBMCs

T cell cultures were carried out in RPMI 1640 medium supplemented with penicillin (100 U/ml) streptomycin (100 µg/ml), L-glutamin (2 mM), 2-β mercapto-ethanol (10^{-5} M) and 10% human pooled serum (HPS). The HPS was a pool of 8 donors, heat inactivated for 30 minutes at 56°C and tissue culture tested. PBMCs (3×10^4/well) were co-cultured with UV irradiated RSV infected autologous B-LCL (10^4/well) in 96 well round bottom plates at 37°C, 5% CO_2. At day 3 recombinant human interleukin-2 (rIL-2; Eurocetus, Amsterdam, The Netherlands) was added to a final concentration of 50 IU/ml. Fresh medium with rIL-2 was added at day 7 and day 11. Twelve to 16 days after this bulk stimulation cells were harvested and counted. To expand further the T cell bulk cultures the cells were restimulated in medium containing 50 IU/ml rIL-2 and 1 µg/ml phytohaemagglutin-L (PHA-L; Boehringer Mannheim, Almere, The Netherlands) 3×10^4 per well in 96 well plates. As feeder cells each well received 10^5 gamma irradiated allogenic PBMCs (a pool of six donors) and 5×10^3 gamma irradiated of both the B-LCL APD (International Histocompatibility Workshop, IHW 9291) and BSM (IHW 9032) as described previously (42). T cell bulks were cultured for another 14 days, adding fresh medium with rIL-2 every 3 to 4 days. Between day 28 and 32 after the first RSV specific stimulation, cells were harvested, counted and used in experiments for cytokine measurements.

Measurement of cytokines in culture supernatants

For measurement of cytokine levels in culture supernatant, cells were restimulated with autologous RSV infected B-LCL, uninfected B-LCL or medium. Culture supernatants were harvested 48 hours after restimulation and tested for the presence of IFN-γ, IL-4 and IL-10. Validity of detection of IFN-γ and IL-4 in culture supernatants was tested using a measles virus specific CD8+ clone known to produce both IFN-γ and IL-4 (R. van Binnendijk, unpublished results) and an RSV specific CD8+ T cell clone known to produce IFN-γ. RSV specific cytokine production was determined by subtracting the cytokine production measured with uninfected B-LCL from that with RSV infected B-LCL. RSV induced cytokine production was considered significant in bulk cultures when levels above 10 pg/ml were found.

Intracellular cytokine immune fluorescence

Intracellular production of IFN-γ and IL-4 in PBMCs bulk cultures, upon RSV stimulation, was measured by intracellular immune fluorescence. Six hours after restimulation with autologous RSV infected B-LCL, control B-LCL or medium, in the presence of monensin (0,5 μM; Sigma, Zwijndrecht, The Netherlands), cells were collected, divided into two portions and stained with RPE-Cy5 anti-CD4+ or anti-CD8+ (DAKO, Glostrup, Denmark) for 1 hour on ice. After washing twice with PBS, cells were fixed in 4% paraformaldehyde for 20 minutes on ice and washed subsequently twice with 0,5% glycine. Cells were then permeabilised with 0.1% saponin in PBS supplemented with 1% normal mouse serum and 5% foetal calf serum. After permeabilising the cells were stained with anti-IFN-γ FITC (DAKO, Glostrup, Denmark) and IL-4-PE (Pharmingen) for 30 minutes on ice. Fluorescence was measured by FACS analysis. Cells were gated on FSC/SSC profile and CD4/CD8 expression. Proportions of CD4+ or CD8+ cells expressing IFN-γ and IL-4 were measured using quadrant markers. Markers were set using the medium control to define 2% IFN-γ and IL-4 positive cells as shown in Figure 5.1.1. Validity of detection of intracellular of IFN-γ and IL-4 was tested using a measles virus specific CD8+ clone known to produce both of IFN-γ and IL-4 and an RSV specific CD8+ T cell clone known to produce IFN-γ.

Statistical analyses

Differences in number of cells, levels of soluble cytokines and percentages of cells expressing IFN-γ between groups of different clinical severity were tested using Mann-Whitney U test (MWU test). For testing of the relation between levels of cytokines in plasma and number of positive cells in immune histochemistry staining Kruskall Wallis one way anova was used.

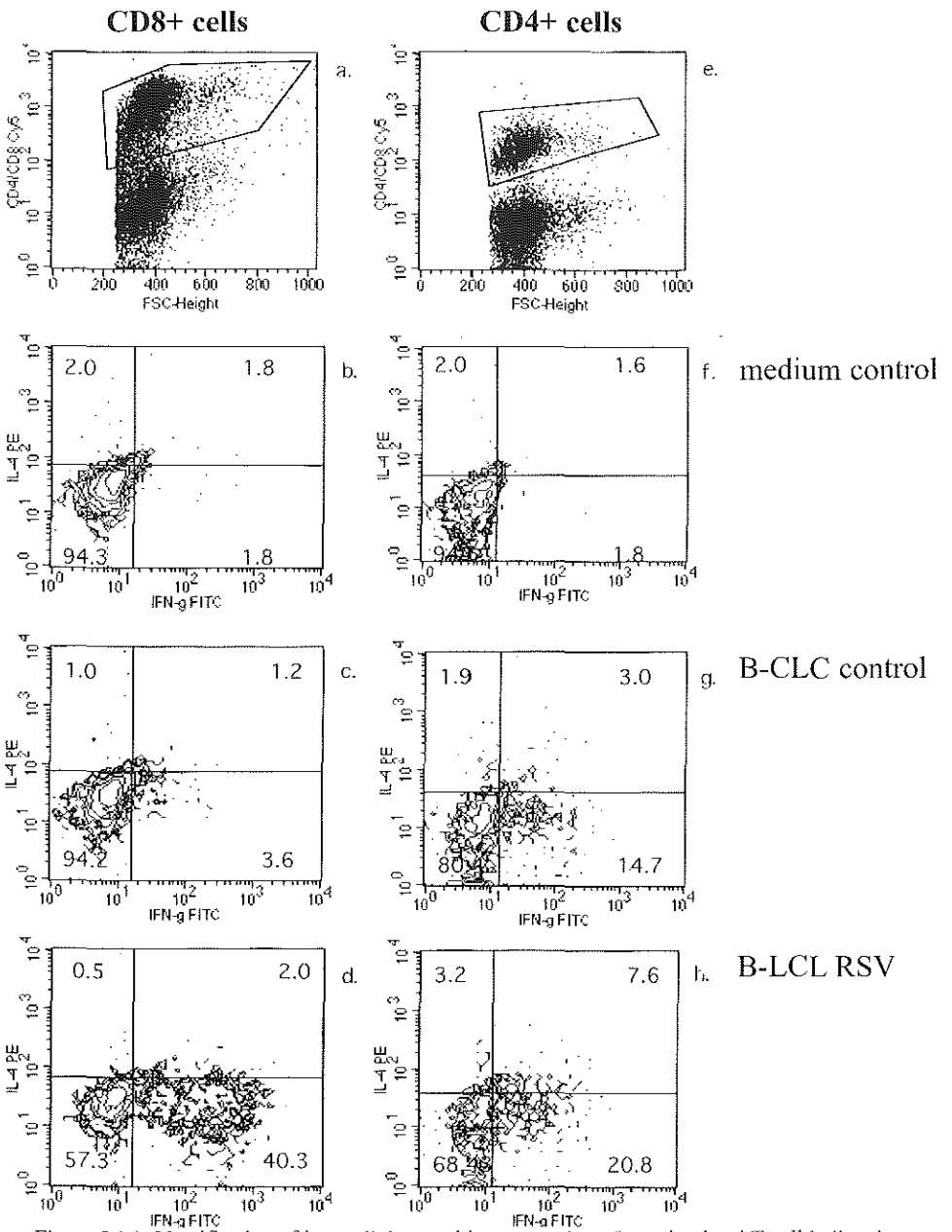

Figure 5.1.1. Identification of intracellular cytokine expression of prestimulated T cell bulk culture from convalescent PBMCs sample of patient no. 5. T cells were restimulated with RSV (d, h), control B-LCL (c, g) and medium control (b, f). Samples were triple stained with either anti-CD8 RPE-Cy5 anti-IFN-γ FITC, IL-4-PE (a-d) or anti-CD4 RPE-Cy5, anti-IFN-γ FITC, IL-4-PE (e-h). Cells were gated on FSC/SSC profile and CD8 (a) or CD4 (e) expression in the medium control. Quadrant markers were set using the medium control defining 2% IFN-γ and IL-4 positive cells. Proportions of CD4+ or CD8+ cells expressing IFN-γ and IL-4 were measured using the same quadrant markers. Percentages of cells in the quadrants are indicated in the figures.

Table 5.1.1. Clinical and virological data of selected patients for detailed T cell responses

Patient No.	Age in days	VI[a]	admission	Artificial ventilation	Respiratory rate/min	pCO_2 (kPa)	SaO_2 (%)	Wheezing	Apnea's	Abnormality on X-ray
Severe RSV infection										
1.	68	RSV	ICUb	yes	ND[c]	10.7	ND	no	no	yes
2.	96	RSV	Normal ward	no	70	6.9	93	yes	yes	yes
3.	65	RSV	ICU	yes	50	7.7	70	no	yes	yes
4.	60	RSV	ICU	no	64	8.1	87	yes	yes	yes
5.	44	RSV	ICU	yes	30	12.2	82	no	yes	yes
6.	59	RSV	ICU	no	30	11.3	ND	no	no	no
7.	198	RSV	Normal ward	no	40	5.0	93	yes	yes	yes
Mild RSV infection										
8.	198	RSV	Not admitted	no	40	ND	ND	no	no	ND
9.	98	RSV	Not admitted	no	32	4.5	96	yes	no	yes
10.	79	RSV	Not admitted	no	56	5.0	96	no	no	no
11.	79	RSV	Not admitted	no	72	5.2	95	no	no	yes
12.	47	RSV	Not admitted	no	72	5.9	98	no	no	ND
13.	161	RSV	Normal ward	no	30	4.8	ND	no	no	ND
RSV negative										
14.	71	negative	Normal ward	no	40	4.7	100	yes	yes	ND
15.	47	pertussis	ICU	no	48	6.3	ND	no	no	no

a: VI = virus isolation
b: ICU = intensive care unit
c: ND = no data recorded or no data available before start of oxygen therapy and/or artificial ventilation

Results

Patients

Of the 111 children included in the study 95 had a RSV infection as diagnosed by direct antigen detection and virus isolation. Of the 16 RSV negative children one suffered from adenovirus infection and one from a *Bordetella pertussis* infection. No causative agent for the other 14 could be identified. Of the RSV positive children fifty were defined as having a severe disease and 45 as having a mild disease by the predetermined criteria. Of the RSV positive children 60 were male and 35 were female. One child had a bronchopulmonary dysplasia, seven had an underlying heart abnormality and four children were born at 33 weeks gestation or less. In 84 children no underlying risk factors were identified. Seventeen needed no hospital admission, 41 were admitted to the medium care unit and 36 were admitted

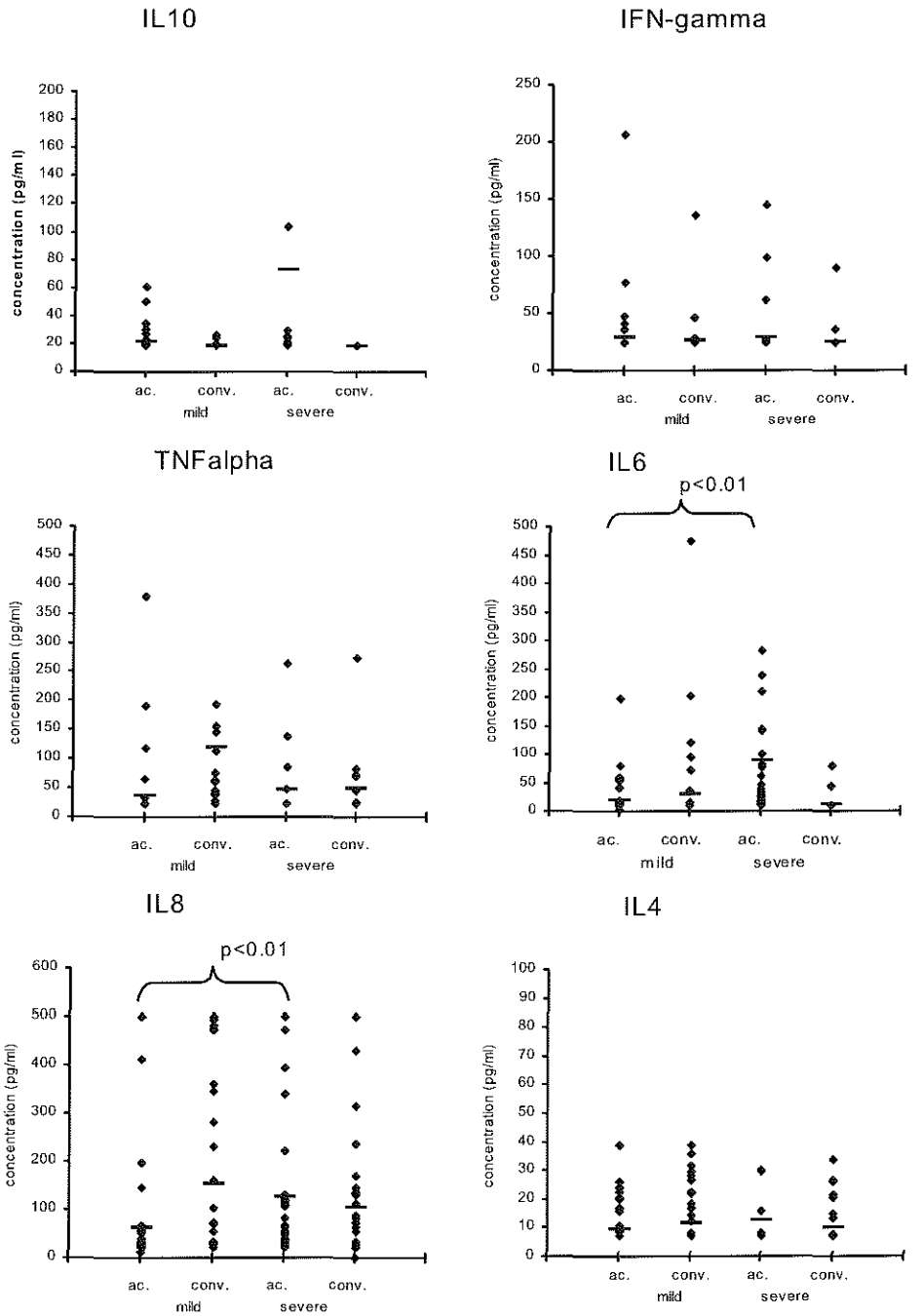

Figure 5.1.2. Plasma concentrations of cytokines in patients with mild vs. severe RSV infection during the acute (ac.) and convalescent (conv.) stage of disease. Individual and mean levels are shown. Levels of IL-6 and IL-8 in the acute phase were significantly higher in the severe group using MWU test.

to the intensive care unit. Of the children admitted to the intensive care twelve needed mechanical ventilation. Twenty-seven children had one or more apnoeas either before or during admission whereas 68 had no history of apnoea.
Plasma levels of cytokines and numbers of positive staining cells in nasopharyngeal washings were compared between RSV positive and negative patients and in RSV positive patients between those with severe versus mild disease.
For characterisation of RSV specific T cell responses fifteen patients without underlying risk factors were selected and matched for age. Seven had a severe RSV infection, six had a mild RSV infection and two had a non-RSV related respiratory infection. Detailed clinical and virological data of these fifteen children are shown in Table 5.1.1.

Cytokines in plasma samples
Plasma levels of cytokines related to a Type 1 or 2 response were either low or undetectable (Figure 5.1.2.). IL-2 and IL-5 could not be detected in any of the plasma samples tested. IL-4 was detected in 18 out of 95 (19%) RSV positive children and one out of 16 (6%) RSV negative children in the acute phase and 22 out of 88 (25%) RSV positive and one out of 14 (7%) RSV negative children in the convalescent phase. IFN-γ was detected in nine out of 93 (10%) RSV positive children and three out of 16 (19%) RSV negative children in the acute phase and five out of 83 (6%) RSV positive and none of the RSV negative children in the convalescent phase. IL-10 was detected in 23 out of 95 (24%) RSV positive children and one out of 16 (6%) RSV negative children in the acute phase and three out of 87 (3%) of RSV positive and none of the RSV negative children in the convalescent phase. Statistically significant differences using MWU test, were not found between RSV positive and negative patients or between RSV positive patients with infection of different clinical severity for plasma levels of cytokines related to a Type 1 or Type 2 response (Figure 5.1.2.). Also no statistically significant differences were found in MWU test when children with underlying risk factors were excluded and when SaO_2, pCO_2, or artificial ventilation was used as the single parameter for clinical severity and also when admission to hospital or admission to the intensive care unit was used as a parameter for clinical severity.
IL-6 was detected in 44 out of 93 (48%) RSV positive children and three out of 16 (19%) RSV negative children in the acute phase and ten out of 84 (12%) RSV positive and two out of 14 (14%) RSV negative children in the convalescent phase. IL-8 was detected in 68 out of 95 (72%) RSV positive children and five out of 15 (33%) RSV negative children in the acute phase and 46 out of 87 (53%) RSV positive and six out of 14 (45%) RSV negative children in the convalescent phase. TNF-α was detected in ten out of 89 (11%) RSV positive children and one out of 15 (7%) RSV negative children in the acute phase and 21 out of 80 (26%) RSV positive and two out of 12 (16%) RSV negative children in the convales-

cent phase. No statistical differences were found between RSV positive and negative children. For RSV positive children IL-6 and IL-8 were found more often in children with more severe disease during the acute phase ($p<0.01$ in MWU test). This finding was confirmed when children with underlying risk factors were excluded and when SaO_2, pCO_2, or artificial ventilation was used as the single parameter for clinical severity and also when admission to hospital or admission to the intensive care unit was used as a parameter for clinical severity. During the convalescent phase and for TNF-α both during the acute and convalescent phase no differences in plasma levels were found (Figure 5.1.2.).

Cells from nasopharyngeal washings

Multispot slides from nasopharyngeal cells obtained in the acute phase of the infection were available from 68 of patients. Of these 59 were RSV positive and 9 were RSV negative. No statistical differences in numbers of inflammatory cells were found between RSV infected and RSV negative children. Results from immune histochemical staining in RSV positive children with severe vs. mild infection are shown in Table 5.1.2.

The cellular infiltrates in RSV infected patients consisted mainly of polymorphonuclear cells (CD15+) and monocytes (CD14+). In the slides for mildly ill patients more BMK-13 positive (eosinophilic granulocytes) and IgE positive cells were found. This was not confirmed however, when SaO_2, pCO_2, or artificial ventilation was used as the single parameter for clinical severity or when hospital admission or admission to the intensive care unit was used as a parameter for clinical severity.

In part of the slides high numbers of cells stained positive for eosinophilic cathionic protein usually considered an eosinophilic marker, whereas only low numbers of BMK-13 positive cells were found. These eosinophilic cathionic protein positive cells were not related to more severe clinical disease and were also not related to the presence of wheezing. In the 26 children with wheezing a median number of 66 eosinophilic cathionic protein positive cells (range 0 – 680) was found. In the 37 children without wheezing a median number of 43 eosinophilic cathionic protein positive cells (range 0 – 680) was found, $p=0.86$ in MWU test.

Comparison of numbers of positive staining cells with plasma levels of IL-6 and IL-8 using Kruskall-Wallis one-way ANOVA showed no relationship.

RSV specific T cell responses

In the fifteen patients selected for characterisation of RSV specific T cell response IFN-γ, IL-4 and IL-10 production in culture supernatants (Figure 5.1.3.) and intracellular expression of IFN-γ and IL-4 (Figure 5.1.4.) were measured upon re-stimulation with RSV infected and control B-LCL in pre-stimulated PBMCs bulk cultures established from samples col-

lected in the acute and the convalescent phases.

RSV-induced IFN-γ was found in the culture supernatant of six of the 13 RSV positive patients in the acute phase (patients 3, 5, 6 and 7 with severe and patients 8 and 11 with mild symptoms) and twelve of 13 patients in the convalescent phase (all seven with severe and patients 8, 10, 11, 12 and 13 with mild symptoms). In patient 9 RSV induced IFN-γ was detected in the culture of the acute phase but not in that of the convalescent phase. In the latter, however, a relatively high background was found upon stimulation with non-infected cells. IL-4 and IL-10 were demonstrated in the culture supernatants at much lower concentrations than IFN-γ. RSV-induced IL-4 above 10 pg/ml was found in one patient (patient 8 with mild symptoms) in the acute phase and in four patients (patients 1, 3, 7 with severe, and patient 8 with mild symptoms) in the convalescent phase. RSV-induced IL-10 above 10 pg/ml was found in 2 patients (patients 8 and 11, both with mild symptoms) in the acute phase and in seven patients (patients 1, 3, 5 and 7 with severe, and patients 8, 11 and 13 with mild symptoms) in the convalescent phase. In the culture of one of the control patients RSV-induced IFN-γ was detected at both time points in low concentrations.

A representative example of a FACS analysis for RSV-induced intracellular IFN-γ and IL-4 is shown in Figure 5.1.1. No RSV specific IL-4 was measured above medium control background in any of these samples, whereas IL-4 is detectable in T cell clones using this method. Results of RSV-induced intracellular IFN-γ expression in acute and convalescent phase samples of all 15 patients studied are shown in Figure 5.1.4. Proportions of RSV-induced IFN-γ positive cells were higher in the CD8+ than in the CD4+ cells. RSV-induced IFN-γ in CD4+ cells was found in four of the 13 patients in the acute phase (patient 5 with severe, and patients 8, 9 and 11 with mild symptoms) and in eleven of the 13 patients in the convalescent phase (patients 1, 2, 4, 5, 6 and 7 with severe, and patients 8, 9, 10, 11 and 12 with mild symptoms). IFN-γ in CD8+ cells was found in six of the 13 patients (patients 6 and 7 with severe, and patient 8, 9, 11 and 12 with mild symptoms) in the acute phase and in all 13 patients in the convalescent phase.

No statistical differences in cytokine concentrations in culture supernatants or proportions of RSV induced IFN-γ positive cells were found between the mild and severe group in the acute or in the convalescent phase.

Discussion

Virus specific T cell responses have been shown to be involved in the clearance of RSV infection, but also in the induction of immune pathology (1,7,9,31,43). In the mouse model this immune pathology is associated with an eosinophilic lung infiltrate and a Type 2 mediated T help response. Young children are prone to develop a type 2 immune response upon infection (11,33) and higher levels of Type 2 cytokines have been found in children with a

Table 5.1.2. Number of inflammatory cells/1000 counted cells found in nasopharyngeal washings of children with RSV infection

	Mild (n=21) median (range)	Severe[a] (n=37) median (range)	P[b]
CD3 (T cells)	30 (4 – 94)	21 (5 – 140)	0.22
CD14 (monocytes)	44 (0 – 156)	57 (0 – 249)	0.78
CD15 (PMN)	325 (10-900)	300 (0 – 900)	0.81
IgE	7 (0 – 64)	1 (0 – 42)	0.04[d]
BMK13 (eosinophils)	1.5 (0 – 9)	0.5 (0 – 11)	0.02[d]
Tryptase (mast cells)	0 (0 – 9)	0 (0 – 32)	0.12
Eg2 (ECP)[c]	42 (0 – 241)	66 (0 – 680)	0.56
CD8	0 (0 – 19)	0 (0 – 23)	0.39

a Severe RSV infection was defined as meeting one or more of the following criteria: pCO_2 > 6,6kPa, SaO_2 < 90%, artificial ventilation.

b p value as tested in Mann-Whitney U test;

c eosinophilic cathionic protein.

d indicates significant difference

RSV infection (34,37). In those studies, however, a comparison was made with healthy non-infected children. In the present study it was shown that infants with clinically more severe RSV infection have higher levels of IL-6 and IL-8 in their plasma, indicating more inflammation in the lungs, but they do not exhibit a more pronounced RSV-specific Type 2 like T cell response than infants with mild RSV infection. Only few eosinophilic granulocytes were detected in nasopharyngeal washings and a Type 1-like T cell response characterised by the expression of mainly IFN-γ and only low levels of IL-4 and IL-10, were found in the supernatants of PBMCs cultures from both groups of infants after RSV-specific stimulation. Intracellular detection of cytokines confirmed these findings. IFN-γ expression proved to be more prominent in CD8+ than in CD4+ cells upon RSV-specific stimulation. No RSV induced expression of IL-4 could be demonstrated while it was measurable in low levels in the culture supernatants. Apparently the intracellular measurement of cytokines, as standardised for measles and RSV-specific T cell clones, was not sensitive enough for the detection of these low levels in the PBMCs bulk cultures.

For the characterisation of specific T cell immunity in these infants we have used a virus specific pre-stimulation protocol to expand the virus-specific T cells in the peripheral blood. Although it may be argued that such a protocol can influence the ratios of the respective cytokines expressed, it is known that Type 2 responses in PBMCs for certain antigens can

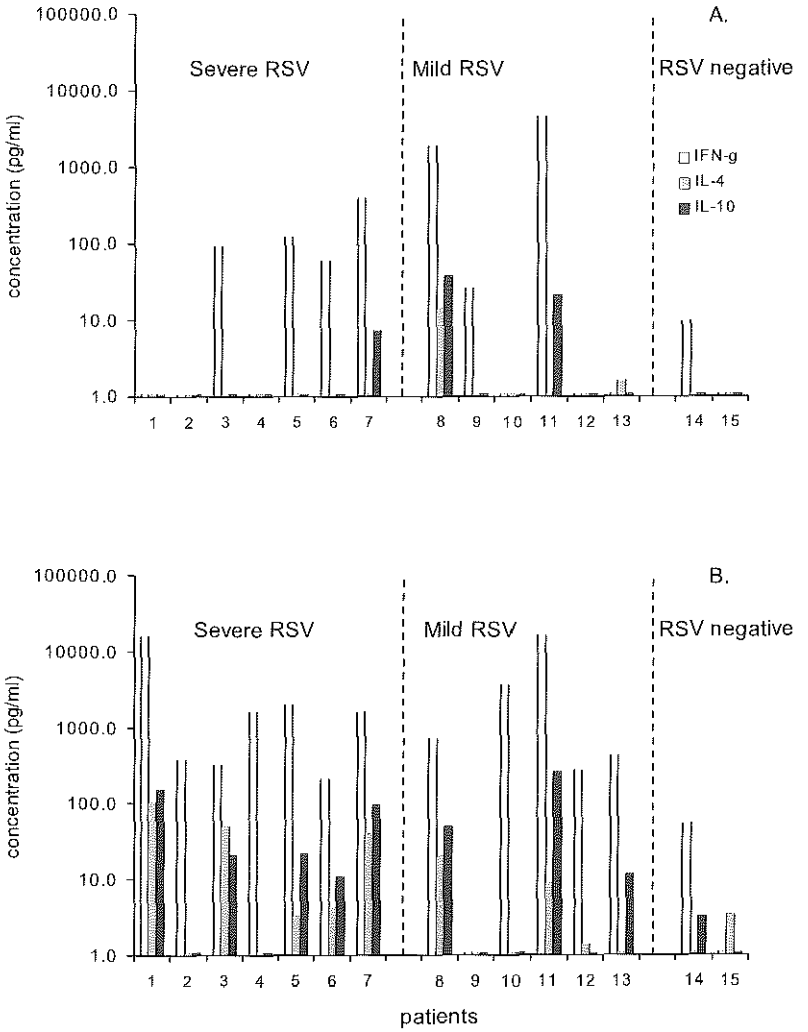

Figure 5.1.3. Concentrations of RSV specific IFN-γ (white), IL-4 (grey) and IL-10 (black) measured in culture supernatants of pre-stimulated T cell bulk cultures of infants with a recent RSV infection of different clinical severity. Samples were from the acute phase (A) and the convalescent phase (B). RSV-specific cytokine production was determined by subtracting the cytokine production in wells stimulated with control B-LCL from the cytokine production in the wells stimulated with RSV infected B-LCL.

only be measured using restimulation protocols that allow for the expansion of T cells (21,28) and that Th1/Th2 responses measured in this way do show a relation to clinical response (12,23,36). In the severely ill patients a less pronounced T cell response was found in the acute phase. This is compatible with the findings of De Weerd et al. (10) that T cells

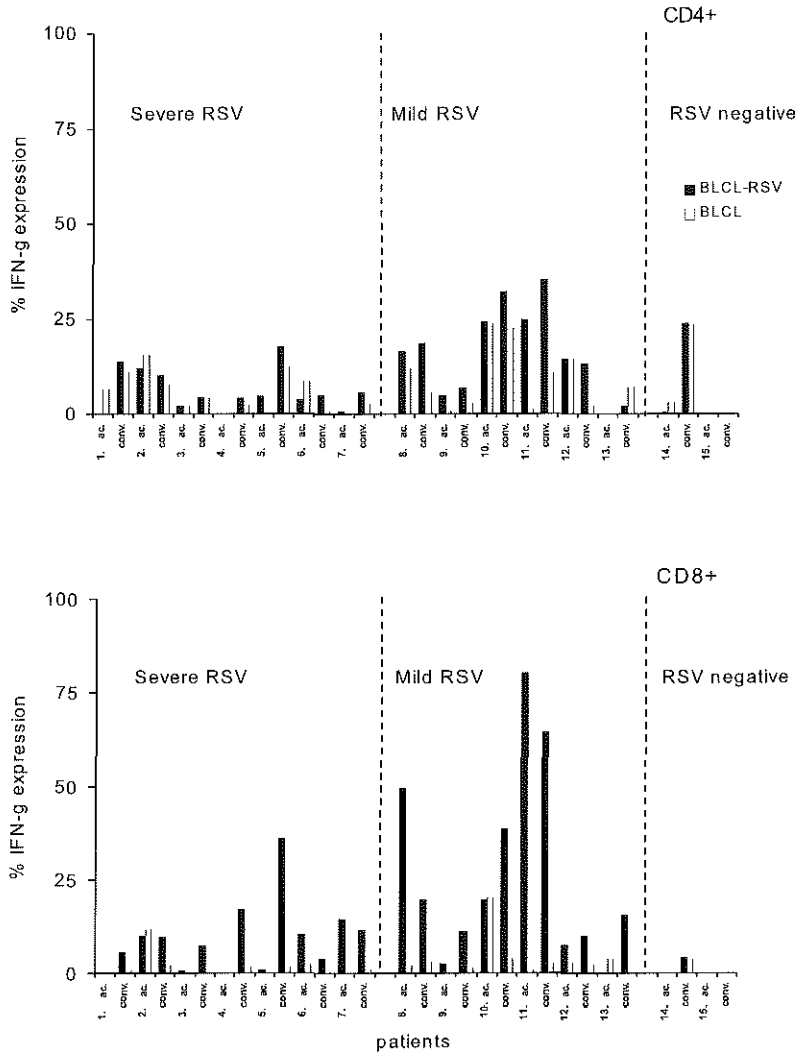

Figure 5.1.4. Proportions of intracellular IFN-γ expression, as found in FACS analysis, in pre-stimulated T cell bulk cultures of infants with a recent RSV infection of different clinical severity. Samples from the acute phase (1) and the convalescent phase (2) were stimulated with RSV infected (black) B-LCL or control B-LCL (white).

are relatively depleted from the peripheral blood in the acute phase and may be accumulated in the respiratory tract.

Epithelial cells and also polymorphonuclear cells have been shown to produce IL-6 and IL-8 upon infection with RSV (2,3,5,26). Our findings that infants with more severe disease have higher levels of IL-6 and IL-8 in their plasma suggests that these infants had more extensive infection and more general inflammation in their respiratory tract. Our data are in

agreement with the recently published data of Bont et al. (6) who found higher plasma IL-8 levels a marker for severe disease. The cellular infiltrates of the nasopharyngeal washings mainly consisted of polymorphonuclear cells and monocytes. This is consistent with the findings of Everard et al. (13) who identified cells in bronchoalveolar lavage fluids and nasopharyngeal washings of RSV infected children. Eosinophilic granulocytes and IgE expressing cells were found more frequently in infants with milder disease (Table 5.1.1.). However, the overall numbers of eosinophils and IgE positive cells were very low and when other criteria for clinical severity were tested, like pCO_2 or artificial ventilation as single determinant or hospital admission no statistical differences were found for these cells. Therefore we conclude that there is no relationship between these parameters and disease severity.

Surprisingly in part of the nasopharyngeal washings relatively high numbers of cells stained positive for eosinophilic cathionic protein, that is known as an activation marker of eosinophilic granulocytes, whereas only few eosinophils were detected in the slides. It has, however, been documented that not only eosinophils but also neutrophilic granulocytes can express eosinophilic cathionic protein (38), that may therefore have been the source for the eosinophilic cathionic protein found. Although higher levels of soluble eosinophilic cathionic protein in nasopharingeal samples have been associated with RSV bronchiolitis and wheezing (15,22), these eosinophilic cathionic protein expressing cells could not be related to clinical severity or wheezing in this study.

In RSV infection there is a wide range of clinical appearance. So using a simple definition of mild versus severe disease may cause considerable overlap between the two groups. Also pre-existing risk factors, like prematurity or cardiopulmonary disease increase the chance of a severe clinical outcome and may influence the analysis. Therefore the data were also analysed excluding children with underlying illnesses, and other parameters related to clinical severity were tested, like need for hospital admission, intensive care unit admission or artificial ventilation, that gave the same results.

For the detailed *in vitro* analysis of RSV-specific T cell responses patients without any of the pre-existing risk factors were selected. Also, because the age at which the first RSV infection takes place may influence clinical severity, the patients selected for these studies were matched for age between the two groups of different clinical severity (Table 5.1.1.). The data of these studies show that, although low levels of Type 2-like cytokines were found in some infants, a predominant Type 1-like RSV-induced T cell response was present in cultures from infants irrespective of the clinical severity of the disease. Genetic variability both in the virus and the host may influence the analysis of the immune response in RSV infection. In the mouse model the Type 2 T cell response inducing lung eosinophilia has been associated with an eleven amino acid domain on the G protein. This response is influenced

by the genetic background and the route of exposure to the antigen (39).

Taken together these data do not support the hypothesis that severe RSV infection is associated with a Type 2-like T cell response in naturally occurring RSV bronchiolitis.

References

1. Alwan, W. H., F. M. Record, and P. J. M. Openshaw. 1992. CD4+ T cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T cells. Clin. Exp. Immunol. 88:527-536.
2. Arnold, R., B. Humbert, H. Werchau, H. Gallati, and W. Konig. 1994. Interleukin-8, interleukin-6, and soluble tumour necrosis factor receptor type I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. Immunology 82:126-133.
3. Arnold, R., B. König, H. Galatti, H. Werchau, and W. König. 1995. Cytokine (IL-8, IL-6, TNF-α) and soluble TNF receptor-I release from human peripheral blood mononuclear cells after respiratory syncytial virus infection. Immunology 85:364-372.
4. Bangham, C. R. and A. J. McMichael. 1986. Specific human cytotoxic T cells recognize B-cell lines persistently infected with respiratory syncytial virus. Proc. Natl. Acad. Sci. U. S. A. 83:9183-9187.
5. Becker, S., H. S. Koren, and D. C. Henke. 1993. Interleukin-8 expression in normal nasal epithelium and its modulation by infection with respiratory syncytial virus and cytokines tumor necrosis factor, interleukin-1, and interleukin-6. Am. J. Respir. Cell Mol. Biol. 8:20-27.
6. Bont, L., C. J. Heijnen, A. Kavelaars, W. M. van Aalderen, F. Brus, J. T. Draaisma, S. M. Geelen, H. J. van Vught, and J. L. Kimpen. 1999. Peripheral blood cytokine responses and disease severity in respiratory syncytial virus bronchiolitis. Eur. Respir. J. 14:144-149.
7. Cannon, M. J., P. J. M. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168:1163-1168.
8. Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette. 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. 89:449-463.
9. Connors, M., A. B. Kulkarni, C.-Y. Firestone, K. L. Holmes, H. C. Morse III, A. V. Sotnikov, and B. R. Murphy. 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J. Virol. 66:7444-7451.
10. De Weerd, W., W. N. Twilhaar, and J. L. Kimpen. 1998. T cell subset analysis in peripheral blood of children with RSV bronchiolitis. Scand. J. Infect. Dis. 30:77-80.
11. Delespesse, G., L. P. Yang, Y. Ohshima, C. Demeure, U. Shu, D. G. Byun, and M. Sarfati. 1998. Maturation of human neonatal CD4+ and CD8+ T lymphocytes into Th1/Th2 effectors. Vaccine 16:1415-1419.
12. Evans, T. G., T. Fitzgerald, D. C. Gibbons, M. C. Keefer, and H. Soucier. 1998. Th1/Th2 cytokine responses following HIV-1 immunization in seronegative volunteers. The AIDS Vaccine Evaluation Group. Clin. Exp. Immunol. 111:243-250.
13. Everard, M. L., A. Swarbrick, M. Wrightham, J. McIntyre, C. Dunkley, P. D. James, H. F. Sewell, and A. D. Milner. 1994. Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection. Arch. Dis. Child 71:428-432.
14. Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn. 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aquaeous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. 89:435-448.
15. Garofalo, R., J. L. Kimpen, R. C. Welliver, and P. L. Ogra. 1992. Eosinophil degranulation in the respiratory tract during naturally acquired respiratory syncytial virus infection. J. Pediatr. 120:28-32.
16. Glezen, W. P., A. Paredes, J. E. Allison, L. H. Taber, and A. L. Frank. 1981. Risk of respiratory syncytial virus infection for infants from low- income families in relationship to age, sex, ethnic group, and maternal antibody level. J. Pediatr. 98:708-715.
17. Green, M., A. F. Brayer, K. A. Schenkman, and E. R. Wald. 1989. Duration of hospitalization in pre-

viously well infants with respiratory syncytial virus infection. Pediatr. Infect. Dis. J. 8:601-605.
18. Groothuis, J. R., K. M. Gutierrez, and B. A. Lauer. 1988. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 82:199-203.
19. Hall, C. B., W. J. Hall, and D. M. Speers. 1979. Clinical and physiological manifestations of bronchiolitis and pneumonia. Outcome of respiratory syncytial virus. Am. J. Dis. Child 133:798-802.
20. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. New England J. Med. 315:77-81.
21. Imada, M., F. E. Simons, F. T. Jay, and K. T. HayGlass. 1995. Antigen mediated and polyclonal stimulation of human cytokine production elicit qualitatively different patterns of cytokine gene expression. Int. Immunol. 7:229-237.
22. Ingram, J. M., G. P. Rakes, G. E. Hoover, T. A. Platts-Mills, and P. W. Heymann. 1995. Eosinophil cationic protein in serum and nasal washes from wheezing infants and children. J. Pediatr. 127:558-564.
23. Kallas, E. G., D. C. Gibbons, H. Soucier, T. Fitzgerald, J. J. Treanor, and T. G. Evans. 1999. Detection of intracellular antigen-specific cytokines in human T cell populations. J. Infect. Dis. 179:1124-1131.
24. Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart. 1969. 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. 89:405-421.
25. Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. E. Jensen, and R. H. Parrott. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89:422-434.
26. Konig, B., T. Krusat, H. J. Streckert, and W. Konig. 1996. IL-8 release from human neutrophils by the respiratory syncytial virus is independent of viral replication. J. Leukoc. Biol. 60:253-260.
27. MacDonald, N. E., C. B. Hall, S. C. Suffin, C. Alexson, P. J. Harris, and J. A. Manning. 1982. Respiratory syncytial viral infection in infants with congenital heart disease. N. Engl. J. Med. 307:397-400.
28. Marshall, J. D., Y. Wen, J. S. Abrams, and D. T. Umetsu. 1993. In vitro synthesis of IL-4 by human CD4+ T cells requires repeated antigenic stimulation. Cell Immunol. 152:18-34.
29. Martin, A. J., P. S. Gardner, and J. McQuillin. 1978. Epidemiology of respiratory viral infection among paediatric inpatients over a six-year period in north-east England. Lancet 2:1035-1038.
30. Navas, L., E. Wang, V. de Carvalho, and J. Robinson. 1992. Improved outcome of respiratory syncytial virus infection in a high- risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J. Pediatr. 121:348-354.
31. Openshaw, P. J. 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am. J. Respir. Crit. Care Med. 152:S59-S62.
32. Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98:289-300.
33. Prescott, S. L., C. Macaubas, B. J. Holt, T. B. Smallacombe, R. Loh, P. D. Sly, and P. G. Holt. 1998. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. J. Immunol. 160:4730-4737.
34. Roman, M., W. J. Calhoun, K. L. Hinton, L. F. Avendano, V. Simon, A. M. Escobar, A. Gaggero, and P. V. Diaz. 1997. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am. J. Respir. Crit. Care Med. 156:190-195.
35. Rothbarth, P. H., J. J. Habova, and N. Masurel. 1988. Rapid diagnosis of infections caused by respiratory syncytial virus. Infection 16:252
36. Secrist, H., C. J. Chelen, Y. Wen, J. D. Marshall, and D. T. Umetsu. 1993. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. J. Exp. Med. 178:2123-2130.
37. Smyth, R. L., J. N. Fletcher, H. M. Thomas, and C. A. Hart. 1997. Immunological responses to respiratory syncytial virus infection in infancy. Arch. Dis. Child 76:210-214.
38. Sur, S., D. G. Glitz, H. Kita, S. M. Kujawa, E. A. Peterson, D. A. Weiler, G. M. Kephart, J. M. Wagner, T. J. George, G. J. Gleich, and K. M. Leiferman. 1998. Localization of eosinophil-derived neurotoxin

and eosinophil cationic protein in neutrophilic leukocytes. J. Leukoc. Biol. 63:715-722.
39. Tebbey, P. W., M. Hagen, and G. E. Hancock. 1998. Atypical pulmonary eosinophilia is mediated by a specific amino acid sequence of the attachment (G) protein of respiratory syncytial virus. J. Exp. Med. 188:1967-1972.
40. van Binnendijk, R. S., M. C. Poelen, P. de Vries, H. O. Voorma, A. D. Osterhaus, and F. G. UytdeHaag. 1989. Measles virus-specific human T cell clones. Characterization of specificity and function of CD4+ helper/cytotoxic and CD8+ cytotoxic T cell clones. J. Immunol. 142:2847-2854.
41. van Binnendijk, R. S., J. P. Versteeg-van Oosten, M. C. Poelen, H. F. Brugghe, P. Hoogerhout, A. D. Osterhaus, and F. G. UytdeHaag. 1993. Human HLA class I- and HLA class II-restricted cloned cytotoxic T lymphocytes identify a cluster of epitopes on the measles virus fusion protein. J. Virol. 67:2276-2284.
42. Verjans, G. M., L. Remeijer, R. S. van Binnendijk, J. G. Cornelissen, H. J. Volker-Dieben, S. G. Baarsma, and A. D. Osterhaus. 1998. Identification and characterization of herpes simplex virus-specific CD4+ T cells in corneas of herpetic stromal keratitis patients. J. Infect. Dis. 177:484-488.
43. Waris, M. E., C. Tsou, D. D. Erdman, S. R. Zaki, and L. J. Anderson. 1996. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J. Virol. 70:2852-2860.

5.2 HLA class I-restricted cytotoxic T cell epitopes of the respiratory syncytial virus fusion protein

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Summary

Virus-specific cytotoxic T lymphocytes (CTL) play a major role in the clearance of respiratory syncytial virus (RSV) infection. We have generated cytotoxic T-cell clones (TCC) from two infants who had just recovered from severe RSV infection. These TCC were functionally characterized and used to identify HLA class I (B57 and C12)-restricted CTL epitopes of RSV.

Respiratory syncytial virus (RSV) is a common cause of upper respiratory tract infections, but may – especially in young infants, the elderly, or immunocompromised individuals – cause severe lower respiratory tract infections (12). In rodent models CD4+, as well as CD8+, RSV-specific T lymphocytes proved to be involved in both recovery from and immune pathogenesis of the infection (1,2,8,19). Therefore, a fine balance must exist in these models between protective and disease-enhancing effects of virus-specific T lymphocytes. RSV-specific CD8+ cytotoxic T lymphocytes (CTL) against virtually all RSV proteins have been demonstrated to circulate in humans after RSV infection (4,7,9). CTL may be expected to play a crucial role in the clearance of RSV infections, but their role in protection and immune pathology remains unclear. Therefore, the identification of CTL epitopes of human RSV may contribute to future studies concerning the role of CTL in pathogenesis and protection from RSV infection. Here we describe the functional characterization of CD8+ cytotoxic T-cell clones (TCC) generated from two infants who had just recovered from severe RSV infection. These TCC were also used to identify, for the first time, HLA class I-restricted CTL epitopes of RSV.

Peripheral blood mononuclear cells (PBMC) were collected from two infants, 4 weeks after a severe, laboratory-confirmed RSV infection. At the time of infection they were 1 and 2 months old and had both been admitted to the intensive care unit. B-lymphoblastic cell lines (B-LCL) were generated by Epstein-Barr virus transformation (22), infected with RSV A2 (ATCC VR1322), and UV irradiated (24) to serve as autologous antigen-presenting cells (APC). PBMC (3×10^4/well in 96-well round-bottom plates) were stimulated with APC (10^4/well), and expanded in RPMI 1640 medium supplemented with antibiotics, 10% pooled and heat-inactivated human serum, and recombinant human interleukin-2 (IL-2, 50 IU/ml). After 2 weeks, T cells were harvested and cloned by limiting dilution, using phytohemagglutinin stimulation as previously described (25). TCC thus generated were expanded and tested for RSV specificity by [^{3}H]thymidine incorporation assays as previously described (22). All of the RSV-specific TCC proved to be of the CD8+ phenotype in fluorescence-activated cell sorter analysis. TCC whose specificity for RSV was confirmed by a second proliferation assay were tested for protein specificity by a gamma interferon (IFN-γ) ELIspot assay (Mabtech AB, Stockholm, Sweden). In this test, paraformaldehyde-fixed autologous B-LCL that had been infected with recombinant vaccinia viruses (rVV) expressing different RSV proteins (F, G, N, P, M2, SH, M, 1B, or 1C) were used as APC. Briefly, TCC (5×10^4/well) were incubated with APC (10^4/well) for 4 h, transferred to anti-human IFN-γ coated plates, and incubated for another 18 h. The ELIspot assay was further performed in accordance to the kit manufacturer's instructions. The results are shown as numbers of IFN-γ producing cells (spots) per well.

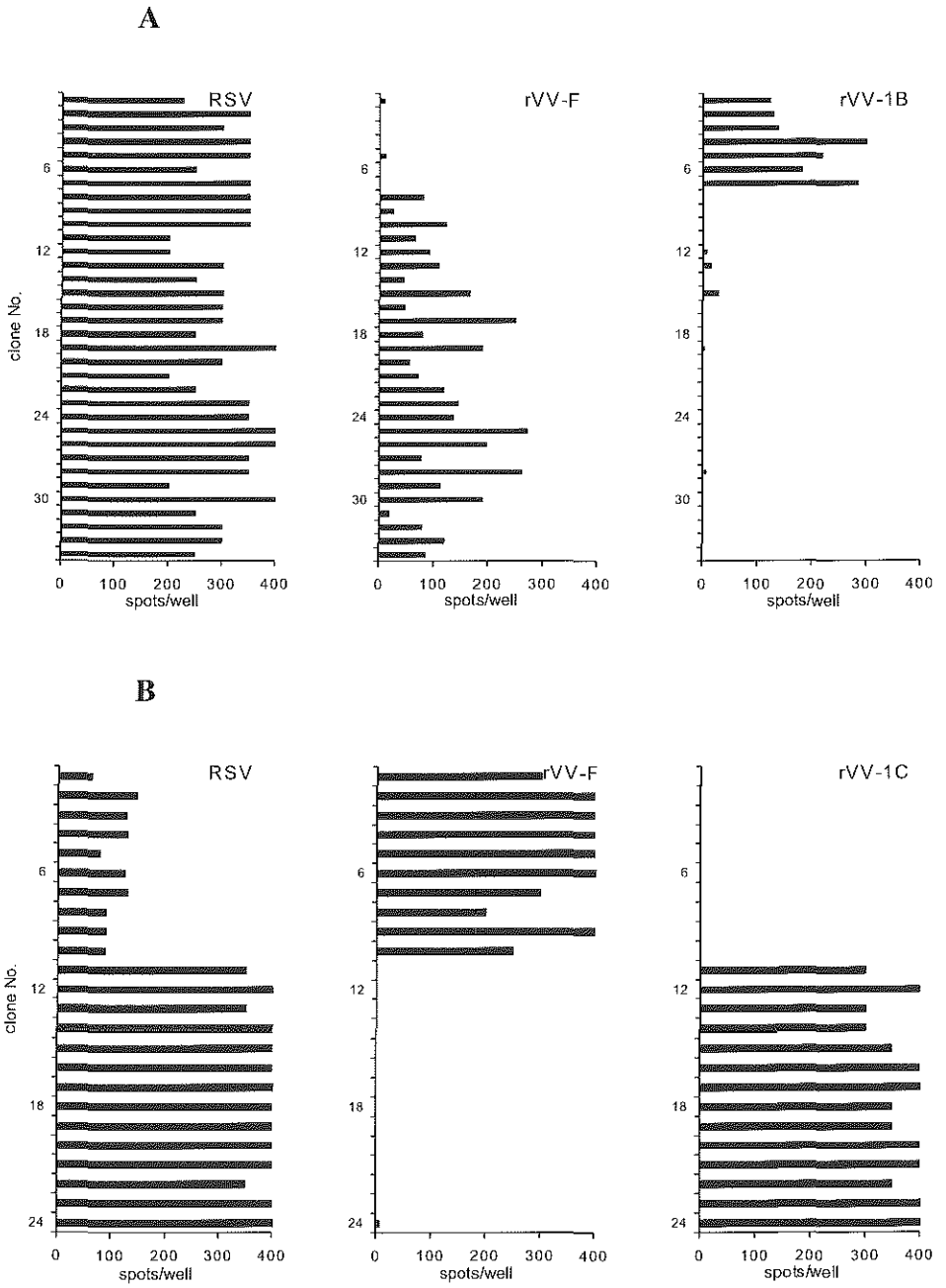

Figure 5.2.1. Responses of RSV-specific TCC from patients 1 (A) and 2 (B) measured by an IFN-γ ELIspot assay using autologous B-LCL infected with RSV A2 or rVV expressing the RSV F, 1B, or 1C protein. Results are indicated as number of spots per well. No responses against the B-LCL infected with the other rVV were found (data not shown).

Thirty-four RSV-specific TCC were generated from the PBMC of patient 1, as detected by a [^{3}H]thymidine incorporation assay. Of these, 27 proved to be RSV-F specific and 7 were RSV-1B specific in an IFN-γ ELIspot assay (Figure 5.2.1.A). Twenty-four RSV-specific TCC were generated from the PBMC of patient 2 as detected by a [^{3}H]thymidine incorporation assay. Of these, 10 were RSV-F specific and 14 proved to be RSV1C-specific in an IFN-γ ELIspot assay (Figure 5.2.1.B). None of the clones detected by the [^{3}H]thymidine incorporation assay was found to be negative by the IFN-γ ELIspot assay. Since the F protein is considered to be a major CTL target (4,7,9), further efforts were focused on the identification of CTL epitopes in the F protein. To this end, 18-mer peptide amides overlapping by 12 amino acids, together spanning the entire F protein of RSV A2 (11,18) (n = 94), were generated in an automated multiple-peptide synthesizer as previously described (26). The purity of the peptides varied between 50 and 90%, as determined by analytical reverse-phase high-performance liquid chromatography (C_8 column; gradient of 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile). Autologous B-LCL of patients 1 and 2 were pulsed overnight with 1 and 3 mM peptide, respectively. RSV F-specific TCC of patient 1 reacted with peptides 17 and 18 (Figure 5.2.2.A). All of the RSV F TCC of patient 2 reacted with peptide 91 and marginally with peptide 90 (Figure 5.2.2.B). Subsequently, two additional sets of partially overlapping 8- to 12-mer peptides (80 to 95% purity) were generated to determine the respective minimal epitopes. Autologous B-LCL of patients 1 and 2 were pulsed for 1 h with the different peptides at 1 and 3 μM respectively. All of the RSV F-specific TCC of patient 1 reacted with one nine-mer peptide (RARREL-PRF) spanning residues 118 to 126 of the F protein (Figure 5.2.2.C). The RSV F TCC of patient 2 all reacted with one nine-mer peptide (IAVGLLLYC) spanning residues 551 to 559 of the F protein (Figure 5.2.2.D).

The HLA restriction of the recognition by the RSV F TCC was also determined by the IFN-γ ELIspot assay using a set of allogeneic B-LCL with partially matched HLA types loaded with the respective minimal peptides, as shown in Figure 5.2.3. Recognition of the RSV F epitope of patient 1 proved to be HLA B57 restricted. Recognition of the epitope of patient 2 proved to be C12 restricted.

Well-growing TCC of both specificities from each of the patients were arbitrarily selected and tested for cytotoxic activity by a chromium release assay as previously described (23). Briefly, autologous ^{51}Cr-labeled, RSV-infected B-LCL and control B-LCL or, in the case of the F-specific TCC of patient 2, minimal-peptide-loaded B-LCL or control (measles virus) peptide-loaded B-LCL were incubated with TCC for 4 h at 37°C at an effector-to-target cell ratio of 10:1, which was found to be the most discriminative ratio in preliminary experiments. Spontaneous ^{51}Cr release (target cells plus medium) and maximum ^{51}Cr release (tar-

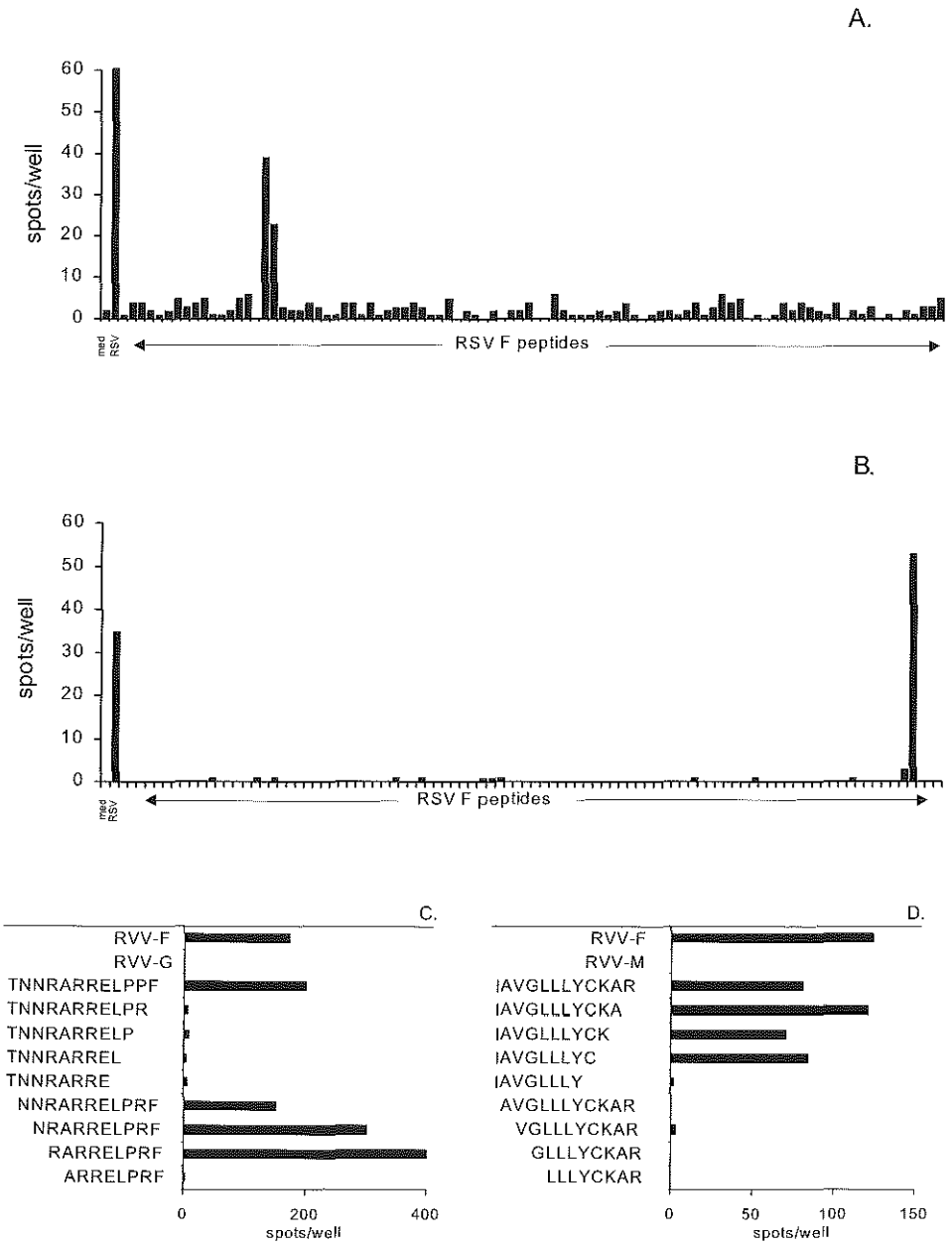

Figure 5.2.2. Fine mapping of T-cell epitopes on the RSV F protein. Responsiveness of TCC from patients 1 (A) and 2 (B) to partially overlapping 18-mer peptide amides (n = 94) spanning the F protein was measured by an IFN-γ ELIspot assay. Minimal peptide activation of TCC from patients 1 (C) and 2 (D) was measured in an IFN-γ ELIspot assay using APC pulsed with two additional sets of overlapping 8- to 12-mer peptides. med, medium.

get cells plus Triton X-100) were determined in 12 identical wells. Supernatants were harvested and analyzed in a gamma counter. Assay results were accepted only when the spontaneous-to-maximum release ratio was <25%. All of the tested TCC showed RSV-specific lysis (Figure 5.2.4.A). Virus-specific production of cytokines in cell-free culture supernatant was measured as previously described (6). TCC were stimulated with RSV-infected B-LCL or control B-LCL or, in the case of the F-specific TCC of patient 2, minimal-peptide-loaded or control B-LCL. for 48 h. The concentrations of the following cytokines were measured using commercially available sandwich enzyme-linked immunosorbent assay systems in accordance with the manufacturers' instructions: IL-4 (CLB, Amsterdam, The Netherlands; detection limit, 7 pg/ml), IFN-γ (Medgenix Diagnostics, Fleurs, Belgium; detection limit, 25 pg/ml), IL-10 (Pharmingen, San Diego, CA; detection limit, 20 pg/ml). All of the TCC produced predominantly IFN-γ, indicating a type 1 phenotype *in vitro* (Figure 5.2.4.B).

In the present study, we identified two nine-mer CTL epitopes on the RSV fusion protein. To our knowledge, these are the first HLA class I-restricted CTL epitopes described for RSV in humans. We found CTL epitopes with an HLA restriction with a low prevalence in the population. But using the techniques described here on samples of more children with a recent RSV infection, CTL epitopes with more prevalent HLA restrictions may be identified.

The TCC found in the children studied were all CD8+ CTL with a Type 1-like cytokine profile. No RSV-specific CD4+ T cells were detected in the samples of these infants, while the use of similar stimulation protocols did result in the identification of such cells in other systems (14,17,20,22). Virus-specific CTL responses play a major role in the clearance of RSV infections but may also be involved in pathogenesis (1,3,5,8,15). In mice, enhanced lung pathology induced by priming with formalin-inactivated RSV has been associated with the absence of a CTL response (13,21). In young children, a CTL response against RSV has

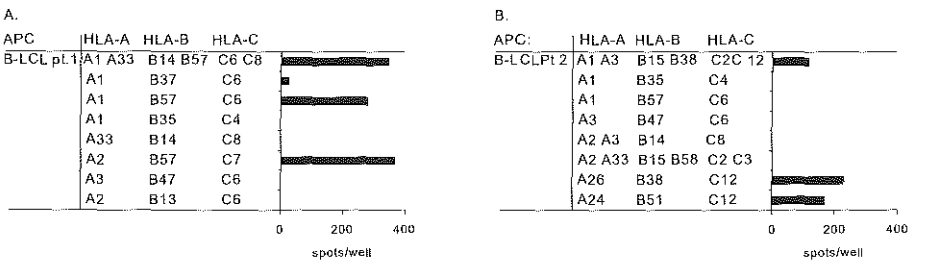

Figure 5.2.3. Determination of HLA restriction of RSV F protein recognition by specific TCC using a set of autologous and partially HLA class I- matched B-LCL for patients 1 (A) and 2 (B), by an IFN-γ ELIspot assay.

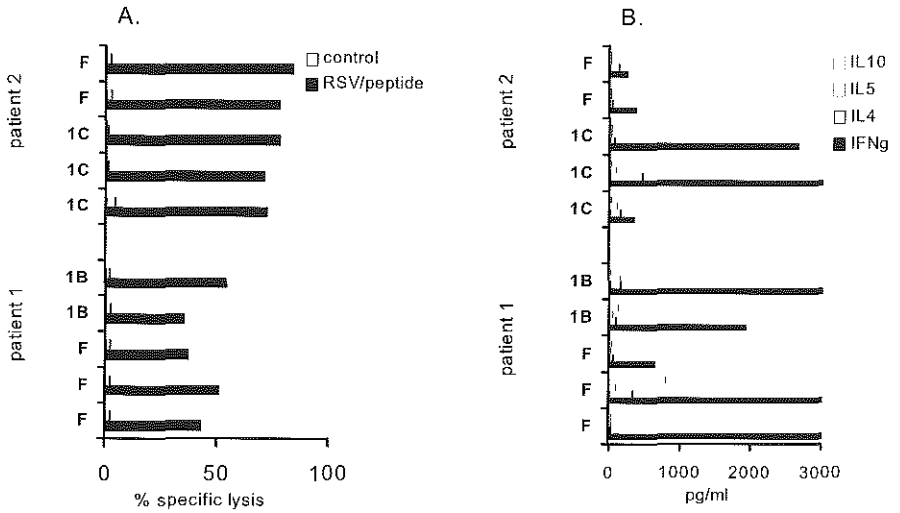

Figure 5.2.4. Cytotoxic responses (A) and cytokine production (B) of selected TCC with different protein specificities from patients 1 and 2. TCC were stimulated with autologous-RSV-infected or control B-LCL and in the case of the F-specific TCC of patient 2, minimal-peptide- or control (measles virus) peptide-loaded B-LCL. Cytotoxic responses were measured in a chromium release assay using an effector-to-target cell ratio of 10. Cytokines in cell-free culture supernatant (IFN-γ, IL-4, IL-5, and IL-10) were measured by enzyme-linked immunosorbent assay. Levels of cytokines in the supernatant of TCC stimulated with control B-LCL were all below the detection limit and are not shown.

been described but poor responses were found in younger and more severely ill patients (10,16). The poor CTL response in young children has been suggested to be one of the possible causes of more severe disease. In these two patients, it was possible to detect RSV-specific cytotoxic TCC, showing that also in very young children with clinically severe infections, priming of a CTL response does occur, although we cannot say anything about the quantitative responses.

In conclusion, use of the IFN-γ ELIspot assay to determine epitope specificity proved to be sensitive and convenient, since only small numbers of T cells and APC were needed. This and similar studies may be important for future studies concerning the role of CTL in the pathogenesis of RSV infection in children.

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References

1. Alwan, W. H., W. J. Kozlowska, and P. J. M. Openshaw. 1994. Distinct types of lung disease caused by functional subsets of antiviral T cells. J. Exp. Med. 179:81-89.
2. Alwan, W. H., F. M. Record, and P. J. M. Openshaw. 1992. CD4+ T cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T cells. Clin. Exp. Immunol. 88:527-536.
3. Bangham, C. R. and A. J. McMichael. 1986. Specific human cytotoxic T cells recognize B-cell lines persistently infected with respiratory syncytial virus. Proc. Natl. Acad. Sci. U. S. A. 83:9183-9187.
4. Bangham, C. R., P. J. Openshaw, L. A. Ball, A. M. King, G. W. Wertz, and B. A. Askonas. 1986. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoprotein (G), expressed by vaccinia virus recombinants. J. Immunol. 137:3973-3977.
5. Bangham, C. R. M., M. J. Cannon, D. T. Karzon, and B. A. Askonas. 1985. Cytotoxic T-cell response to respiratory syncytial virus in mice. J. Virol. 56:55-59.
6. Brandenburg, A. H., A. Kleinjan, B. van het Land, H. A. Moll, H. H. Timmerman, R. L. de Swart, H. J. Neijens, W. Fokkens, and A. D. M. E. Osterhaus. 2000. A type 1 like immune response is found in children with RSV infection regardless of clinical severity. J. Med. Virol. 62: (in press)
7. Cannon, M. J. and C. R. M. Bangham. 1989. Recognition of Respiratory Syncytial Virus Fusion Protein by Mouse cytotoxic T Cell Clones and a Human Cytotoxic T Cell Line. J. Gen. Virol. 70:79-87.
8. Cannon, M. J., P. J. M. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168:1163-1168.
9. Cherrie, A. H., K. Anderson, G. W. Wertz, and P. J. M. Openshaw. 1992. Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J. Virol. 66:2102-2110.
10. Chiba, Y., Y. Higashidate, K. Suga, K. Honjo, H. Tsatsumi, and P. L. Ogra. 1989. Development of cell-mediated cytotoxic immunity to respiratory syncytial virus in human infants following naturally acquired infection. J. Med. Virol. 28:133-139.
11. Collins, P. L., Y. T. Huang, and G. W. Wertz. 1984. Nucleotide sequence of the gene encoding the fusion (F) glycoprotein of human respiratory syncytial virus. Proc. Natl. Acad. Sci. U. S. A. 81:7683-7687.
12. Collins, P. L., K. Mcintosh, and R. M. Chanock. 1996. Respiratory syncytial virus, p. 1313-1351. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology. Lippincott - Raven Publishers, Philadelphia.
13. Connors, M., A. B. Kulkarni, C.-Y. Firestone, K. L. Holmes, H. C. Morse III, A. V. Sotnikov, and B. R. Murphy. 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J. Virol. 66:7444-7451.
14. Evans, T. G., T. Fitzgerald, D. C. Gibbons, M. C. Keefer, and H. Soucier. 1998. Th1/Th2 cytokine responses following HIV-1 immunization in seronegative volunteers. The AIDS Vaccine Evaluation Group. Clin. Exp. Immunol. 111:243-250.
15. Fishaut, M., D. Tubergen, and K. Mcintosh. 1980. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J. Pediatr. 96:179-186.
16. Isaacs, D., C. R. M. Bangham, and A. J. McMichael. 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet ii:769-771.
17. Kallas, E. G., D. C. Gibbons, H. Soucier, T. Fitzgerald, J. J. Treanor, and T. G. Evans. 1999. Detection of intracellular antigen-specific cytokines in human T cell populations. J. Infect. Dis. 179:1124-1131.
18. Olmsted, R. A., N. Elango, G. A. Prince, B. R. Murphy, P. R. Johnson, B. Moss, R. M. Chanock, and P. L. Collins. 1986. Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc. Natl. Acad. Sci. U. S. A. 83:7462-7466.
19. Openshaw, P. J. 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am. J. Respir. Crit. Care Med. 152:S59-S62.
20. Secrist, H., C. J. Chelen, Y. Wen, J. D. Marshall, and D. T. Umetsu. 1993. Allergen immunotherapy

decreases interleukin 4 production in CD4+ T cells from allergic individuals. J. Exp. Med. 178:2123-2130.

21. Srikiatkhachorn, A. and T. J. Braciale. 1997. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. J. Exp. Med. 186:421-432.
22. van Binnendijk, R. S., M. C. Poelen, P. de Vries, H. O. Voorma, A. D. Osterhaus, and F. G. UytdeHaag. 1989. Measles virus-specific human T cell clones. Characterization of specificity and function of CD4+ helper/cytotoxic and CD8+ cytotoxic T cell clones. J. Immunol. 142:2847-2854.
23. van Binnendijk, R. S., M. C. Poelen, K. C. Kuijpers, A. D. Osterhaus, and F. G. UytdeHaag. 1990. The predominance of CD8+ T cells after infection with measles virus suggests a role for CD8+ class I MHC-restricted cytotoxic T lymphocytes (CTL) in recovery from measles. Clonal analyses of human CD8+ class I MHC-restricted CTL. J. Immunol. 144:2394-2399.
24. van Binnendijk, R. S., J. P. M. Versteeg-van Oosten, M. C. M. Poelen, H. F. Brugghe, P. Hoogerhout, A. D. M. E. Osterhaus, and F. G. C. M. UytdeHaag. 1993. Human HLA class I- and HLA class II-restricted cloned cytotoxic T lymphocytes identify a cluster of epitopes on the measles virus fusion protein. J. Virol. 67:2276-2284.
25. Verjans, G. M., L. Remeijer, R. S. van Binnendijk, J. G. Cornelissen, H. J. Volker-Dieben, S. G. Baarsma, and A. D. Osterhaus. 1998. Identification and characterization of herpes simplex virus-specific CD4+ T cells in corneas of herpetic stromal keratitis patients. J. Infect. Dis. 177:484-488.
26. Wiertz, E. J., J. A. van Gaans-van den Brink, H. Gausepohl, A. Prochnicka-Chalufour, P. Hoogerhout, and J. T. Poolman. 1992. Identification of T cell epitopes occurring in a meningococcal class 1 outer membrane protein using overlapping peptides assembled with simultaneous multiple peptide synthesis. J. Exp. Med. 176:79-88.

Chapter 6
General discussion

6.1 Pathogenesis of RSV lower respiratory tract infection: implications for vaccine development

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Invited review in Vaccine

Summary

Respiratory syncytial virus (RSV) infection is the most prevalent cause of severe respiratory disease in infants. It also causes considerable morbidity in older children and adults with underlying risk factors. RSV vaccine development has been complicated by the need to administer the vaccine at a very young age, and by enhanced disease observed after vaccination with formalin inactivated RSV.

For infants live attenuated vaccines, which may not be expected to predispose for vaccine induced enhanced pathology, hold the greatest promise. However the balance between attenuation and immunogenicity appears to be delicate. For older risk groups results with subunit vaccines are most promising.

Introduction

Infection with respiratory syncytial virus (RSV) is the most common cause of severe respiratory disease in young children, leading to the hospitalisation of up to 2% of children in their first year of life (56,58,63,96,111). In fact up to 70% of hospital admissions of babies for respiratory infections during the winter season may be caused by RSV (54). Currently no licensed vaccine is available for this major cause of infant morbidity.

The majority of RSV infections run a mild clinical course and only a minority of infected children (0,5 – 2%) has to be admitted to hospital. However, since RSV infections are so prevalent with an estimated 50% of children being infected in their first year of life, RSV bronchiolitis is a common reason for hospital admission of young infants. Why RSV infection is complicated by lower respiratory tract involvement in part of the infected children is not understood, but an immune pathological mechanism has long been suspected.

A better understanding of protective, but also of putative disease enhancing mechanisms in RSV infection – upon both natural and vaccine induced responses – is crucial to prevent the recurrence of the vaccination debacle with a formalin inactivated RSV (FI-RSV) vaccine (47,90,108) and to determine approaches which result in the induction of a safe and effective immune response against RSV.

The virus

RSV is a member of the genus Pneumovirus, of the family Paramyxoviridae. Other members of this genus are bovine RSV – which causes respiratory tract disease in calves similar to human RSV in infants – pneumonia virus of mice and the turkey rhino-tracheitis virus (27).

The viruses of the genus Pneumovirus differ from the other Paramyxoviridae in that they have a second matrix protein (M2) (27) and envelope glycoproteins with no hemagglutinin or neuraminidase activity. One of the surface glycoproteins, the fusion (F) protein, mediates both viral penetration and cell to cell spread by fusion of membranes. This protein contains N-linked oligosacharide groups and is composed of two disulphide-linked subunits of 47kD (F1) and 20kD (F2) respectively (76). The larger envelope protein G serves a function corresponding to the host cell attachment of the hemagglutinin of other paramyxoviruses. It is heavily glycosylated with both O- and N-linked oligosacharide groups. In fact more than 60% of the molecular weight of the G protein consists of sugar moieties.

Antigenic diversity in RSV isolates is largely determined by variation in the F and G glycoproteins. RSV can be divided into two major antigenic groups, A and B, by their reactivities with monoclonal antibodies (5,52,106) and nucleotide sequence differences (20,82,137,140). These two groups circulate independently in the population with group A being the most prevalent (3,70,105,148). Sequence diversity within the two groups can also

be extensive. This diversity is mainly found in the G protein (83). G protein amino acid homology between different G proteins can be as low as 53% and antigenic homology as low as 5%, making the G protein, the protein most tolerable to mutations without loss of function, known in nature (27). Several lineages within the groups A and B also seem to co-circulate independently (18,137). Studies on heterogeneity in RSV strains show an accumulation of amino acid changes over the years, suggesting antigenic drift based immune mediated selection (19,20,48,81,117).

Epidemiology

RSV infections have a world-wide distribution. In temperate climates infections are largely confined to the winter season. In tropical climates RSV infections may be found during the whole year, with a concentration in the rainy season in some geographical areas (77,133,152). Although infection can be established in several laboratory animals, natural infection with RSV seems to be limited to humans and apes (17,27). Transmission occurs through direct close contact or contact with surfaces contaminated with respiratory secretions (62). The incubation time for RSV infection ranges between 2 and 7 days (62,102). RSV infections are highly prevalent in the population and reinfections are common. In a study of Hall et al. about 25% of adult volunteers could be reinfected with RSV of the same group two months after natural infection (65). Over 50% of children are infected in their first winter season. By two years of age almost all children have been infected and over 50% will have been infected twice (69).

RSV causes respiratory tract infections in patients of all ages. The highest morbidity of RSV disease is seen below the age of 6 months (56,58,63,116) and in children with underlying risk factors such as prematurity (111), bronchopulmonary dysplasia (59), congenital heart disease with increased pulmonary circulation (95) or immune deficiency (64). Clinically severe RSV infections occur mainly in the very young but also in immunocompromised adults (42,159). In the elderly significant morbidity induced by RSV infection has been found (45,67).

An estimated 0.5 – 2% of all infants with RSV infection are hospitalised (56,58,63,96,111) and 7% – 21% of these hospitalised infants will develop respiratory insufficiency and require respiratory support (96,145). The proportion of infants, in the industrialised world, eventually dying from RSV infection has been estimated at 0.5-1.5% of hospitalised RSV infected infants. However, higher mortality rates have been found in infants with underlying disease (93-95).

Factors influencing clinical severity

Although recent data suggest that RSV is also an important pathogen for the elderly

(45,159), severe problems related to RSV infections are mainly found in young infants probably during their first infection. In up to 40% of infected children, involvement of the lower respiratory tract may be detected (57,87,135) but only in a minority this leads to problems necessitating hospitalisation. Many factors related to the virus, the environment and the patient involved, may influence the clinical outcome. This renders studies concerning the relative importance of these different factors in naturally occurring RSV bronchiolitis complicated.

Identification of the two virus groups (RSV-A and -B) has led to studies investigating the possible relationship between virus group and clinical severity. In about half of these studies group A was found to cause more severe disease, whereas no such differences were found by others (91,153). Only two studies ever reported group B to be more severe (72,138). Strain variation within groups A and B, as defined by nucleotide sequence analysis, have also been suggested to influence disease severity (15,46,153). However, no conclusive data to support this notion have been published.

Also environmental circumstances may influence the outcome of RSV infection. The age at which first RSV infection takes place influences the chance of developing lower respiratory tract infection and thus factors which increase the chance of early exposure, like number of siblings, attendance of day care centres and socio-economic status (116), may all increase the risk of developing lower respiratory tract disease. In addition factors like geographical area (13), parental smoking (98,131,162) and use of wood-burning stoves (131) have been associated with an increased risk of severe RSV infections.

Individual patient factors like underlying illnesses associated with compromised respiratory function like bronchopulmonary dysplasia (BPD) (59) or congenital heart disease with increased pulmonary circulation (95) significantly enhance the risk to develop severe RSV infection. However, also subclinical, reduced lung function has been associated with an increased risk for the development of lower respiratory tract disease in young children (97).

Finally, RSV specific immunity may, besides being protective, also be involved in an immune pathological mechanism leading to severe lower respiratory tract disease. This hypothesis is largely based on experimental animal data and the observation that vaccination of infants with FI-RSV candidate vaccines resulted in an enhanced susceptibility to develop severe lower respiratory tract involvement upon subsequent natural infection (25,47,90).

Humoral immune response

In response to RSV infection antibodies to most RSV proteins are generated (53,149,154). Antibodies against the F- and G-proteins are probably most important in the host defence against RSV infection. Protection is however incomplete and reinfection, especially of the

upper respiratory tract, is readily possible even in the presence of high levels of virus neutralising (VN) antibodies (65). In young infants the antibody response to RSV is limited, because of immaturity of the immune system and interference by maternal antibodies (12). Local antibodies in the respiratory tract appear to be related to protection against reinfection: presence of VN antibodies in the respiratory tract correlates with relative protection against experimental infection in adults (102,110) and production of local IgA coincides with virus clearance (99).

Severe RSV lower respiratory tract infection is predominantly seen in infants between six weeks and six months of age, when most of them still have RSV specific maternal serum antibodies. Vaccination with FI-RSV vaccine, inducing high titres of RSV specific antibodies, gave rise to more severe clinical disease upon subsequent exposure to the virus (25,108). Therefore, in the past, antibodies have often been incriminated as a factor in more severe clinical outcome of the infection. However it has become clear that VN antibodies are in fact protective against RSV infection provided that high titres are present (123): children with higher maternally derived antibody titres were found to be more protected against developing bronchiolitis (56). Although virtually all children have measurable RSV specific antibodies at birth, titres drop relatively fast to unprotective levels (12). Intravenous administration of normal gamma globulin did not induce protection against severe RSV infection (100) whereas administration of immune globulins with a high titre of VN antibodies against RSV (RSVIG) decreased the incidence of severe lower respiratory tract involvement in high-risk children (61,68,129). RSVIG has been licensed for prophylaxis in high-risk children and similarly, a VN monoclonal antibody preparation directed against the RSV-F protein has been registered for prophylaxis in high-risk children (1,2,101,126,139).

Cellular immune response

Virus specific T cell responses have a major impact on the clinical outcome of RSV infection. T cell responses have been shown to play a role in clearance of the virus (7,9). Whilst in normal children, virus excretion stops within 1-3 weeks, children who have no adequate cellular immune response may shed the virus for prolonged periods of time (44). It has been shown that in humans after RSV infection virus specific HLA class-I restricted CD8+ CTL are present in circulation (7,8,21,24) and that RSV infection in mice can be aborted by transfer of RSV-specific CD4+ and CD8+ T cells (4,22,23). In contrast to this apparent protective role, RSV specific cellular immunity has also been shown to be involved in the induction of enhanced pathology in rodent models. Vaccination of rodents with FI-RSV preparations was associated with enhanced lung pathology upon challenge. This pathology was characterised by an eosinophilic infiltrate, which correlated with a type 2 immune response (22,112,155). Passively transferred CD4+ and to a lesser extent CD8+ cell lines from thus

vaccinated animals to RSV infected mice, reduced virus titres in the lungs but also augmented lung pathology (4,33). This lung pathology could be abrogated by the administration of anti-IL-4 and anti-IL-10 but not of anti-IFN-γ or IL-2 (32). The inability to induce virus specific CTL with FI-RSV has been associated with this type 2 mediated enhanced pathology in a mouse model (33,134).

Much less is known about the natural immune response to RSV infection in humans. Whether a similar immune pathological mechanism does indeed play a role in naturally occurring RSV bronchiolitis in infants, as has often been suggested, remains unclear. A study concerning the RSV specific memory T cell response in normal adults and children showed a predominant type 1 like cytokine pattern (6). We have recently shown that IFN-γ is the most prevalent cytokine produced by RSV specific T cells in young infants, regardless of clinical severity (14). Also comparison of cytokine profiles in nasopharyngeal washings between children with mild and severe disease showed increased production of IFN-γ in severely affected children (151). However, type 2 cytokines have been detected in PBMC of normal adults when stimulated *in vitro* with RSV. Il-5 was for instance measured at higher levels in RSV infected than in uninfected PBMC cultures (144). *In vitro* stimulation of PBMC with different RSV antigen preparations showed a type 1 like response upon stimulation with live RSV and RSV-F protein, whereas a type 2 like response was found after stimulation with FI-RSV or RSV-G protein (80).

Collectively these data indicate that RSV infections may induce both type 1 and type 2 like T cell responses. However, whether indeed a skewing towards type 2 like response in part of infected infants is at the basis of the more severe manifestations of RSV related disease remains elusive.

Pathogenesis

After infection, RSV primarily replicates in the epithelial cells of the nasopharynx (66). The exact mechanism by which RSV may spread to the lower respiratory tract is unknown. This may either occur through direct cell-to-cell spread or by aspiration of nasopharyngeal secretions. RSV infection is considered to be limited to the respiratory tract (27,49). No culturable virus is found in blood samples of immunologically normal subjects, although the presence of RSV RNA may be demonstrable in blood (39,124). Signs of lower respiratory tract involvement may develop 1 to 3 days after the onset of upper respiratory symptoms (27).

Severe lower respiratory tract disease in RSV infection is, at least in part, caused by direct cytopathic changes. An aberrant immunological response, occurring in some of the infected children, has been suspected to contribute to the severity of the inflammatory process. The role played by the virus specific immune response in the pathogenesis of lower respiratory tract infection is still unclear. Type 2 T cell mediated enhanced lung pathology has

been described in rodent models after vaccination with FI-RSV or RSV-G protein expressing vectors (112,134,155). RSV lower respiratory tract infection in infants is thought to occur predominantly in the course of their first RSV infection, when they have not been exposed to RSV antigens before. This argues against an early virus specific cellular immune pathological mechanism in naturally occurring severe RSV infection, as has been found in the FI-RSV vaccinees. On the other hand, several observations in favour of the involvement of an immune pathological mechanism in humans have been reported. Very young children are known to be more prone to produce a type 2 like immune response upon infection (38). Higher type 2 cytokines in supernatants of PBMC from children who experienced a RSV bronchiolitis at an early age have been found (125), although in these cases RSV infected patients were compared with non-infected patients. Furthermore type 2 cytokines have also been found *in vitro* in culture supernatants of PBMC from normal adults when stimulated with FI-RSV or RSV-G protein (80).

Direct measurement of cytokines in plasma samples has shown higher levels of systemic sCD25 and ICAM in RSV infected than in control children, but no relation with clinical severity was found (132). In a recent study we also failed to relate clinical severity of RSV disease in young infants to a predominant type 1 or 2 like cytokine profile in plasma samples (14). Not unexpectedly the general pro-inflammatory cytokines IL-6 and IL-8 were found more often in children with more severe disease (11,14). However, because RSV infections are usually restricted to the respiratory tract, the amount of cytokines measured systemically, may not be a reliable reflection of the local process. Direct measurement of cytokines and other soluble factors in samples from the respiratory tract, as has been studied by certain groups (50,128,143,151) may be more informative in studying RSV pathogenesis. Finally, in the mouse model enhanced disease upon RSV infection is characterised by a striking eosinophilia in the lungs. In studies investigating cellular infiltrates of respiratory secretions of children with naturally occurring RSV infection, the cellular infiltrates mainly consisted of polymorphonuclear granulocytes and/or monocytes (14,43,128). This observation indicates that the pathogenesis of natural RSV lower respiratory tract infection in infants does not parallel that of the enhanced disease observed in rodent models after FI-RSV priming.

Animal models in RSV research

Over the past decades several animal models, using rodents, calves, sheep, ferrets, hamsters, dogs and several primate species including chimpanzees have been used in RSV research with varying degrees of success (for review see (17)). Perhaps with the exception of RSV infection of chimpanzees, no animal model combines all aspects of RSV related disease in humans.

Many studies on the mechanism of FI-RSV mediated enhanced disease have been carried out in mice and cotton rats (78,79,109,112,119,155). The mouse model has been shown to be useful for studying RSV specific immune responses and RSV-related immunopathogenesis. Although this was largely due to the absence of alternative animal models in which the pathogenesis of RSV infection more closely resembled that in man, the model has contributed substantially to our current understanding of FI-RSV-related immune pathogenesis. Cotton rats are more permissive to RSV than mice and show higher RSV specific antibody responses. Studies in the RSV-cotton rat model showed that high titres of VN antibodies were protective against RSV lower respiratory tract disease (122,123). This ultimately resulted in the development of the passive immunisation strategies for high-risk children (1,61,68). However, lack of reagents to characterise the immune response of cotton rats have considerably hampered studies of the cellular immune response and immune pathogenesis in cotton rats.

Bovine RSV induces a disease in calves that is quite similar to that caused by human RSV in infants. The major advantage of the bovine RSV infection model is the use of a natural infection in its natural host. Also the observation of immunopathological effects in calves after FI- bovine RSV vaccination and subsequent challenge with bovine RSV makes this an attractive, although expensive model (51,158). However, until recently it has been impossible to perform detailed immunological studies such as those carried out in the mouse model, due to lack of reagents (92,103,161).

The close genetic relatedness between humans and other primates has led to the development of several primate models, using chimpanzees (28,160), African green monkeys (86) and macaques (157). Although chimpanzees are naturally infected with RSV, and develop a disease that closely resembles that in humans, their use is largely limited by financial and ethical constraints. RSV infection in African green monkeys is also quite similar to that in humans and enhanced lung pathology has been observed following FI-RSV vaccination (86). However, due to lack of specific immunological reagents, this model also has clear limitations. The description of a human RSV challenge model in macaques (157), for which more immunological reagents are available, does offer some promise for the future. However, no clinical disease has been observed in this species upon experimental RSV infection.

Vaccine development

Since RSV is the most prevalent infectious agent causing severe respiratory disease in infants, the development of a vaccine against RSV is high on the list of priorities of the Global Program for Vaccines of the World Health Organisation (35). However, the development of a safe and effective RSV vaccine has been severely hampered by a combination

of complicating factors. Severe RSV infections occur at a very young age. Therefore, vaccination should preferably be carried out almost immediately after birth. This implies that a RSV vaccine would have to induce an effective response in a largely immature immune system and usually in the presence of maternal antibodies. Another discouraging observation is that frequent reinfections occur throughout life (57,65). Therefore it is probably not realistic to expect that a candidate RSV-vaccine would induce sterile immunity. Protection of the lower respiratory tract and therefore prevention of severe complications should probably be the primary goal. Perhaps the most worrying complication in RSV vaccine development at present is related to the enhanced disease that was observed after experimental vaccination with FI-RSV and subsequent natural RSV infection (25,47,88,90). For any future candidate RSV vaccine it should convincingly be shown that its use would not predispose for this enhanced disease upon infection with the virus. This may be hard to show since the underlying mechanism of enhanced disease is not understood. A preventive vaccine that could be incorporated in the current pediatric vaccination schedules would definitely be the approach of choice for protection of infants.

Besides young infants, other groups at risk of developing complications when infected with RSV are the elderly, older children with chronic lung disease and the immune compromised. Requirements for RSV candidate vaccines may vary for the respective risk groups. Also vaccination of pregnant mothers has been suggested, aiming to enhance transplacentally acquired VN antibodies in the new-born and thereby prolonging the period during which protective levels of antibodies are present (41,55).

Virtually all modern approaches for vaccine development are still being pursued currently for the development of a safe and effective RSV vaccine (Table 6.1.1.).

Subunit vaccines

Several candidate subunit vaccines based on the RSV-F and/or G proteins, the main targets for induction of neutralising antibodies, have been developed. These vaccines aim primarily at the induction of an adequate VN antibody response resulting in significant protection of the lower respiratory tract. This goal has indeed been achieved with a number of such candidate vaccines in clinical trials in older children and adults belonging to several risk groups (10,31,156). RSV-F subunit vaccines PFP-1 and 2 have been tested in healthy seropositive children (10,115,146) and cystic fibrosis- (118) as well as older BPD (60) patients. These vaccines were found to be safe and to reduce respiratory problems upon RSV infection.

A recently developed candidate peptide vaccine, BBG2Na, a fusion protein between the 130-230 residues of the G protein and the albumin-binding domain (BB) of streptococcal G protein (34,120) has proven highly immunogenic in mice (130) and is currently under eval-

Table 6.1.1. RSV vaccination approaches

Vaccine candidates	Major advantages	Major disadvantages
Non-replicating		
formaldehyde inactivated RSV		predisposition for enhanced disease
Subunits:		
- purified proteins (PFP 1/2)	controlled manufacturing	predisposition for enhanced disease not ruled out
	safe in the elderly	no CTL response
- peptide (BBG2Na)	controlled manufacturing	predisposition for enhanced disease not ruled out
	safe in the elderly	no CTL response
- iscom	induction of VN antibody and CTL, also in the presence of maternal antibodies	only preclinical data
Replicating		
live attenuated	mimics natural infection	over/under attenuation
		reversion to wild type
vector delivery systems:		
- adenoviruses	induction of VN antibody and CTL	limited immunogenicity in primates
- poxviruses:		
classical vaccinia viruses	induction of VN antibody and CTL	limited immunogenicity in primates
		safety concerns
replication deficient vaccinia viruses (ALVAC®, NYVAC®, MVA)	induction of VN antibody and CTL	only preclinical data

uation in phase 1 and 2 human clinical trials.

Since subunit vaccines may generally not be expected to induce a HLA class-I restricted CTL response the induction by these vaccines of enhanced pathology related to the absence of CTL in immune naïve children cannot fully be excluded at present. In rodent models both the presence and absence of enhanced pathology to certain subunit vaccines containing the G protein has been reported (31,109,156). Although also the BBG2Na peptide vaccine has been shown to induce a type 2 like response in mice, subsequent challenge with wild type RSV did not result in lung pathology or type 2 responses (34,121). Therefore such subunit and peptide vaccines may probably be expected to be useful in immune non-naïve individuals at high risk of developing pulmonary complications upon RSV infection.

Antigens presented in immune stimulatory complexes (iscoms) have been described to induce both good antibody and T cell mediated immune responses including HLA class-1 restricted CTL, even in the presence of maternally derived antibodies (for review see (114)). Because the lack of development of CD8+ CTL has been associated with enhanced disease in RSV infection (33,134), iscom vaccines expressing RSV proteins may be of major interest for evaluation, even in immune naïve children. Iscoms presenting both human and bovine RSV glycoproteins have been shown to induce both VN antibodies and a CTL response in rodent models (74,75,147).

Live attenuated vaccines

Live attenuated vaccines have the advantage of mimicking the natural infection most closely. Therefore antigen delivery takes place by the natural route inducing a response resembling the natural response after RSV infection. Consequently, no enhanced pathology may be expected. If the vaccine is delivered via the mucosal route, also a balanced, both systemic and local immune response will develop including mucosal antibodies and a cytotoxic T cell response, which are both known to be important in protection against infection and clearance of the virus. A disadvantage of the live attenuated vaccine approach however, is that maternal antibodies may interfere with induction of immunity by neutralising the vaccine virus. This may preclude an effective response in the very young upon parenteral administration, as is observed after vaccination with live attenuated measles vaccine in children less than one year of age (113).

The classical Jennerian approach has initially been evaluated for RSV, by using bovine RSV as a vaccine. However bovine RSV did not protect chimpanzees against RSV infection rendering this approach unsuitable for further evaluation in humans (35).

Attenuated RSV strains of both groups (A and B) have been generated by several research groups in the past by repeated passages of the virus at low temperature followed by passage in the presence of mutagenic agents (36,107). In pre-clinical and clinical trials with these

candidate vaccines it became clear that the balance between attenuation and immunogenicity is delicate. These vaccines proved to be either over- or under-attenuated and also reversion to wild type was not uncommon. Enhanced disease however, was never found in chimpanzees or infants upon infection after vaccination with such candidate vaccines (40). More recently so-called further attenuated cold passaged temperature sensitive (cpts) RSV preparations were developed and tested in chimpanzees, adult volunteers and also in RSV seropositive and seronegative children (89). These candidate vaccines proved to be more stable and no reversion to the wild type has been detected. Importantly, no enhanced disease was found upon infection in the subsequent season but respiratory symptoms upon vaccination were not uncommon. Protective efficacy against lower respiratory tract involvement upon repeated vaccination, in combination with other pediatric vaccines is currently evaluated.

Recently it has become possible to recover infectious virus from cDNA clones of RSV (26). This technology may have a major impact on RSV vaccine development. It has become possible to identify the exact mutations responsible for attenuation by introducing point mutations, and studying these mutant viruses *in vivo* (84,85). Also induction of combinations of mutations may make it possible to rationally design a genetically stable candidate vaccine combining sufficient attenuation with adequate immunogenic potential (29). Moreover it may become possible to prepare viruses expressing glycoproteins from the different RSV groups or, as has recently been shown, a vaccine candidate based on bovine RSV that expresses human-RSV F and G proteins (16).

Live vector vaccines

Live vectors presenting RSV antigens may be expected to induce both good VN antibody and HLA class-I restricted CTL responses. Vaccinia- and adenoviruses expressing RSV proteins have in the past been developed and encouraging responses were found in several non-primate animal models (30,73). However the poor responses found in chimpanzees for both vector delivery systems (28,37,73) and concerns about safety of vaccinia virus make them less suitable vector candidates to deliver RSV antigens. More recently, recombinant poxvirus constructs have been developed based on replication-deficient poxviruses, like canarypox (ALVAC®), highly attenuated vaccinia (NYVAC®) virus and modified vaccinia virus Ankara (MVA). These are all known to replicate not at all, or only limitedly in mammalian cells (104). Compared with replication competent strains of vaccinia virus, MVA is found safe (136), allows similar expression levels of the recombinant genes (141), and can induce equal or better antibody and T cell responses, including CD8+ CTL responses, in animals (71,127,142,163). MVA expressing RSV glycoproteins has been shown to be stable and to induce good serological responses in mice (164).

Concluding remarks

Although recently encouraging results have been obtained with preventive passive immune therapy against RSV infection in high-risk children, the most cost effective and practical approach to prevent RSV lower respiratory tract infection would undoubtedly be preventive vaccination.

In the mouse model pathogenesis of enhanced disease after vaccination with FI-RSV is related to a type 2 mediated T cell (112) reaction, which is also associated with the virtual absence of a CTL response (134). Data in mice (150) and humans (14) however, did so far not convincingly show that a similar pathogenesis occurs in naturally occurring severe manifestations of RSV infection. Other factors, like disturbed respiratory functions and epidemiological as well as geographical factors may play a major role in RSV pathogenesis. Studies aimed at a better understanding of the pathogenesis of RSV lower respiratory tract disease in previously healthy infants are of importance for the rational design of pediatric RSV candidate vaccines.

Because even naturally occurring RSV infection fails to induce complete protection, the most realistic aim for a RSV vaccine should be the prevention of lower respiratory tract disease. For risk groups, like the elderly and older children with BPD or cystic fibrosis, the priming for enhanced disease upon natural RSV infection is not expected to be a major problem, because of the pre-existing immunity. Boosting of the VN antibody titre is associated with a reduced risk of developing complications upon infection with RSV infection and current results with subunit vaccines are promising in this respect. Moreover, in the future, the combination of RSV subunit vaccines with influenza vaccines may be attractive for these risk groups.

For the induction of protective immunity by vaccination in immune naïve children, the issue of predisposition for enhanced pathology upon natural RSV infection is of great concern. Since at present for candidate subunit vaccines the induction of enhanced disease cannot convincingly be ruled out, these non-replicating vaccines are not likely to be used first in immune naïve children. Recent data, however, obtained with new generations of adjuvant and antigen presentation systems – like the iscom presentation system – indicate that also with subunit preparations even in the presence of neutralising antibodies, good VN antibody and HLA class-1 restricted CD8+ CTL responses may be induced (113,114). Live attenuated vaccines may be the most promising in the ultimate target group: newborn children. With these vaccines antigen delivery resembles natural infection most closely and consequently, no enhanced pathology upon re-infection may be expected. Moreover, taking advantage of the recently developed reversed genetics technology it may be possible to rationally design sufficiently attenuated and immunogenic vaccine viruses, expressing antigens from both RSV A and B (29). Similar results may be obtained with the novel generation of live vector

vaccines, based on replication deficient poxviruses. A problem associated with the live attenuated and live vector vaccine approaches in very young infants may be the relative immaturity of the immune system of the infant and the presence of maternal antibodies, which may interfere with replication of the vaccine virus. The latter problem may perhaps be overcome by application via the mucosal route.

New insights in the pathogenesis of RSV infections, as well as the rapid developments in the molecular and immunological fields of modern vaccinology, will be of major importance for the further development of safe and efficacious RSV vaccination approaches. These approaches may be expected to differ for different target groups.

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References

1. Anonymous1998. Palivizumab, a Humanized Respiratory Syncytial Virus Monoclonal Antibody, Reduces Hospitalization From Respiratory Syncytial Virus Infection in High-risk Infants. Pediatrics 102:531-537.
2. Anonymous1998. Prevention of respiratory syncytial virus infections: indications for the use of palivizumab and update on the use of RSV-IGIV. American Academy of Pediatrics Committee on Infectious Diseases and Committee of Fetus and Newborn. Pediatrics 102:1211-1216.
3. Akerlind, B. and E. Norrby. 1986. Occurrence of respiratory syncytial virus subtypes A and B strains in Sweden. J. Med. Virol. 19:241-247.
4. Alwan, W. H., F. M. Record, and P. J. M. Openshaw. 1992. CD4+ T cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T cells. Clin. Exp. Immunol. 88:527-536.
5. Anderson, L. J., J. C. Hierholzer, C. Tsou, R. M. Hendry, B. F. Fernie, Y. Stone, and K. Mcintosh. 1985. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J. Infect. Dis. 151:626-633.
6. Anderson, L. J., C. Tsou, C. Potter, H. L. Keyserling, T. F. Smith, G. Ananaba, and C. R. M. Bangham. 1994. Cytokine response to respiratory syncytial virus stimulation of human peripheral blood mononuclear cells. J. Infect. Dis. 170:1201-1208.
7. Bangham, C. R. and A. J. McMichael. 1986. Specific human cytotoxic T cells recognize B-cell lines persistently infected with respiratory syncytial virus. Proc. Natl. Acad. Sci. U. S. A. 83:9183-9187.
8. Bangham, C. R., P. J. Openshaw, L. A. Ball, A. M. King, G. W. Wertz, and B. A. Askonas. 1986. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoprotein (G), expressed by vaccinia virus recombinants. J. Immunol. 137:3973-3977.
9. Bangham, C. R. M., M. J. Cannon, D. T. Karzon, and B. A. Askonas. 1985. Cytotoxic T-cell response to respiratory syncytial virus in mice. J. Virol. 56:55-59.
10. Belshe, R. B., E. L. Anderson, and E. E. Walsh. 1993. Immunogenicity of purified F glycoprotein of respiratory syncytial virus: clinical and immune responses to subsequent natural infection in children. J. Infect. Dis. 168:1024-1029.
11. Bont, L., C. J. Heijnen, A. Kavelaars, W. M. van Aalderen, F. Brus, J. T. Draaisma, S. M. Geelen, H. J. van Vught, and J. L. Kimpen. 1999. Peripheral blood cytokine responses and disease severity in respiratory syncytial virus bronchiolitis. Eur. Respir. J. 14:144-149.
12. Brandenburg, A. H., J. Groen, H. A. Van Steensel-Moll, E. J. C. Claas, P. H. Rothbarth, H. J. Neijens, and A. D. M. E. Osterhaus. 1997. Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. J. Med. Virol. 52:97-104.

13. Brandenburg, A. H., P. Y. Jeannet, H. A. Steensel-Moll, A. Ott, P. H. Rothbarth, W. Wunderli, S. Suter, H. J. Neijens, A. D. Osterhaus, and C. A. Siegrist. 1997. Local variability in respiratory syncytial virus disease severity. Arch. Dis Child 77:410-414.
14. Brandenburg, A. H., A. Kleinjan, B. van het Land, H. A. Moll, H. H. Timmerman, R. L. de Swart, H. J. Neijens, W. Fokkens, and A. D. M. E. Osterhaus. 2000. A type 1 like immune response is found in children with RSV infection regardless of clinical severity. J. Med. Virol. (Abstract)
15. Brandenburg, A. H., R. van Beek, H. A. Moll, A. D. M. E. Osterhaus, and E. C. J. Claas. 2000. G protein variation in respiratory syncytial virus group A: no correlation with clinical severity. (UnPub)
16. Buchholz, U. J., H. Granzow, K. Schuldt, S. S. Whitehead, B. R. Murphy, and P. L. Collins. 2000. Chimeric bovine respiratory syncytial virus with glycoprotein gene substitutions from human respiratory syncytial virus (HRSV): effects on host range and evaluation as a live-attenuated HRSV vaccine. J. Virol. 2000. Feb;74(3):1187-99. 74:1187-1199.
17. Byrd, L. G. and G. A. Prince. 1997. Animal models of respiratory syncytial virus infection. Clin. Infect. Dis. 25:1363-1368.
18. Cane, P. A., D. A. Matthews, and C. R. Pringle. 1992. Analysis of relatedness of subgroup A respiratory syncytial viruses isolated worldwide. Virus Res. 25:15-22.
19. Cane, P. A., D. A. Matthews, and C. R. Pringle. 1994. Analysis of respiratory syncytial virus strain variation in successive epidemics in one city. J. Clin. Microbiol. 32:1-4.
20. Cane, P. A. and C. R. Pringle. 1995. Evolution of subgroup A respiratory syncytial virus: evidence for progressive accumulation of amino acid changes in the attachment protein. J. Virol. 69:2918-2925.
21. Cannon, M. J. and C. R. M. Bangham. 1989. Recognition of Respiratory Syncytial Virus Fusion Protein by Mouse cytotoxic T Cell Clones and a Human Cytotoxic T Cell Line. J. Gen. Virol. 70:79-87.
22. Cannon, M. J., P. J. M. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168:1163-1168.
23. Cannon, M. J., E. J. Stott, G. Taylor, and B. A. Askonas. 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. Immunology 62:133-138.
24. Cherrie, A. H., K. Anderson, G. W. Wertz, and P. J. M. Openshaw. 1992. Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J. Virol. 66:2102-2110.
25. Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette. 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. 89:449-463.
26. Collins, P. L., M. G. Hill, E. Camargo, H. Grosfeld, R. M. Chanock, and B. R. Murphy. 1995. 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. 92:11563-11567.
27. Collins, P. L., K. Mcintosh, and R. M. Chanock. 1996. Respiratory syncytial virus, p. 1313-1351. In B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Fields virology. Lippincott - Raven Publishers, Philadelphia.
28. Collins, P. L., R. H. Purcell, W. T. London, L. A. Lawrence, R. M. Chanock, and B. R. Murphy. 1990. Evaluation in chimpanzees of vaccinia virus recombinants that express the surface glycoproteins of human respiratory syncytial virus. Vaccine 8:164-168.
29. Collins, P. L., S. S. Whitehead, A. Bukreyev, R. Fearns, M. N. Teng, K. Juhasz, R. M. Chanock, and B. R. Murphy. 1999. Rational design of live-attenuated recombinant vaccine virus for human respiratory syncytial virus by reverse genetics. Adv. Virus Res. 54:423-51:423-451.
30. Connors, M., P. L. Collins, C. Y. Firestone, and B. R. Murphy. 1991. Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. J. Virol. 65:1634-1637.
31. Connors, M., P. L. Collins, C. Y. Firestone, A. V. Sotnikov, A. Waitze, A. R. Davis, P. P. Hung, R. M. Chanock, and B. R. Murphy. 1992. Cotton rats previously immunized with a chimeric RSV FG glycoprotein develop enhanced pulmonary pathology when infected with RSV, a phenomenon not encountered following immunization with vaccinia--RSV recombinants or RSV. Vaccine 10:475-484.

32. Connors, M., N. A. Giese, A. B. Kulkarni, C.-Y. Firestone, H. C. Morse III, and B. R. Murphy. 1994. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. J. Virol. 68:5321-5325.
33. Connors, M., A. B. Kulkarni, C.-Y. Firestone, K. L. Holmes, H. C. Morse III, A. V. Sotnikov, and B. R. Murphy. 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J. Virol. 66:7444-7451.
34. Corvaia, N., P. Tournier, T. N. Nguyen, J. F. Haeuw, U. F. Power, H. Binz, and C. Andreoni. 1997. Challenge of BALB/c mice with respiratory syncytial virus does not enhance the Th2 pathway induced after immunization with a recombinant G fusion protein, BBG2NA, in aluminum hydroxide. J. Infect. Dis. 176:560-569.
35. Crowe, J. E., Jr. 1995. Current approaches to the development of vaccines against disease caused by respiratory syncytial virus (RSV) and parainfluenza virus (PIV). A meeting report of the WHO Programme for Vaccine Development. Vaccine 13:415-421.
36. Crowe, J. E., Jr. 1998. Immune responses of infants to infection with respiratory viruses and live attenuated respiratory virus candidate vaccines. Vaccine 16:1423-1432.
37. Crowe, J. E., Jr., P. L. Collins, W. T. London, R. M. Chanock, and B. R. Murphy. 1993. A comparison in chimpanzees of the immunogenicity and efficacy of live attenuated respiratory syncytial virus (RSV) temperature-sensitive mutant vaccines and vaccinia virus recombinants that express the surface glycoproteins of RSV. Vaccine 11:1395-1404.
38. Delespesse, G., L. P. Yang, Y. Ohshima, C. Demeure, U. Shu, D. G. Byun, and M. Sarfati. 1998. Maturation of human neonatal CD4+ and CD8+ T lymphocytes into Th1/Th2 effectors. Vaccine 16:1415-1419.
39. Domurat, F., N. J. Roberts,Jr., E. E. Walsh, and R. Dagan. 1985. Respiratory syncytial virus infection of human mononuclear leukocytes in vitro and in vivo. J. Infect. Dis. 152:895-902.
40. Dudas, R. A. and R. A. Karron. 1998. Respiratory syncytial virus vaccines. Clin. Microbiol. Rev. 11:430-439.
41. Englund, J., W. P. Glezen, and P. A. Piedra. 1998. Maternal immunization against viral disease. Vaccine 16:1456-1463.
42. Englund, J. A., C. J. Sullivan, M. C. Jordan, L. P. Dehner, G. M. Vercellotti, and H. H. Balfour,Jr. 1988. Respiratory syncytial virus infection in immunocompromised adults. Ann. Intern. Med. 109:203-208.
43. Everard, M. L., A. Swarbrick, M. Wrightham, J. McIntyre, C. Dunkley, P. D. James, H. F. Sewell, and A. D. Milner. 1994. Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection. Arch. Dis. Child 71:428-432.
44. Fishaut, M., D. Tubergen, and K. Mcintosh. 1980. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J. Pediatr. 96:179-186.
45. Fleming, D. M. and K. W. Cross. 1993. Respiratory syncytial virus or influenza? Lancet 342:1507-1510.
46. Fletcher, J. N., R. L. Smyth, H. M. Thomas, D. Ashby, and C. A. Hart. 1997. Respiratory syncytial virus genotypes and disease severity among children in hospital. Arch. Dis. Child 77:508-511.
47. Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn. 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aquacous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. 89:435-448.
48. Garcia, O., M. Martin, J. Dopazo, J. Arbiza, S. Frabasile, J. Russi, M. Hortal, P. Perez-Brena, I. Martinez, and B. Garcia-Barreno. 1994. Evolutionary pattern of human respiratory syncytial virus (subgroup A): cocirculating lineages and correlation of genetic and antigenic changes in the G glycoprotein. J. Virol. 68:5448-5459.
49. Gardner, P. S., J. McQuillin, and S. D. Court. 1970. Speculation on pathogenesis in death from respiratory syncytial virus infection. Br. Med. J. 1:327-330.
50. Garofalo, R., J. L. Kimpen, R. C. Welliver, and P. L. Ogra. 1992. Eosinophil degranulation in the respiratory tract during naturally acquired respiratory syncytial virus infection. J. Pediatr. 120:28-32.
51. Gershwin, L. J., E. S. Schelegle, R. A. Gunther, M. L. Anderson, A. R. Woolums, D. R. Larochelle, G. A. Boyle, K. E. Friebertshauser, and R. S. Singer. 1998. A bovine model of vaccine enhanced respira-

tory syncytial virus pathophysiology. Vaccine 16:1225-1236.
52. Gimenez, H. B., N. Hardman, H. M. Keir, and P. Cash. 1986. Antigenic variation between human respiratory syncytial virus isolates. J. Gen. Virol. 67:863-870.
53. Gimenez, H. B., H. M. Keir, and P. Cash. 1987. Immunoblot analysis of the human antibody response to respiratory syncytial virus infection. J. Gen. Virol. 68:1267-1275.
54. Glezen, P. and F. W. Denny. 1973. Epidemiology of acute lower respiratory disease in children. N. Engl. J. Med. 288:498-505.
55. Glezen, W. P. and M. Alpers. 1999. Maternal immunization. Clin. Infect. Dis. 28:219-224.
56. Glezen, W. P., A. Paredes, J. E. Allison, L. H. Taber, and A. L. Frank. 1981. Risk of respiratory syncytial virus infection for infants from low- income families in relationship to age, sex, ethnic group, and maternal antibody level. J. Pediatr. 98:708-715.
57. Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child 140:543-546.
58. Green, M., A. F. Brayer, K. A. Schenkman, and E. R. Wald. 1989. Duration of hospitalization in previously well infants with respiratory syncytial virus infection. Pediatr. Infect. Dis. J. 8:601-605.
59. Groothuis, J. R., K. M. Gutierrez, and B. A. Lauer. 1988. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 82:199-203.
60. Groothuis, J. R., S. J. King, D. A. Hogerman, P. R. Paradiso, and E. A. Simoes. 1998. Safety and immunogenicity of a purified F protein respiratory syncytial virus (PFP-2) vaccine in seropositive children with bronchopulmonary dysplasia. J. Infect. Dis. 177:467-469.
61. Groothuis, J. R., E. A. Simoes, M. J. Levin, C. B. Hall, C. E. Long, W. J. Rodriguez, J. Arrobio, H. C. Meissner, D. R. Fulton, and R. C. Welliver. 1993. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. The Respiratory Syncytial Virus Immune Globulin Study Group. N. Engl. J. Med. 329:1524-1530.
62. Hall, C. B. and R. G. Douglas,Jr. 1981. Modes of transmission of respiratory syncytial virus. J. Pediatr. 99:100-103.
63. Hall, C. B., W. J. Hall, and D. M. Speers. 1979. Clinical and physiological manifestations of bronchiolitis and pneumonia. Outcome of respiratory syncytial virus. Am. J. Dis. Child 133:798-802.
64. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315:77-81.
65. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis 163:693-698.
66. Hall, W. J., C. B. Hall, and D. M. Speers. 1978. Respiratory syncytial virus infection in adults: clinical, virologic, and serial pulmonary function studies. Ann. Intern. Med. 88:203-205.
67. Han, L. L., J. P. Alexander, and L. J. Anderson. 1999. Respiratory syncytial virus pneumonia among the elderly: an assessment of disease burden. J. Infect. Dis. 179:25-30.
68. Hemming, V. G., G. A. Prince, J. R. Groothuis, and G. R. Siber. 1995. Hyperimmune globulins in prevention and treatment of respiratory syncytial virus infections. Clin. Microbiol. Rev. 8:22-33.
69. Henderson, F. W., A. M. Collier, W. A. Clyde,Jr., and F. W. Denny. 1979. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N. Engl. J. Med. 300:530-534.
70. Hendry, R. M., A. L. Talis, E. Godfrey, L. J. Anderson, B. F. Fernie, and K. Mcintosh. 1986. Concurrent circulation of antigenically distinct strains of respiratory syncytial virus during community outbreaks. J. Infect. Dis. 153:291-297.
71. Hirsch, V. M., T. R. Fuerst, G. Sutter, M. W. Carroll, L. C. Yang, S. Goldstein, M. Piatak,Jr., W. R. Elkins, W. G. Alvord, D. C. Montefiori, B. Moss, and J. D. Lifson. 1996. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. J. Virol. 70:3741-3752.
72. Hornsleth, A., B. Klug, M. Nir, J. Johansen, K. S. Hansen, L. S. Christensen, and L. B. Larsen. 1998. Severity of respiratory syncytial virus disease related to type and genotype of virus and to cytokine values in nasopharyngeal secretions. Pediatr. Infect. Dis. J. 17:1114-1121.
73. Hsu, K. H., M. D. Lubeck, A. R. Davis, R. A. Bhat, B. H. Selling, B. M. Bhat, S. Mizutani, B. R. Murphy, P. L. Collins, and R. M. Chanock. 1992. Immunogenicity of recombinant adenovirus-respiratory syncytial virus vaccines with adenovirus types 4, 5, and 7 vectors in dogs and a chimpanzee. J.

Infect. Dis. 166:769-775.
74. Hu, K. F., J. Ekstrom, M. Merza, K. Lovgren-Bengtsson, and B. Morein. 1999. Induction of antibody responses in the common mucosal immune system by respiratory syncytical virus immunostimulating complexes. Med. Microbiol. Immunol. (Berl) 187:191-198.
75. Hu, K. F., M. Elvander, M. Merza, L. Akerblom, A. Brandenburg, and B. Morein. 1998. The immunostimulating complex (ISCOM) is an efficient mucosal delivery system for respiratory syncytial virus (RSV) envelope antigens inducing high local and systemic antibody responses. Clin. Exp. Immunol. 113:235-243.
76. Huang, Y. T., P. L. Collins, and G. W. Wertz. 1985. Characterization of the 10 proteins of human respiratory syncytial virus: identification of a fourth envelope-associated protein. Virus Res. 2:157-173.
77. Huq, F., M. Rahman, N. Nahar, A. Alam, M. Haque, D. A. Sack, T. Butler, and R. Haider. 1990. Acute lower respiratory tract infection due to virus among hospitalized children in Dhaka, Bangladesh. Rev. Infect. Dis. 12 Suppl 8:S982-7:S982-7.
78. Hussell, T., A. Georgiou, T. E. Sparer, S. Matthews, P. Pala, and P. J. Openshaw. 1998. Host genetic determinants of vaccine-induced eosinophilia during respiratory syncytial virus infection. J. Immunol. 161:6215-6222.
79. Hussell, T. and P. J. Openshaw. 1998. Intracellular IFN-gamma expression in natural killer cells precedes lung CD8+ T cell recruitment during respiratory syncytial virus infection. J. Gen. Virol. 79:2593-2601.
80. Jackson, M. and R. Scott. 1996. Different patterns of cytokine induction in cultures of respiratory syncytial (RS) virus-specific human Th cell lines following stimulation with RS virus and RS virus proteins. J. Med. Virol. 49:161-169.
81. Johansen, J., L. S. Christensen, A. Hornsleth, B. Klug, K. S. Hansen, and M. Nir. 1997. Restriction pattern variability of respiratory syncytial virus during three consecutive epidemics in Denmark. APMIS 105:303-308.
82. Johnson, P. R., M. K. Spriggs, R. A. Olmsted, and P. L. Collins. 1987. The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: extensive sequence divergence between antigenically related proteins. Proc. Natl. Acad. Sci. USA 84:5625-5629.
83. Johnson, P. R., Jr., R. A. Olmsted, G. A. Prince, B. R. Murphy, D. W. Alling, E. E. Walsh, and P. L. Collins. 1987. Antigenic relatedness between glycoproteins of human respiratory syncytial virus subgroups A and B: evaluation of the contributions of F and G glycoproteins to immunity. J. Virol. 61:3163-3166.
84. Juhasz, K., S. S. Whitehead, C. A. Boulanger, C. Y. Firestone, P. L. Collins, and B. R. Murphy. 1999. The two amino acid substitutions in the L protein of cpts530/1009, a live-attenuated respiratory syncytial virus candidate vaccine, are independent temperature-sensitive and attenuation mutations. Vaccine 17:1416-1424.
85. Juhasz, K., S. S. Whitehead, P. T. Bui, J. M. Biggs, J. E. Crowe, C.A.Boulanger, P. L. Collins, and B. R. Murphy. 1997. The temperature-sensitive (ts) phenotype of a cold-passaged (cp) live attenuated respiratory syncytial virus vaccine candidate, designated cpts530, results from a single amino acid substitution in the L protein [published erratum XXears in J Virol 1997 Nov; 71(11):8953]. J. Virol. 71:5814-5819.
86. Kakuk, T. J., K. Soike, R. J. Brideau, R. M. Zaya, S. L. Cole, J.-Y. Zhang, E. D. Roberts, P. A. Wells, and M. W. Wathen. 1993. A human respiratory syncytial virus (RSV) primate model of enhanced pulmonary pathology induced with a formalin-inactivated RSV vaccine bu not a recombinant FG subunit vacine. J. Infect. Dis. 167:553-561.
87. Kapikian, A. Z., J. A. Bell, F. M. Mastrota, K. M. Johnson, R. J. Huebner, and R. M. Channock. 1961. An outbreak of febrile illnessand pneumonia associated with respiratory syncytial virus infection. Am. J. Hyg. 74:234-248.
88. Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart. 1969. 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. 89:405-421.
89. Karron, R. A., P. F. Wright, J. E. Crowe Jr., M. L. Clements-Mann, J. Thompson, M. Makhene, R. Casey, and B. R. Murphy. 1997. Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial vaccines in chimpanzees and in human adults, infants, and children. J. Infect. Dis. 176:1428-1436.

90. Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. E. Jensen, and R. H. Parrott. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89:422-434.
91. Kneyber, M. C., A. H. Brandenburg, P. H. Rothbarth, R. de Groot, A. Ott, and H. A. Van Steensel-Moll. 1996. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch. Dis. Child 75:137-140.
92. Knott, I., V. Weynants, K. Walravens, W. H. van der Poel, J. A. Kramps, and J. J. Letesson. 1998. Immune response of calves experimentally infected with non-cell-culture- passaged bovine respiratory syncytial virus. Arch. Virol. 143:1119-1128.
93. La Via, W. V., S. W. Grant, H. R. Stutman, and M. I. Marks. 1993. Clinical profile of pediatric patients hospitalized with respiratory syncytial virus infection. Clin. Pediatr. 32:450-454.
94. La Via, W. V., M. I. Marks, and H. R. Stutman. 1992. Respiratory syncytial virus puzzle: clinical features, pathophysiology, treatment, and prevention. J. Pediatr. 121:503-510.
95. MacDonald, N. E., C. B. Hall, S. C. Suffin, C. Alexson, P. J. Harris, and J. A. Manning. 1982. Respiratory syncytial viral infection in infants with congenital heart disease. N. Engl. J. Med. 307:397-400.
96. Martin, A. J., P. S. Gardner, and J. McQuillin. 1978. Epidemiology of respiratory viral infection among paediatric inpatients over a six-year period in north-east England. Lancet 2:1035-1038.
97. Martinez, F. D., W. J. Morgan, A. L. Wright, C. J. Holberg, and L. M. Taussig. 1988. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. N. Engl. J. Med. 319:1112-1117.
98. McConnochie, K. M. and K. J. Roghmann. 1986. Parental smoking, presence of older siblings, and family history of asthma increase risk of bronchiolitis. Am. J. Dis. Child 140:806-812.
99. Mcintosh, K., H. B. Masters, I. Orr, R. K. Chao, and R. M. Barkin. 1978. The immunologic response to infection with respiratory syncytial virus in infants. J. Infect. Dis. 138:24-32.
100. Meissner, H. C., D. R. Fulton, J. R. Groothuis, R. L. Geggel, G. R. Marx, V. G. Hemming, T. Hougen, and D. R. Snydman. 1993. Controlled trial to evaluate protection of high-risk infants against respiratory syncytial virus disease by using standard intravenous immune globulin. Antimicrob. Agents Chemother. 37:1655-1658.
101. Meissner, H. C., R. C. Welliver, S. A. Chartrand, B. J. Law, L. E. Weisman, H. L. Dorkin, and W. J. Rodriguez. 1999. Immunoprophylaxis with palivizumab, a humanized respiratory syncytial virus monoclonal antibody, for prevention of respiratory syncytial virus infection in high risk infants: a consensus opinion. Pediatr. Infect. Dis. J. 18:223-231.
102. Mills, J., 5th, J. E. Van Kirk, P. F. Wright, and R. M. Chanock. 1971. Experimental respiratory syncytial virus infection of adults. Possible mechanisms of resistance to infection and illness. J. Immunol. 107:123-130.
103. Morrison, W. I., G. Taylor, R. M. Gaddum, and S. A. Ellis. 1999. Contribution of advances in immunology to vaccine development. Adv. Vet. Med. 41:181-95:181-195.
104. Moss, B., M. W. Carroll, L. S. Wyatt, J. R. Bennink, V. M. Hirsch, S. Goldstein, W. R. Elkins, T. R. Fuerst, J. D. Lifson, M. Piatak, N. P. Restifo, W. Overwijk, R. Chamberlain, S. A. Rosenberg, and G. Sutter. 1996. Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. Adv. Exp. Med. Biol. 397:7-13:7-13.
105. Mufson, M. A., R. B. Belshe, C. Orvell, and E. Norrby. 1988. Respiratory syncytial virus epidemics: variable dominance of subgroups A and B strains among children, 1981-1986. J. Infect. Dis. 157:143-148.
106. Mufson, M. A., C. Orvell, B. Rafnar, and E. Norrby. 1985. Two distinct subtypes of human respiratory syncytial virus. J. Gen. Virol. 66:2111-2124.
107. Murphy, B. R., S. L. Hall, A. B. Kulkarni, J. E. Crowe Jr., P. L. Collins, M. Connors, R. A. Karron, and R. M. Chanock. 1994. An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. Virus Res. 32:13-36.
108. Murphy, B. R., G. A. Prince, E. E. Walsh, H. W. Kim, R. H. Parrott, V. G. Hemming, W. J. Rodriguez, and R. M. Chanock. 1986. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J. Clin. Microbiol. 24:197-202.
109. Murphy, B. R., A. V. Sotnikov, L. A. Lawrence, S. M. Banks, and G. A. Prince. 1990. Enhanced pul-

monary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3-6 months after immunization. Vaccine 8:497-502.
110. Nadal, D. and P. L. Ogra. 1990. Development of local immunity: role in mechanisms of protection against or pathogenesis of respiratory syncytial viral infections. Lung 168 Suppl:379-87:379-387.
111. Navas, L., E. Wang, V. de Carvalho, and J. Robinson. 1992. Improved outcome of respiratory syncytial virus infection in a high- risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J. Pediatr. 121:348-354.
112. Openshaw, P. J. 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am. J. Respir. Crit. Care Med. 152:S59-S62.
113. Osterhaus, A., G. van Amerongen, and R. van Binnendijk. 1998. Vaccine strategies to overcome maternal antibody mediated inhibition of measles vaccine. Vaccine 16:1479-1481.
114. Osterhaus, A. D. M. E. and G. F. Rimmelzwaan. 1997. Iscoms: Materials, preparations, antigen delivery and immune response. p. 123-138. In B. Gander, H. P. Merkle, and G. Corradin (eds.), Antigen Delivery Systems. Harwood Academic Publishers,
115. Paradiso, P. R., S. W. Hildreth, D. A. Hogerman, D. J. Speelman, E. B. Lewin, J. Oren, and D. H. Smith. 1994. Safety and immunogenicity of a subunit respiratory syncytial virus vaccine in children 24 to 48 months old. Pediatr. Infect. Dis. J. 13:792-798.
116. Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98:289-300.
117. Peret, T. C., C. B. Hall, K. C. Schnabel, J. A. Golub, and L. J. Anderson. 1998. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J. Gen. Virol. 79:2221-2229.
118. Piedra, P. A., S. Grace, A. Jewell, S. Spinelli, D. Bunting, D. A. Hogerman, F. Malinoski, and P. W. Hiatt. 1996. Purified fusion protein vaccine protects against lower respiratory tract illness during respiratory syncytial virus season in children with cystic fibrosis. Pediatr. Infect. Dis. J. 15:23-31.
119. Piedra, P. A., P. R. Wyde, W. L. Castleman, M. W. Ambrose, A. M. Jewell, D. J. Speelman, and S. W. Hildreth. 1993. Enhanced pulmonary pathology associated with the use of formalin-inactivated respiratory syncytial virus vaccine in cotton rats is not a unique viral phenomenon. Vaccine 11:1415-1423.
120. Plotnicky-Gilquin, H., L. Goetsch, T. Huss, T. Champion, A. Beck, J. F. Haeuw, T. N. Nguyen, J. Y. Bonnefoy, N. Corvaia, and U. F. Power. 1999. Identification of multiple protective epitopes (Protectopes) in the central conserved domain of a prototype human respiratory syncytial virus G protein. J. Virol. 73:5637-5645.
121. Plotnicky-Gilquin, H., T. Huss, J. P. Aubry, J. F. Haeuw, A. Beck, J. Y. Bonnefoy, T. N. Nguyen, and U. F. Power. 1999. Absence of lung immunopathology following respiratory syncytial virus (RSV) challenge in mice immunized with a recombinant RSV G protein fragment. Virology 258:128-140.
122. Prince, G. A., V. G. Hemming, R. L. Horswood, and R. M. Chanock. 1985. Immunoprophylaxis and immunotherapy of respiratory syncytial virus infection in the cotton rat. Virus Res. 3:193-206.
123. Prince, G. A., R. L. Horswood, and R. M. Chanock. 1985. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J. Virol. 55:517-520.
124. Rohwedder, A., O. Keminer, J. Forster, K. Schneider, E. Schneider, and H. Werchau. 1998. Detection of respiratory syncytial virus RNA in blood of neonates by polymerase chain reaction. J. Med. Virol. 54:320-327.
125. Roman, M., W. J. Calhoun, K. L. Hinton, L. F. Avendano, V. Simon, A. M. Escobar, A. Gaggero, and P. V. Diaz. 1997. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am. J. Respir. Crit. Care Med. 156:190-195.
126. Saez-Llorens, X., E. Castano, D. Null, J. Steichen, P. J. Sanchez, O. Ramilo, F. H. Top,Jr., and E. Connor. 1998. Safety and pharmacokinetics of an intramuscular humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. The MEDI-493 Study Group. Pediatr. Infect. Dis. J. 17:787-791.
127. Schneider, J., S. C. Gilbert, T. J. Blanchard, T. Hanke, K. J. Robson, C. M. Hannan, M. Becker, R. Sinden, G. L. Smith, and A. V. Hill. 1998. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus

Ankara. Nat. Med. 4:397-402.
128. Sheeran, P., H. Jafri, C. Carubelli, J. Saavedra, C. Johnson, K. Krisher, P. J. Sanchez, and O. Ramilo. 1999. Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease. Pediatr. Infect. Dis. J. 18:115-122.
129. Siber, G. R., J. Leszcynski, V. Pena-Cruz, C. Ferren-Gardner, R. Anderson, V. G. Hemming, E. E. Walsh, J. Burns, K. Mcintosh, and R. Gonin. 1992. Protective activity of a human respiratory syncytial virus immune globulin prepared from donors screened by microneutralization assay. J. Infect. Dis. 165:456-463.
130. Siegrist, C. A., H. Plotnicky-Gilquin, M. Cordova, M. Berney, J. Y. Bonnefoy, T. N. Nguyen, P. H. Lambert, and U. F. Power. 1999. Protective efficacy against respiratory syncytial virus following murine neonatal immunization with BBG2Na vaccine: influence of adjuvants and maternal antibodies. J. Infect. Dis. 179:1326-1333.
131. Singleton, R. J., K. M. Petersen, J. E. Berner, E. Schulte, K. Chiu, C. M. Lilly, E. A. Hughes, L. R. Bulkow, and T. L. Nix. 1995. Hospitalizations for respiratory syncytial virus infection in Alaska Native children. Pediatr. Infect. Dis. J. 14:26-30.
132. Smyth, R. L., J. N. Fletcher, H. M. Thomas, and C. A. Hart. 1997. Immunological responses to respiratory syncytial virus infection in infancy. Arch. Dis. Child 76:210-214.
133. Spence, L. and N. Barratt. 1968. Respiratory syncytial virus associated with acute respiratory infections in Trinidadian patients. Am. J. Epidemiol. 88:257-266.
134. Srikiatkhachorn, A. and T. J. Braciale. 1997. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. J. Exp. Med. 186:421-432.
135. Sterner, G., S. Wolontis, B. Bloth, and G. DeHevesy. 1966. Respiratory syncytial virus: an outbreak of acute respiratory illnesses in a home fore infants. Acta Paediatr. Scan. 55:273-279.
136. Stittelaar, K. J., R. L. de Swart, T. Kuiken, G. van Amerongen, H. W. Vos, P. van Schalkwijk, T. van der Kwast, L. S. Wyatt, B. Moss, and A. D. M. E. Osterhaus. 2000. Safety of Modified Vaccinia virus Ankara (MVA) in immune-suppressed macaques. Vaccine (in press)
137. Storch, G. A., L. J. Anderson, C. S. Park, C. Tsou, and D. E. Dohner. 1991. Antigenic and genomic diversity within group A respiratory syncytial virus. J. Infect. Dis. 163:858-861.
138. Straliotto, S. M., B. Roitman, J. B. Lima, G. B. Fischer, and M. M. Siqueira. 1994. Respiratory syncytial virus (RSV) bronchiolitis: comparative study of RSV groups A and B infected children. Rev. Soc. Bras. Med. Trop. 27:1-4.
139. Subramanian, K. N., L. E. Weisman, T. Rhodes, R. Ariagno, P. J. Sanchez, J. Steichen, L. B. Givner, T. L. Jennings, F. H. Top,Jr., D. Carlin, and E. Connor. 1998. Safety, tolerance and pharmacokinetics of a humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. MEDI-493 Study Group. Pediatr. Infect. Dis. J. 17:110-115.
140. Sullender, W. M., M. A. Mufson, L. J. Anderson, and G. W. Wertz. 1991. Genetic diversity of the attachment protein of subgroup B respiratory syncytial viruses. J. Virol. 65:5425-5434.
141. Sutter, G. and B. Moss. 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc. Natl. Acad. Sci. U. S. A. 89:10847-10851.
142. Sutter, G., L. S. Wyatt, P. L. Foley, J. R. Bennink, and B. Moss. 1994. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine 12:1032-1040.
143. Tang, Y. W., K. M. Neuzil, J. E. Fischer, F. W. Robinson, R. A. Parker, and B. S. Graham. 1997. Determinants and kinetics of cytokine expression patterns in lungs of vaccinated mice challenged with respiratory syncytial virus. Vaccine 15:597-602.
144. Thurau, A. M., H. J. Streckert, C. H. Rieger, and U. Schauer. 1998. Increased number of T cells committed to IL-5 production after respiratory syncytial virus (RSV) infection of human mononuclear cells in vitro. Clin. Exp. Immunol. 113:450-455.
145. Tissing, W. J. E., H. A. Van Steensel-Moll, and M. Offringa. 1993. Risk factors for mechanical ventilation in respiratory syncytial virus infection. Eur. J. pediatr. 152:125-127.
146. Tristram, D. A., R. C. Welliver, C. K. Mohar, D. A. Hogerman, S. W. Hildreth, and P. Paradiso. 1993. Immunogenicity and safety of respiratory syncytial virus subunit vaccine in seropositive children 18-36 months old. J. Infect. Dis. 167:191-195.
147. Trudel, M., F. Nadon, C. Séguin, C. Simard, and G. Lussier. 1989. Experimental polyvalent ISCOMs

subunit vaccine induces antibodies that neutralize human and bovine respiratory syncytial virus. Vaccine 7:12-16.
148. Tsutsumi, H., M. Onuma, K. Suga, T. Honjo, Y. Chiba, S. Chiba, and P. L. Ogra. 1988. Occurrence of respiratory syncytial virus subgroup A and B strains in Japan, 1980 to 1987. J. Clin. Microbiol. 26:1171-1174.
149. Vainionpaa, R., O. Meurman, and H. Sarkkinen. 1985. Antibody response to respiratory syncytial virus structural proteins in children with acute respiratory syncytial virus infection. J. Virol. 53:976-979.
150. Van Schaik, S. M., G. Enhorning, I. Vargas, and R. C. Welliver. 1998. Respiratory syncytial virus affects pulmonary function in BALB/c mice. J. Infect. Dis 177:269-276.
151. Van Schaik, S. M., D. A. Tristram, I. S. Nagpal, K. M. Hintz, R. C. Welliver,2nd, and R. C. Welliver. 1999. Increased production of IFN-gamma and cysteinyl leukotrienes in virus- induced wheezing. J. Allergy Clin. Immunol. 103:630-636.
152. Vardas, E., D. Blaauw, and J. McAnerney. 1999. The epidemiology of respiratory syncytial virus (RSV) infections in South African children. S. Afr. Med. J. 89:1079-1084.
153. Walsh, E. E., K. M. McConnochie, C. E. Long, and C. B. Hall. 1997. Severity of respiratory syncytial virus infection is related to virus strain. J. Infect. Dis. 175:814-820.
154. Ward, K. A., P. R. Lambden, M. M. Ogilvie, and P. J. Watt. 1983. Antibodies to respiratory syncytial virus polypeptides and their significance in human infection. J. Gen. Virol. 64:1867-1876.
155. Waris, M. E., C. Tsou, D. D. Erdman, S. R. Zaki, and L. J. Anderson. 1996. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J. Virol. 70:2852-2860.
156. Wathen, M. W., T. J. Kakuk, R. J. Brideau, E. C. Hausknecht, S. L. Cole, and R. M. Zaya. 1991. Vaccination of cotton rats with a chimeric FG glycoprotein of human respiratory syncytial virus induces minimal pulmonary pathology on challenge. J. Infect. Dis. 163:477-482.
157. Weltzin, R., V. Traina-Dorge, K. Soike, J.-Y. Zhang, P. Mack, G. Soman, G. Drabik, and T. P. Monath. 1996. Intranasal monoclonal IgA antibody to respiratory syncytial virus protects rhesus monkeys against upper and lower respiratory tract infection. J. Infect. Dis. 174:256-261.
158. West, K., L. Petrie, C. Konoby, D. M. Haines, V. Cortese, and J. A. Ellis. 1999. The efficacy of modified-live bovine respiratory syncytial virus vaccines in experimentally infected calves. Vaccine 18:907-919.
159. Whimbey, E., R. B. Couch, J. A. Englund, M. Andreeff, J. M. Goodrich, I. I. Raad, V. Lewis, N. Mirza, M. A. Luna, and B. Baxter. 1995. Respiratory syncytial virus pneumonia in hospitalized adult patients with leukemia. Clin. Infect. Dis. 21:376-379.
160. Whitehead, S. S., A. Bukreyev, M. N. Teng, C. Y. Firestone, M. St Claire, W. R. Elkins, P. L. Collins, and B. R. Murphy. 1999. Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. J. Virol. 73:3438-3442.
161. Woolums, A. R., R. S. Singer, G. A. Boyle, and L. J. Gershwin. 1999. Interferon gamma production during bovine respiratory syncytial virus (BRSV) infection is diminished in calves vaccinated with formalin- inactivated BRSV. Vaccine 17:1293-1297.
162. Wright, A. L., C. Holberg, F. D. Martinez, and L. M. Taussig. 1991. Relationship of parental smoking to wheezing and nonwheezing lower respiratory tract illnesses in infancy. Group Health Medical Associates. J. Pediatr. 118:207-214.
163. Wyatt, L. S., S. T. Shors, B. R. Murphy, and B. Moss. 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. Vaccine 14:1451-1458.
164. Wyatt, L. S., S. S. Whitehead, K. A. Venanzi, B. R. Murphy, and B. Moss. 1999. Priming and boosting immunity to respiratory syncytial virus by recombinant replication-defective vaccinia virus MVA. Vaccine 18:392-397.

6.2 Concluding remarks and summary

Concluding remarks and summary

For more than 40 years RSV has been recognized as the most prevalent cause of severe respiratory infection in young infants. Despite considerable research still no causative therapeutic strategy and no effective vaccine are available. The limited knowledge on the pathogenesis of lower respiratory tract involvement in RSV infection, both in naturally occurring and in enhanced disease following experimental FI-RSV vaccination, has hampered the development of therapeutic and preventive strategies. It has been shown in recent years that enhanced pulmonary disease after FI-RSV vaccination – at least in the mouse model – is associated with an aberrant type 2 like immune response. Whether this also is the case in naturally occurring RSV associated lower respiratory tract disease remains elusive. Studies on the immune-pathogenesis of RSV associated lower respiratory tract infection in human infants are more complicated, since several factors, related to the virus, the environment or the individual patient may also have an impact on the clinical outcome of naturally occurring RSV infection in young children (see table 6.2.1.).

Variation in the RSV strain involved may be a factor influencing clinical severity by as yet unknown virulence factors in certain strains or by less cross protective maternal antibodies, thus increasing the chance of becoming severely infected at a younger age. In chapter 2 we studied RSV strain variation in relation to clinical severity. Differences in clinical outcome between infections with the two RSV groups A and B were studied in a group of 232 children with a RSV infection, who were either admitted to, or visited the outpatient department of the Sophia Children's Hospital Rotterdam between 1992 and 1995. By analyzing a set of standardized clinical data collected from these children we found no relationship between clinical severity and the group of RSV involved.

Several studies have been published concerning the association between clinical severity and the RSV group involved. In about half of these studies infections with group A were found to be more severe (26,27,40,45,53,58,60) whereas in other studies no differences were detected between the infections with either of the groups (30,35,42,44,52,59-61). Only two studies ever reported group B to be more severe (31,57). Whether certain strains within the two groups are more virulent is not known but it has been suggested that some virus variants within group A may induce more severe disease (15,26,60). To address this question we selected 28 group A strains from three consecutive winters and subjected them to sequence analyses of part of the G protein. In the season 1994/95 a high incidence of RSV infections during a relatively short period was found which were all group A viruses. We studied whether this peak represented infection with one, possibly more virulent, strain of RSV. Several lineages circulated repeatedly and simultaneously during the respective seasons. This was also the case during the 1994/95 season and no one dominant strain was

Table 6.2.1. Factors influencing the clinical outcome of RSV infection.

Factors related to the	Disease severity	references
Virus		
Strain variation	no influence on disease severity	(30,35,42,44,52,59-61)
	group A more severe	(26,27,39,40,45,53,58,60)
Environment		
crowding (siblings, day care centers)	increased disease severity	(50)
socio-economic status	increased disease severity	(18)
parental smoking	increased disease severity	(41,55,63)
wood-burning stoves	increased disease severity	(55)
geographic area	varying disease severity	(6,32,61)
Individual patient		
young age	increased disease severity	(18,20,23,46,50)
gender	boys > girls	(13,17,18)
prematurity	increased disease severity	(47)
underlying illnesses		
- lung disease (BPD, cystic fibrosis)	increased disease severity	(21,47)
- congenital heart defects	increased disease severity	(36-38,47)
- T cell immune deficiency	increased disease severity	(24)
Immune response		
- Humoral: maternal/passively administered	protective	(18,22,28,51,54)
- Cellular	protective	(3-5,7,8,10)
	enhanced in the mouse model	(9,48,49,62)

found. Also no relationships were found between circulating RSV lineages on the one hand and clinical severity or age of the infected infants on the other hand. Taken together, the studies in chapter 2 showed no relationship between clinical severity and the RSV strain involved.

Environmental factors have been described to influence the clinical outcome of RSV infection. Circumstances related to crowding like number of siblings, attendance of day care centers and socioeconomic status, which increase the chance of early infection, are all associated with a higher chance of becoming severely affected (18,50). In addition factors, possibly influencing lung function, like parental smoking (41,55,63) and use of wood-burning stoves (55) have been associated with an increased risk of severe RSV infections. Difference in severity of RSV associated disease has been reported for different areas. In chapter 3 we assessed the possible influence of geographical differences in disease severity between two university hospitals in cities in different parts of Europe. RSV infections are considered a serious disease in the Sophia Children's Hospital (Rotterdam, The Netherlands) but generally more benign in the Geneva Children's Hospital (Switzerland). We studied 151 infants, 55 from Geneva and 96 from Rotterdam, primarily admitted for a RSV infection. Analysis of clinical data collected in a standardized way confirmed that the course of RSV infections was indeed more benign in Geneva than in Rotterdam: infants admitted in the Geneva Children's Hospital less often presented with apnea or respiratory insufficiency and thus less often required ICU admission or respiratory support. This was also reflected by a shorter length of hospitalization. We could not identify epidemiological factors explaining the differences between the two cities. Studies including more different geographical areas will probably be required to identify factors responsible for the differences in disease severity in different areas. We did conclude that geographical differences might influence the RSV associated disease pattern and thus may complicate the interpretation of multi-center studies aiming at the development of prophylactic and therapeutic strategies.

Individual patient characteristics are well known to influence the chance of becoming severely infected with RSV. Most severe RSV infections are seen when children are between six weeks and six months of age. The younger a child encounters its first RSV infection, the higher is its chance of lower respiratory tract involvement and apnea. Also boys have a higher hospitalization rate for RSV infection (13,17,18). Prematurely born children are at higher risk of becoming severely affected by RSV, possibly because they receive less maternal antibodies (47). Several underlying illnesses such as bronchopulmonary dysplasia, congenital heart disease with increased pulmonary circulation and immune deficiency significantly increase the risk of becoming severely ill when infected with RSV

(21,24,36-38,47). Also subclinical, reduced lung function has been associated with an increased risk for the development of lower respiratory tract disease in young children (39). So apart from the individual's immune responsiveness, anatomical and physiological conditions in the lungs influence the clinical outcome of RSV infection.
Immunity to RSV infection is incomplete and/or very short-lived and reinfections occur frequently throughout life (25,29). The way in which RSV evades the immune response is still unknown but the frequent reinfections in the whole population enable RSV to reach infants at an early age, when they are most vulnerable for severe lower respiratory tract involvement.

Antibodies have often been incriminated as playing a role in the pathology of RSV lower respiratory tract infection mainly on the basis of two observations. (i) Most severe infections are seen at an age when most children still have maternally derived antibodies (19,33), and (ii) the FI-RSV candidate vaccine – which predisposed for more severe disease upon subsequent infection – induced high levels of antibodies (11,16,34). However, it has become clear that virus neutralizing antibodies – albeit at high titers – are in fact protective against RSV lower respiratory tract disease (18,51).
In chapter 4 we studied the decline of maternally derived antibodies in and the response to RSV infection in young infants. In all children RSV neutralizing antibodies could be demonstrated at birth, which declined with a half-life of about 26 days. Given this half-life time and the relatively high titers necessary for protection, the observed antibody levels may be expected to drop relatively fast to non-protective levels. Furthermore, in a group of 38 children with a suspected RSV infection, all younger than six months of age upon admission, the value of serological assays to diagnose a RSV infection was evaluated. In 32 of these children RSV infection was confirmed by virus isolation, direct immune fluorescence and RT-PCR analyses. All serological tests together studied in chapter 4a identified only 13 (41%) of the 32 RSV infected infants. In chapter 4b a subtype specific ELISA using peptides from the central conserved region of the G protein detected 67% of infections making this peptide based ELISA more sensitive than all other serological tests combined, for this age group. Taken together we concluded that in children under six months of age the diagnostic value of RSV serology is limited and by far inferior to the direct detection methods for RSV antigen or viral RNA.
With the exception of pCO_2 levels, which were higher in children with higher VN titers upon admission, none of the parameters of clinical severity correlated with antibody levels upon admission. The correlation of pCO_2 levels with VN titers can be explained by the higher pCO_2 usually found in younger infants with RSV infection (46), at which age also higher maternal antibody levels are expected. Thus no causal relationship between RSV specif-

ic antibody titers and severity of infection was detected.

Virus specific T cell responses have been described to be involved in the clearance of RSV infection, as well as in the induction of immune pathology in mouse models: upon vaccination with FI-RSV, a predominant type 2 T help response was found (2,9,12,49,62). Moreover, in this respect it should be considered that young children, having a relatively immature immune system, are prone to develop a type 2 like immune response upon infection. Whether a type 2 like immune response does indeed play a role in naturally occurring RSV lower respiratory tract infection in infants remains elusive. In chapter 5.1 we studied inflammatory responses in infants, younger than six months of age, with RSV infections of different clinical severities. We found that infants with clinically more severe RSV infections have higher plasma levels of IL-6 and IL-8, which is probably a reflection of more extensive inflammation of the lungs in these children. The cellular components of nasopharyngeal washings of these infants mainly consisted of polymorphonuclear cells and monocytes and only few eosinophilic granulocytes were detected. A type 1 like T cell response characterized by the expression of mainly IFN-γ and only low levels of IL4 and IL10 was found in PBMC cultures of 15 children after RSV specific stimulation, regardless of clinical severity. Using FACS analysis, RSV specific IFN-γ expression proved to be more prominent in CD8+ than in CD4+ cells, indicating a virus specific HLA class-1 restricted CTL response in these children, which is usually associated with virus clearance (3,4,14). Moreover, in the mouse model, the absence of a CTL response was associated with the induction of enhanced disease following vaccination with FI-RSV (12,56). On the other hand CD8+ T cells – although to a lesser extent than CD4+ T cells – were also found to be associated with more lung inflammation in the mouse model (1,2). Therefore detailed identification and characterization of CTL responses against RSV in humans will contribute to the development of candidate vaccine targets and also to future studies concerning the role of CTL in the immune pathogenesis of RSV infection. In chapter 5.2 T cell clones (TCC), generated from two infants who had just recovered from severe RSV infection, were studied. These TCC were used to identify, for the first time, HLA class-I restricted CTL epitopes on the RSV fusion protein in humans. In the samples from these two children, only HLA class-1 restricted CD8+ TCC and no CD4+ TCC were detected, whereas high frequencies of type 2 cytokine producing CD4+ T cells have been found in different animal models of FI-RSV mediated immune-pathogenesis.

Taken together the data presented in chapter 5 do not support the hypothesis that severe RSV infection is associated with a type 2 like T cell response in naturally occurring RSV bronchiolitis.

Future directions

Recently passive immune prophylaxis against RSV infection has proven useful in high-risk children. However, the most cost effective and practical approach to prevent RSV lower respiratory tract infection will undoubtedly be vaccination. As has been discussed in chapter 6.1 the development of a safe and effective vaccine will require detailed knowledge of both protective and disease enhancing mechanisms in RSV infection.

In recent years several new techniques have become available, which will allow studying the immune response in more detail. For instance ELIspot techniques have shown to be sensitive and convenient tools to study T cell responses using only small amounts of patient material. Also DNA array assisted mRNA profiling technology is becoming available, making it possible to determine for example the expression levels of many mRNAs of cytokines and their receptors in a parallel fashion.

These and other techniques will significantly contribute to our knowledge of the RSV immune response in young infants and will hopefully show the way towards the most effective approach for the induction of safe and effective preventive and therapeutic strategies.

References

1. Alwan, W. H., W. J. Kozlowska, and P. J. M. Openshaw. 1994. Distinct types of lung disease caused by functional subsets of antiviral T cells. J. Exp. Med. 179:81-89.
2. Alwan, W. H., F. M. Record, and P. J. M. Openshaw. 1992. CD4+ T cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T cells. Clin. Exp. Immunol. 88:527-536.
3. Bangham, C. R. and A. J. McMichael. 1986. Specific human cytotoxic T cells recognize B-cell lines persistently infected with respiratory syncytial virus. Proc. Natl. Acad. Sci. U. S. A 83:9183-9187.
4. Bangham, C. R. M., M. J. Cannon, D. T. Karzon, and B. A. Askonas. 1985. Cytotoxic T-cell response to respiratory syncytial virus in mice. J. Virol. 56:55-59.
5. Bangham, C. R. M., P. J. M. Openshaw, L. A. Ball, A. M. Q. King, G. W. Wertz, and B. A. Askonas. 1986. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoproteins (G), expressed by vaccinia virus recombinants. J. Immunol. 137:3973-3977.
6. Brandenburg, A. H., P. Y. Jeannet, H. A. Steensel-Moll, A. Ott, P. H. Rothbarth, W. Wunderli, S. Suter, H. J. Neijens, A. D. Osterhaus, and C. A. Siegrist. 1997. Local variability in respiratory syncytial virus disease severity. Arch. Dis Child 77:410-414.
7. Brandenburg, A. H., A. Kleinjan, B. van het Land, H. A. Moll, H. H. Timmerman, R. L. de Swart, H. J. Neijens, W. Fokkens, and A. D. M. E. Osterhaus. 2000. A type 1 like immune response is found in children with RSV infection regardless of clinical severity. J. Med. Virol.
8. Cannon, M. J. and C. R. M. Bangham. 1989. Recognition of Respiratory Syncytial Virus Fusion Protein by Mouse cytotoxic T Cell Clones and a Human Cytotoxic T Cell Line. J. Gen. Virol. 70:79-87.
9. Cannon, M. J., P. J. M. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168:1163-1168.
10. Cherrie, A. H., K. Anderson, G. W. Wertz, and P. J. M. Openshaw. 1992. Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J. Virol. 66:2102-2110.
11. Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette. 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population.

Am. J. Epidemiol. 89:449-463.
12. Connors, M., A. B. Kulkarni, C.-Y. Firestone, K. L. Holmes, H. C. Morse III, A. V. Sotnikov, and B. R. Murphy. 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J. Virol. 66:7444-7451.
13. Denny, F. W., A. M. Collier, F. W. Henderson, and W. A. Clyde,Jr. 1977. The epidemiology of bronchiolitis. Pediatr. Res. 11:234-236.
14. Fishaut, M., D. Tubergen, and K. Mcintosh. 1980. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J. Pediatr. 96:179-186.
15. Fletcher, J. N., R. L. Smyth, H. M. Thomas, D. Ashby, and C. A. Hart. 1997. Respiratory syncytial virus genotypes and disease severity among children in hospital. Arch. Dis. Child 77:508-511.
16. Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn. 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. 89:435-448.
17. Glezen, P. and F. W. Denny. 1973. Epidemiology of acute lower respiratory disease in children. N. Engl. J. Med. 288:498-505.
18. Glezen, W. P., A. Paredes, J. E. Allison, L. H. Taber, and A. L. Frank. 1981. Risk of respiratory syncytial virus infection for infants from low- income families in relationship to age, sex, ethnic group, and maternal antibody level. J. Pediatr. 98:708-715.
19. Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child 140:543-546.
20. Green, M., A. F. Brayer, K. A. Schenkman, and E. R. Wald. 1989. Duration of hospitalization in previously well infants with respiratory syncytial virus infection. Pediatr. Infect. Dis. J. 8:601-605.
21. Groothuis, J. R., K. M. Gutierrez, and B. A. Lauer. 1988. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 82:199-203.
22. Groothuis, J. R., E. A. Simoes, M. J. Levin, C. B. Hall, C. E. Long, W. J. Rodriguez, J. Arrobio, H. C. Meissner, D. R. Fulton, and R. C. Welliver. 1993. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. The Respiratory Syncytial Virus Immune Globulin Study Group. N. Engl. J. Med. 329:1524-1530.
23. Hall, C. B., W. J. Hall, and D. M. Speers. 1979. Clinical and physiological manifestations of bronchiolitis and pneumonia. Outcome of respiratory syncytial virus. Am. J. Dis. Child 133:798-802.
24. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315:77-81.
25. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. 163:693-698.
26. Hall, C. B., E. E. Walsh, K. C. Schnabel, C. E. Long, K. M. McConnochie, S. W. Hildreth, and L. J. Anderson. 1990. 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. 162:1283-1290.
27. Heikkinen, T., M. Waris, O. Ruuskanen, A. Putto-Laurila, and J. Mertsola. 1995. Incidence of acute otitis media associated with group A and B respiratory syncytial virus infections. Acta Paediatr. 84:419-423.
28. Hemming, V. G., G. A. Prince, J. R. Groothuis, and G. R. Siber. 1995. Hyperimmune globulins in prevention and treatment of respiratory syncytial virus infections. Clin. Microbiol. Rev. 8:22-33.
29. Henderson, F. W., A. M. Collier, W. A. Clyde,Jr., and F. W. Denny. 1979. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N. Engl. J. Med. 300:530-534.
30. Hendry, R. M., A. L. Talis, E. Godfrey, L. J. Anderson, B. F. Fernie, and K. Mcintosh. 1986. Concurrent circulation of antigenically distinct strains of respiratory syncytial virus during community outbreaks. J. Infect. Dis. 153:291-297.
31. Hornsleth, A., B. Klug, M. Nir, J. Johansen, K. S. Hansen, L. S. Christensen, and L. B. Larsen. 1998. Severity of respiratory syncytial virus disease related to type and genotype of virus and to cytokine values in nasopharyngeal secretions. Pediatr. Infect. Dis. J. 17:1114-1121.

32. Kapikian, A. Z., J. A. Bell, F. M. Mastrota, K. M. Johnson, R. J. Huebner, and R. M. Channock. 1961. An outbreak of febrile illnessand pneumonia associated with respiratory syncytial virus infection. Am. J. Hyg. 74:234-248.
33. Kim, H. W., J. O. Arrobio, C. D. Brandt, B. C. Jeffries, G. Pyles, J. L. Reid, R. M. Chanock, and R. H. Parrott. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. Am. J. Epidemiol. 98:216-225.
34. Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. E. Jensen, and R. H. Parrott. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89:422-434.
35. Kneyber, M. C., A. H. Brandenburg, P. H. Rothbarth, R. de Groot, A. Ott, and H. A. Van Steensel-Moll. 1996. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch. Dis. Child 75:137-140.
36. La Via, W. V., S. W. Grant, H. R. Stutman, and M. I. Marks. 1993. Clinical profile of pediatric patients hospitalized with respiratory syncytial virus infection. Clin. Pediatr. 32:450-454.
37. La Via, W. V., M. I. Marks, and H. R. Stutman. 1992. Respiratory syncytial virus puzzle: clinical features, pathophysiology, treatment, and prevention. J. Pediatr. 121:503-510.
38. MacDonald, N. E., C. B. Hall, S. C. Suffin, C. Alexson, P. J. Harris, and J. A. Manning. 1982. Respiratory syncytial viral infection in infants with congenital heart disease. N. Engl. J. Med. 307:397-400.
39. Martinez, F. D., W. J. Morgan, A. L. Wright, C. J. Holberg, and L. M. Taussig. 1988. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. N. Engl. J. Med. 319:1112-1117.
40. McConnochie, K. M., C. B. Hall, E. E. Walsh, and K. J. Roghmann. 1990. Variation in severity of respiratory syncytial virus infections with subtype. J. Pediatr. 117:52-62.
41. McConnochie, K. M. and K. J. Roghmann. 1986. Parental smoking, presence of older siblings, and family history of asthma increase risk of bronchiolitis. Am. J. Dis. Child 140:806-812.
42. McIntosh, E. D., L. M. De Silva, and R. K. Oates. 1993. Clinical severity of respiratory syncytial virus group A and B infection in Sydney, Australia. Pediatr. Infect. Dis. J. 12:815-819.
43. Meehan, J. T., R. C. Cutlip, H. D. Lehmkuhl, J. P. Kluge, and M. R. Ackermann. 1994. Infected cell types in ovine lung following exposure to bovine respiratory syncytial virus. Vet. Pathol. 31:229-236.
44. Monto, A. S. and S. Ohmit. 1990. Respiratory syncytial virus in a community population: circulation of subgroups A and B since 1965. J. Infect. Dis. 161:781-783.
45. Mufson, M. A., R. B. Belshe, C. Orvell, and E. Norrby. 1988. Respiratory syncytial virus epidemics: variable dominance of subgroups A and B strains among children, 1981-1986. J. Infect. Dis. 157:143-148.
46. Mulholland, E. K., A. Olinsky, and F. A. Shann. 1990. Clinical findings and severity of acute bronchiolitis. Lancet 335:1259-1261.
47. Navas, L., E. Wang, V. de Carvalho, and J. Robinson. 1992. Improved outcome of respiratory syncytial virus infection in a high- risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J. Pediatr. 121:348-354.
48. Openshaw, P. J. 1995. Immunopathological mechanisms in respiratory syncytial virus disease. Springer Semin. Immunopathol. 17:187-201.
49. Openshaw, P. J. 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am. J. Respir. Crit. Care Med. 152:S59-S62.
50. Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98:289-300.
51. Prince, G. A., R. L. Horswood, and R. M. Chanock. 1985. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J. Virol. 55:517-520.
52. Russi, J. C., H. Chiparelli, A. Montano, P. Etorena, and M. Hortal. 1989. Respiratory syncytial virus subgroups and pneumonia in children. Lancet 2:1039-1040.
53. Salomon, H. E., M. M. Avila, M. C. Cerqueiro, C. Orvell, and M. Weissenbacher. 1991. Clinical and epidemiologic aspects of respiratory syncytial virus antigenic variants in Argentinian children. J.

Infect. Dis. 163:1167

54. Siber, G. R., J. Leszcynski, V. Pena-Cruz, C. Ferren-Gardner, R. Anderson, V. G. Hemming, E. E. Walsh, J. Burns, K. Mcintosh, and R. Gonin. 1992. Protective activity of a human respiratory syncytial virus immune globulin prepared from donors screened by microneutralization assay. J. Infect. Dis. 165:456-463.
55. Singleton, R. J., K. M. Petersen, J. E. Berner, E. Schulte, K. Chiu, C. M. Lilly, E. A. Hughes, L. R. Bulkow, and T. L. Nix. 1995. Hospitalizations for respiratory syncytial virus infection in Alaska Native children. Pediatr. Infect. Dis. J. 14:26-30.
56. Srikiatkhachorn, A. and T. J. Braciale. 1997. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. J. Exp. Med. 186:421-432.
57. Straliotto, S. M., B. Roitman, J. B. Lima, G. B. Fischer, and M. M. Siqueira. 1994. Respiratory syncytial virus (RSV) bronchiolitis: comparative study of RSV groups A and B infected children. Rev. Soc. Bras. Med. Trop. 27:1-4.
58. Taylor, C. E., S. Morrow, M. Scott, B. Young, and G. L. Toms. 1989. Comparative virulence of respiratory syncytial virus subgroups A and B. Lancet 1:777-778.
59. Tsutsumi, H., M. Onuma, K. Nagai, H. Yamazaki, and S. Chiba. 1991. Clinical characteristics of respiratory syncytial virus (RSV) subgroup infections in Japan. Scand. J. Infect. Dis. 23:671-674.
60. Walsh, E. E., K. M. McConnochie, C. E. Long, and C. B. Hall. 1997. Severity of respiratory syncytial virus infection is related to virus strain. J. Infect. Dis. 175:814-820.
61. Wang, E. E., B. J. Law, and D. Stephens. 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.
62. Waris, M. E., C. Tsou, D. D. Erdman, S. R. Zaki, and L. J. Anderson. 1996. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J. Virol. 70:2852-2860.
63. Wright, A. L., C. Holberg, F. D. Martinez, and L. M. Taussig. 1991. Relationship of parental smoking to wheezing and nonwheezing lower respiratory tract illnesses in infancy. Group Health Medical Associates. J. Pediatr. 118:207-214.

Abbreviations

µl	microliter
B-LCL	B lymfoblastoid celline
BPD	bronchopulmonary dysplasia
BSA	bovine serum albumin
cDNA	complementary desoxy ribonucleic acid
CF-assay	complement fixation assay
CHD	congenital heart disease
comp-F ELISA	competition ELISA against RSV-F protein
comp-G ELISA	competition ELISA against RSV-G protein
cpts	cold passaged temperature sensitive
CTL	cytotoxic T lymphocytes
DIFA	direct immune fluorescence
DNA	desoxy ribonucleic acid
EBV	Epstein-Barr virus
ECP	eosinophilic cationic protein
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FIO_2	fraction of inspired oxygen
FI-RSV	formalin inactivated respiratory syncytial virus
FITC	fluorescein isothiocyanate
FSC	forward scatter
GMT	geometric mean titer
HLA	human leukocyte antigen
HPS	human pooled serum
HRPO	horse radish peroxidase
HRSV	human respiratory syncytial virus
ICAM	intercellular adhesion molecule
ICU	intensive care unit
IFN-γ	interferon gamma
IgA	immuneglobulin class A
IgG	immuneglobulin class G
IgM	immuneglobulin class M
IHW	International Histocompatibility Workshop
IL	interleukin

iscom	immune stimulating complex
IU	international units
kPa	kilopascal
LRTI	lower respiratory tract infection
MAB	monoclonal antibody
ml	milliliter
MVA	modified vaccinia virus Ankara
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pCO_2	carbon dioxide pressure
PCR	polymerase chain reaction
PFP	purified F protein
pg	picogram
PMN	polymorphonuclear cells
RNA	ribonucleic acid
RPE	R.Phycoerythrin
RPE-Cy5	R-phycoerythrin-Cyanine 5
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
SaO_2	oxygen saturation
sCD25	soluble CD25
SSC	side scatter
TCC	T cell clones
TNF-α	tumor necrosis factor alpha
URTI	upper respiratory tract infection
VI	virus isolation
VN	virus neutralizing

Algemene conclusies en samenvatting

RSV is de meest voorkomende verwekker van ernstige luchtweginfecties bij jonge kinderen. De meeste RSV-infecties verlopen mild en maar een kleine minderheid van geïnfecteerde kinderen (0,5 – 2%) behoeft ziekenhuisopname. RSV-infecties komen echter veelvuldig in de populatie voor en geschat wordt dat minstens 50% van alle kinderen hun eerste RSV-infectie oploopt tijdens het eerste levensjaar (31). Hierdoor is ernstig verlopende RSV-infectie een belangrijke oorzaak van ziekenhuisopname bij baby's.
Infecties met RSV komen wereldwijd voor. In gematigde streken komen RSV-infecties alleen in de winterperiode voor, waar ieder jaar een epidemiologische verheffing gezien wordt met de piek rond december/januari. In tropische gebieden worden infecties het hele jaar door gevonden met in sommige streken pieken tijdens het natte seizoen (33,57,61).
De overdracht van het virus vindt plaats via direct contact met een geïnfecteerde persoon, via grote druppelinfectie of via contact met besmette oppervlakken (23). RSV kan enkele uren overleven op bijvoorbeeld tafeloppervlakken, handschoenen, stethoscopen en kleding en ongeveer een half uur op huid (6,24). Infectie via kleine druppelaerosolen over grotere afstanden zoals bij influenza, is voor RSV nooit gevonden.

Op dit moment is er geen veilig en effectief vaccin tegen RSV beschikbaar. In de zestiger jaren toen het virus pas ontdekt was is een formaline-geïnactiveerd RSV-vaccin (FI-RSV) ontwikkeld. Dit vaccin induceerde hoge antistoftiters tegen RSV, echter na toediening van dit vaccin aan een groep kinderen bleek dat ze niet beschermd waren tegen RSV, integendeel, ze hadden een veel grotere kans op het ontwikkelen van een ernstig verloop van de infectie en er zijn zelfs enkele gevaccineerde kinderen overleden (12,17,35).
De beperkte kennis van de pathogenese van lagere luchtweginfecties met RSV, zowel bij natuurlijke infecties als bij vaccin geïnduceerde versterkte pathologie, hebben de ontwikkeling van preventieve en therapeutische strategieën bij RSV-infecties in de weg gestaan. Recentelijk is in muizenexperimenten gevonden dat door vaccin geïnduceerde versterkte pathologie geassocieerd is met een type 2-achtige cellulaire immuunrespons (49). Of dit bij natuurlijk optredende RSV-infecties van de lagere luchtwegen bij kinderen ook het geval is, is onbekend. Evaluatie van onderzoek naar een mogelijke immunologische component in het ontstaan van ernstige RSV-infecties bij kinderen is gecompliceerd omdat meerdere factoren, gerelateerd aan het virus, aan de omgeving en aan de omstandigheden in de individuele patiënt van grote invloed kunnen zijn op de ernst van de infectie (zie ook tabel 1).

Variatie in circulerende virusstammen zou de ernst van de RSV-infectie kunnen beïnvloeden door op dit moment onbekende virulentiefactoren in het virus of door maternale anti-

Tabel 1. Factoren die de ernst van de RSV-infectie kunnen beïnvloeden.

Factoren gerelateerd aan	**Ernst van de ziekte**	**referenties**
Het virus		
Variatie in virusstam	Geen invloed op ernst van de ziekte	(32,36,43,45,53,60,62,63)
	Groep A ernstiger	(28,29,40,41,46,54,59,62)
De omgeving		
Crowding (meer broertjes en zusjes, crèchebezoek, lagere sociaal-economische status)	Ernstiger ziektebeeld	(19,51)
Rokende ouders	Ernstiger ziektebeeld	(42,56,65)
Gebruik van houtkachels	Ernstiger ziektebeeld	(56)
Geografisch gebied	Variërende ernst van de ziekte	(7,34,63)
De individuele patiënt		
jonge leeftijd	Ernstiger ziektebeeld	(19,20,25,47,51)
geslacht	jongens ernstiger dan meisjes	(14,18,19)
prematuriteit	Ernstiger ziektebeeld	(48)
onderliggende ziekte		
- long ziekte (BPD, Cystische fibrose)	Ernstiger ziektebeeld	(21,48)
- congenitale hartafwijking	Ernstiger ziektebeeld	(37-39,48)
- T cel immuun deficiëntie	Ernstiger ziektebeeld	(26)
Immunologische respons		
- Humoraal: maternaal/passief toegediend	Beschermend	(19,22,30,52,55)
- Cellulair	Beschermend	(3-5,8,9,11)
	Ernstiger ziektebeeld in muizenmodel	(10,49,50,64)

stoffen die minder kruisreageren met andere virusstammen waardoor kinderen al jonger gevoelig zijn voor lagere luchtweginfecties. In hoofdstuk 2 wordt de variatie in virusstam vergeleken met de ernst van infectie. Verschillen in ernst tussen de twee bekende RSV groepen A en B werden bestudeerd in een groep van 232 kinderen met een RSV-infectie die gezien werden in het Sophia kinderziekenhuis Rotterdam gedurende de periode 1992 – 1995. Er werd geen relatie gevonden tussen de ernst van de infectie en de RSV-groep waarmee de kinderen geïnfecteerd waren.
In de literatuur zijn verschillende studies gepubliceerd waarin de relatie tussen de ernst van infectie en de twee RSV-groepen werd vergeleken. In ongeveer de helft van deze studies werd gevonden dat infectie met RSV-A ernstiger verliep dan infectie met RSV-B (28,29,41,46,54,59,62). In de andere helft werd geen verschil in ernst gevonden (32,36,43,45,53,60,62,63). Of er bepaalde stammen binnen de 2 groepen zijn die ernstiger infecties veroorzaken is onbekend maar er is gesuggereerd dat bepaalde RSV-A stammen meer pathogeen zijn (16,28,62).
Gedurende het winterseizoen 1994/95 werd een groot aantal kinderen opgenomen met een RSV-infectie, welke allemaal tot groep-A behoorden, gedurende een relatief korte periode. Om te bestuderen of deze piek één enkele, mogelijk meer pathogene stam, vertegenwoordigde, werden 28 RSV-A stammen geselecteerd uit de 3 seizoenen 1992 – 1995 en bestudeerd via sequentieanalyse van een variabel deel van het RSV-G eiwit. Gedurende alle 3 seizoenen circuleerden er verschillende stammen gelijktijdig. Ook gedurende het seizoen 1994/95 werd niet één enkele, dominante virusstam gevonden. Er werd ook geen relatie gevonden tussen de klinische ernst van de infectie en de verschillende stammen. Samenvattend geven de studies in hoofdstuk 2 geen aanwijzing voor een relatie tussen de ernst van infectie en de betreffende virusstam.

Omgevingsfactoren kunnen van invloed zijn op de ernst van RSV-infecties. Omstandigheden die gerelateerd zijn aan "crowding", zoals oudere broertjes en zusjes, crèche bezoek en lagere sociaal-economische status verhogen de kans op een ernstiger RSV-infectie (19,51). Ook factoren die van invloed kunnen zijn op de longfunctie, zoals rokende ouders en het gebruik van houtkachels zijn geassocieerd met een hogere kans op een ernstiger verloop van een RSV-infectie (42,56,65). In hoofdstuk 3 wordt de mogelijk invloed van het geografisch gebied op de ernst van de ziekte bestudeerd. Er werd een gestandaardiseerde set klinische gegevens verzameld van RSV-geïnfecteerde kinderen, jonger dan 12 maanden, in twee universiteitsziekenhuizen in verschillende delen van Europa. In het Sophia kinderziekenhuis (Rotterdam, Nederland) worden RSV-infecties beschouwd als een ernstige ziekte terwijl in het kinderziekenhuis Genève RSV-infecties als een milde ziekte worden beschouwd. Er werden 151 kinderen, 55 uit Genève (Zwitserland) en 96 uit

Rotterdam, primair opgenomen voor een RSV-infectie, in de studie geïncludeerd. Analyse van de verzamelde klinische data van de kinderen bevestigde dat het klinisch verloop van de infectie inderdaad milder was in kinderen uit Genève dan in kinderen uit Rotterdam. Kinderen in Genève hadden minder vaak apneu's en hoefden minder vaak beademd te worden. Zij waren gemiddeld ook minder lang opgenomen dan Rotterdamse kinderen. Er werden geen epidemiologische factoren gevonden die dit verschil konden verklaren. Waarschijnlijk zijn studies die meer verschillende geografische gebieden binnen één studie bestuderen noodzakelijk om onderliggende factoren, verantwoordelijk voor verschil in ernst van de ziekte, te detecteren. Er werd wel geconcludeerd dat een verschil in klinische ernst kan bestaan tussen verschillende gebieden en dat dit van invloed kan zijn op de interpretatie van uitkomsten van trials die mogelijke profylactische of therapeutische strategieën bestuderen.

Individuele patiënt-factoren zijn duidelijk van invloed op de kans op ernstige ziekte bij een RSV-infectie. De meest ernstige infecties worden gezien bij zeer jonge kinderen, wanneer zij tussen 6 weken en 6 maanden oud zijn. Hoe jonger een kind is bij de eerste RSV-infectie hoe groter de kans op een ernstig verloop. Jongetjes lopen een hogere kans om opgenomen te worden met een RSV-infectie dan meisjes (14,18,44). Prematuur geboren kinderen hebben een hogere kans op een ernstige infectie, mogelijk omdat zij minder maternale antistoffen meegekregen hebben (48). Verschillende onderliggende ziektes zoals bronchopulmonale dysplasie, congenitale hartafwijkingen met verhoogde bloedcirculatie in de longen en T-cel-immuunstoornissen verhogen allemaal de kans op een ernstig verloop van een RSV-infectie (21,26,39,48). Behalve de individuele immuunrespons zijn anatomische en fysiologische condities in de patiënt dus zeker van invloed op het klinisch verloop van een RSV-infectie.

De natuurlijke immuniteit tegen RSV is incompleet. Herinfecties komen veelvuldig voor gedurende het hele leven (27,31). Hierdoor kan het virus zich zeer effectief onder de bevolking verspreiden en het overgrote deel van de baby's al in het eerste levensjaar besmetten, op de leeftijd dat ze het meest gevoelig zijn voor RSV-infectie. De manier waarop RSV het immuunsysteem omzeilt en steeds herinfecties kan veroorzaken is onbekend.

Lang is gedacht dat antistoffen een rol spelen in de pathogenese van lagere luchtweginfecties met RSV. Dit werd voornamelijk gedacht op basis van twee observaties: (i) De meest ernstige infecties worden gezien op een leeftijd dat kinderen nog maternale antistoffen bezitten en (ii) het formaline-geïnactiveerde RSV-vaccin induceerde hoge antistoftiters en predisponeerde ook voor een ernstige verloop van een RSV-infectie. Het is de laatste jaren

echter duidelijk geworden dat virus-neutraliserende antistoffen juist beschermend werken tegen lagere luchtweginfectie met RSV. Hiervoor zijn wel relatief hoge virus-neutraliserende antistoftiters nodig.
In hoofdstuk 4 wordt het verloop van maternaal verkregen antistoftiters in kinderen bestudeerd. In alle kinderen werden bij geboorte RSV-specifieke antistoffen aangetoond. Deze antistoftiters verminderden met een halfwaardetijd van ongeveer 26 dagen. Bij deze halfwaardetijd en de relatief hoge titers die nodig zijn voor bescherming van de lagere luchtwegen, is het waarschijnlijk dat antistoftiters snel tot beneden de beschermende waarde dalen.
Verder werd de waarde van serologische testen bij RSV infecties onderzocht in een groep van 38 kinderen jonger dan 6 maanden, met een acute luchtweginfectie, waarbij RSV als verwekker waarschijnlijk werd geacht. Bij 32 van deze kinderen werd een acute RSV-infectie vastgesteld met directe antigeendetectie, virale kweek en RT-PCR. In alle serologische testen samen in hoofdstuk 4A werd maar in 13 (41%) van de kinderen een significante antistofrespons gevonden. In hoofdstuk 4B werd een subtype specifieke ELISA getest, waarbij peptiden van het centraal geconserveerde deel van het G-eiwit als antigeen werden gebruikt. In deze ELISA werd bij 67% een significante respons gevonden. Ook differentieerde de peptide ELISA goed tussen RSV-A en B infecties. Geconcludeerd werd dat de peptide ELISA zoals beschreven gevoeliger was dan de hoofdstuk 4A beschreven serologische tests. Echter serologie als diagnostiek bij RSV-infecties is duidelijk ongevoeliger dan detectie van het virus of componenten hiervan bij jonge kinderen.
Met uitzondering van de pCO_2, die gemiddeld hoger was bij kinderen met een hogere titer aan virus neutraliserende antistoffen bij opname, werd er geen verband gevonden tussen klinische parameters en antistoftiters. De correlatie tussen pCO_2 en virus-neutraliserende antistoftiters kan worden verklaard door de bekende relatie tussen jongere leeftijd en hogere pCO_2 bij RSV-infectie (47), op welke leeftijd ook gemiddeld hoger antistoftiters gevonden worden. In hoofdstuk 4 werd dus geen relatie tussen virus-neutraliserende antistoftiter en ernst van de infectie aangetoond.

De virusspecifieke T-cel-respons speelt een rol in zowel bescherming tegen infectie als bij de inductie van immuunpathologische respons in het muizenmodel. Na vaccinatie met FI-RSV werd een type 2 cellulaire immuunrespons gevonden in muizen (2,10,13,49,64). Zeer jonge kinderen hebben een onrijp immuunsysteem en een predispositie tot het vormen van een type 2-achtige immuunrespons. Of een type 2-cellulaire immuunrespons een rol speelt bij het ontstaan van een ernstig klinisch beeld bij een deel van de RSV-geïnfecteerde jonge kinderen is echter onbekend. In hoofdstuk 5.1 werd de afweerrespons gemeten in relatie tot de klinische ernst bij kinderen, jonger dan 6 maanden, met een acute RSV-infectie. In plas-

mamonsters van kinderen met een ernstiger klinisch beeld werden hogere concentraties IL6 en IL8 gevonden, passend bij meer ontstekingsreactie in de longen van deze kinderen. De cellulaire component van nasopharynx-spoelsels bestond voornamelijk uit polymorfkernige granulocyten en monocyten bij zowel ernstig zieke kinderen als bij kinderen met een mild ziektebeeld. Er werden slechts weinig eosinofiele granulocyten gevonden, ook bij de ernstig zieke kinderen. In een subgroep van 15 kinderen werd de virusspecifieke T-cel-respons gemeten in perifere bloedmonsters. Er werd een type 1-achtige T-cel-respons gevonden, gekarakteriseerd door expressie van voornamelijk IFN-γ en slechts weinig IL4 en IL10, bij zowel kinderen met een ernstig als met een mild ziektebeeld. Met gebruik van FACS-analyse werd expressie van IFN-γ meer in CD8+ T-cellen gevonden dan in CD4+ T-cellen. Dit impliceert een virusspecifieke cytotoxische-T-cel-respons (CTL-respons), over het algemeen geassocieerd met klaring van de virusinfectie (3,4,15). In het muizenmodel wordt het niet induceren van een CTL-respons na vaccinatie met FI-RSV geassocieerd met de versterkte ontstekingsreaktie in de longen (13,58). Aan de andere kant worden virusspecifieke CD8+ T- cellen, hoewel minder sterk dan CD4+ T-cellen, geassocieerd met meer ontsteking in de longen bij muizen (1,2). Gedetailleerde identificatie en karakterisering van de CTL-respons tegen RSV-infectie kan daarom van belang zijn voor studies die de mogelijk immuunpathogenese van RSV bestuderen en voor de ontwikkeling van mogelijke vaccins tegen RSV. In hoofdstuk 5.2 worden T-cel kloons bestudeerd, die gegenereerd zijn uit bloedmonsters van 2 kinderen die recentelijk hersteld waren van een ernstige RSV-infectie. Deze T-celkloons werden gebruikt om HLA klasse-I gerestricteerde CTL-epitopen op het RSV fusie proteïne, voor het eerst bij de mens te identificeren. In de bloedmonsters van deze kinderen werden alleen CD8+ en geen CD4+ T cel kloons gedetecteerd, terwijl in diermodellen na FI-RSV-vaccinatie hoge frequenties van type 2-cytokine producerende CD4+ cellen gedetecteerd werden.

Gezamenlijk zijn de data in hoofdstuk 5 geen ondersteuning voor de hypothese dat een abberante, type 2-achtige, immuunrespons ten grondslag ligt aan de pathogenese van ernstige natuurlijk optredende RSV-infecties van de lagere luchtwegen.

Toekomstige richtingen voor RSV research

Recentelijk is passieve immunoprofylaxe bij kinderen met een hoog risico zinvol gebleken in de vorm van maandelijkse toediening van intraveneus gammaglobuline of een gehumaniseerd anti-RSV-monoklonaal. De meest kosten-effectieve en praktische oplossing ter preventie van ernstige RSV-infecties blijft natuurlijk de ontwikkeling van een effectief vaccin. Zoals bediscussieerd in hoofdstuk 6.1 is voor het ontwikkelen van een veilig en effectief vaccin gedetailleerde kennis noodzakelijk van beschermende en ziekte versterkende mechanismen bij RSV-infecties.

Er komen steeds meer technieken beschikbaar waarmee de immuunrespons in detail bestudeerd kan worden. De ELIspot techniek is bijvoorbeeld een gevoelige en technisch goed bruikbare methode om de T cel respons te bestuderen wanneer maar kleine hoeveelheden patiëntmateriaal beschikbaar zijn. De recent beschikbaar gekomen. Een andere voorbeeld is de DNA-array technologie. Hiermee is het mogelijk om messenger-RNA-profielen van vele cytokines en hun receptoren uit één monster te bepalen, waarmee de immunologische respons gedetailleerd gekarakteriseerd kan worden.

Deze en andere technieken kunnen een bijdrage leveren aan onze kennis van de RSV-specifieke immuunrespons en aan het bepalen van de meest veelbelovende richting voor het ontwikkelen van veilige en effectieve strategieën ter voorkoming van RSV-infecties.

Literatuur

1. Alwan, W. H., W. J. Kozlowska, and P. J. M. Openshaw. 1994. Distinct types of lung disease caused by functional subsets of antiviral T cells. J. Exp. Med. 179:81-89.
2. Alwan, W. H., F. M. Record, and P. J. M. Openshaw. 1992. CD4+ T cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T cells. Clin. Exp. Immunol. 88:527-536.
3. Bangham, C. R. and A. J. McMichael. 1986. Specific human cytotoxic T cells recognize B-cell lines persistently infected with respiratory syncytial virus. Proc. Natl. Acad. Sci. U. S. A 83:9183-9187.
4. Bangham, C. R. M., M. J. Cannon, D. T. Karzon, and B. A. Askonas. 1985. Cytotoxic T-cell response to respiratory syncytial virus in mice. J. Virol. 56:55-59.
5. Bangham, C. R. M., P. J. M. Openshaw, L. A. Ball, A. M. Q. King, G. W. Wertz, and B. A. Askonas. 1986. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoproteins (G), expressed by vaccinia virus recombinants. J. Immunol. 137:3973-3977.
6. Blydt-Hansen, T., K. Subbarao, P. Quennec, and J. McDonald. 1999. Recovery of respiratory syncytial virus from stethoscopes by conventional viral culture and polymerase chain reaction. Pediatr. Infect. Dis. J. 18:164-165.
7. Brandenburg, A. H., P. Y. Jeannet, H. A. Steensel-Moll, A. Ott, P. H. Rothbarth, W. Wunderli, S. Suter, H. J. Neijens, A. D. Osterhaus, and C. A. Siegrist. 1997. Local variability in respiratory syncytial virus disease severity. Arch. Dis Child 77:410-414.
8. Brandenburg, A. H., A. Kleinjan, B. van het Land, H. A. Moll, H. H. Timmerman, R. L. de Swart, H. J. Neijens, W. Fokkens, and A. D. M. E. Osterhaus. 2000. A type 1 like immune response is found in children with RSV infection regardless of clinical severity. J. Med. Virol. (Abstract)
9. Cannon, M. J. and C. R. M. Bangham. 1989. Recognition of Respiratory Syncytial Virus Fusion Protein by Mouse cytotoxic T Cell Clones and a Human Cytotoxic T Cell Line. J. Gen. Virol. 70:79-87.
10. Cannon, M. J., P. J. M. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168:1163-1168.
11. Cherrie, A. H., K. Anderson, G. W. Wertz, and P. J. M. Openshaw. 1992. Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J. Virol. 66:2102-2110.
12. Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette. 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. 89:449-463.
13. Connors, M., A. B. Kulkarni, C.-Y. Firestone, K. L. Holmes, H. C. Morse III, A. V. Sotnikov, and B. R. Murphy. 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J. Virol. 66:7444-7451.

14. Denny, F. W., A. M. Collier, F. W. Henderson, and W. A. Clyde,Jr. 1977. The epidemiology of bronchiolitis. Pediatr. Res. 11:234-236.
15. Fishaut, M., D. Tubergen, and K. Mcintosh. 1980. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J. Pediatr. 96:179-186.
16. Fletcher, J. N., R. L. Smyth, H. M. Thomas, D. Ashby, and C. A. Hart. 1997. Respiratory syncytial virus genotypes and disease severity among children in hospital. Arch. Dis. Child 77:508-511.
17. Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn. 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aquaeous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. 89:435-448.
18. Glezen, P. and F. W. Denny. 1973. Epidemiology of acute lower respiratory disease in children. N. Engl. J. Med. 288:498-505.
19. Glezen, W. P., A. Paredes, J. E. Allison, L. H. Taber, and A. L. Frank. 1981. Risk of respiratory syncytial virus infection for infants from low- income families in relationship to age, sex, ethnic group, and maternal antibody level. J. Pediatr. 98:708-715.
20. Green, M., A. F. Brayer, K. A. Schenkman, and E. R. Wald. 1989. Duration of hospitalization in previously well infants with respiratory syncytial virus infection. Pediatr. Infect. Dis. J. 8:601-605.
21. Groothuis, J. R., K. M. Gutierrez, and B. A. Lauer. 1988. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 82:199-203.
22. Groothuis, J. R., E. A. Simoes, M. J. Levin, C. B. Hall, C. E. Long, W. J. Rodriguez, J. Arrobio, H. C. Meissner, D. R. Fulton, and R. C. Welliver. 1993. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. The Respiratory Syncytial Virus Immune Globulin Study Group [see comments]. N. Engl. J. Med. 329:1524-1530.
23. Hall, C. B. and R. G. Douglas,Jr. 1981. Modes of transmission of respiratory syncytial virus. J. Pediatr. 99:100-103.
24. Hall, C. B., R. G. Douglas,Jr., and J. M. Geiman. 1980. Possible transmission by fomites of respiratory syncytial virus. J. Infect. Dis. 141:98-102.
25. Hall, C. B., W. J. Hall, and D. M. Speers. 1979. Clinical and physiological manifestations of bronchiolitis and pneumonia. Outcome of respiratory syncytial virus. Am. J. Dis. Child 133:798-802.
26. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315:77-81.
27. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. 163:693-698.
28. Hall, C. B., E. E. Walsh, K. C. Schnabel, C. E. Long, K. M. McConnochie, S. W. Hildreth, and L. J. Anderson. 1990. 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. 162:1283-1290.
29. Heikkinen, T., M. Waris, O. Ruuskanen, A. Putto-Laurila, and J. Mertsola. 1995. Incidence of acute otitis media associated with group A and B respiratory syncytial virus infections. Acta Paediatr. 84:419-423.
30. Hemming, V. G., G. A. Prince, J. R. Groothuis, and G. R. Siber. 1995. Hyperimmune globulins in prevention and treatment of respiratory syncytial virus infections. Clin. Microbiol. Rev. 8:22-33.
31. Henderson, F. W., A. M. Collier, W. A. Clyde,Jr., and F. W. Denny. 1979. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N. Engl. J. Med. 300:530-534.
32. Hendry, R. M., A. L. Talis, E. Godfrey, L. J. Anderson, B. F. Fernie, and K. Mcintosh. 1986. Concurrent circulation of antigenically distinct strains of respiratory syncytial virus during community outbreaks. J. Infect. Dis. 153:291-297.
33. Huq, F., M. Rahman, N. Nahar, A. Alam, M. Haque, D. A. Sack, T. Butler, and R. Haider. 1990. Acute lower respiratory tract infection due to virus among hospitalized children in Dhaka, Bangladesh. Rev. Infect. Dis. 12 Suppl 8:S982-7:S982-7.
34. Kapikian, A. Z., J. A. Bell, F. M. Mastrota, K. M. Johnson, R. J. Huebner, and R. M. Channock. 1961. An outbreak of febrile illnessand pneumonia associated with respiratory syncytial virus infection. Am. J. Hyg. 74:234-248.

35. Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. E. Jensen, and R. H. Parrott. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89:422-434.
36. Kneyber, M. C., A. H. Brandenburg, P. H. Rothbarth, R. de Groot, A. Ott, and H. A. Van Steensel-Moll. 1996. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch. Dis. Child 75:137-140.
37. La Via, W. V., S. W. Grant, H. R. Stutman, and M. I. Marks. 1993. Clinical profile of pediatric patients hospitalized with respiratory syncytial virus infection. Clin. Pediatr. 32:450-454.
38. La Via, W. V., M. I. Marks, and H. R. Stutman. 1992. Respiratory syncytial virus puzzle: clinical features, pathophysiology, treatment, and prevention. J. Pediatr. 121:503-510.
39. MacDonald, N. E., C. B. Hall, S. C. Suffin, C. Alexson, P. J. Harris, and J. A. Manning. 1982. Respiratory syncytial viral infection in infants with congenital heart disease. N. Engl. J. Med. 307:397-400.
40. Martinez, F. D., W. J. Morgan, A. L. Wright, C. J. Holberg, and L. M. Taussig. 1988. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. N. Engl. J. Med. 319:1112-1117.
41. McConnochie, K. M., C. B. Hall, E. E. Walsh, and K. J. Roghmann. 1990. Variation in severity of respiratory syncytial virus infections with subtype. J. Pediatr. 117:52-62.
42. McConnochie, K. M. and K. J. Roghmann. 1986. Parental smoking, presence of older siblings, and family history of asthma increase risk of bronchiolitis. Am. J. Dis. Child 140:806-812.
43. McIntosh, E. D., L. M. De Silva, and R. K. Oates. 1993. Clinical severity of respiratory syncytial virus group A and B infection in Sydney, Australia. Pediatr. Infect. Dis. J. 12:815-819.
44. Meehan, J. T., R. C. Cutlip, H. D. Lehmkuhl, J. P. Kluge, and M. R. Ackermann. 1994. Infected cell types in ovine lung following exposure to bovine respiratory syncytial virus. Vet. Pathol. 31:229-236.
45. Monto, A. S. and S. Ohmit. 1990. Respiratory syncytial virus in a community population: circulation of subgroups A and B since 1965. J. Infect. Dis. 161:781-783.
46. Mufson, M. A., R. B. Belshe, C. Orvell, and E. Norrby. 1988. Respiratory syncytial virus epidemics: variable dominance of subgroups A and B strains among children, 1981-1986. J. Infect. Dis. 157:143-148.
47. Mulholland, E. K., A. Olinsky, and F. A. Shann. 1990. Clinical findings and severity of acute bronchiolitis. Lancet 335:1259-1261.
48. Navas, L., E. Wang, V. de Carvalho, and J. Robinson. 1992. Improved outcome of respiratory syncytial virus infection in a high- risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J. Pediatr. 121:348-354.
49. Openshaw, P. J. 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am. J. Respir. Crit. Care Med. 152:S59-S62.
50. Openshaw, P. J. 1995. Immunopathological mechanisms in respiratory syncytial virus disease. Springer Semin. Immunopathol. 17:187-201.
51. Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock. 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98:289-300.
52. Prince, G. A., R. L. Horswood, and R. M. Chanock. 1985. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J. Virol. 55:517-520.
53. Russi, J. C., H. Chiparelli, A. Montano, P. Etorena, and M. Hortal. 1989. Respiratory syncytial virus subgroups and pneumonia in children. Lancet 2:1039-1040.
54. Salomon, H. E., M. M. Avila, M. C. Cerqueiro, C. Orvell, and M. Weissenbacher. 1991. Clinical and epidemiologic aspects of respiratory syncytial virus antigenic variants in Argentinian children. J. Infect. Dis. 163:1167
55. Siber, G. R., J. Leszcynski, V. Pena-Cruz, C. Ferren-Gardner, R. Anderson, V. G. Hemming, E. E. Walsh, J. Burns, K. Mcintosh, and R. Gonin. 1992. Protective activity of a human respiratory syncytial virus immune globulin prepared from donors screened by microneutralization assay. J. Infect. Dis. 165:456-463.
56. Singleton, R. J., K. M. Petersen, J. E. Berner, E. Schulte, K. Chiu, C. M. Lilly, E. A. Hughes, L. R. Bulkow, and T. L. Nix. 1995. Hospitalizations for respiratory syncytial virus infection in Alaska Native

children. Pediatr. Infect. Dis. J. 14:26-30.
57. Spence, L. and N. Barratt. 1968. Respiratory syncytial virus associated with acute respiratory infections in Trinidadian patients. Am. J. Epidemiol. 88:257-266.
58. Srikiatkhachorn, A. and T. J. Braciale. 1997. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. J. Exp. Med. 186:421-432.
59. Taylor, C. E., S. Morrow, M. Scott, B. Young, and G. L. Toms. 1989. Comparative virulence of respiratory syncytial virus subgroups A and B [letter] [see comments]. Lancet 1:777-778.
60. Tsutsumi, H., M. Onuma, K. Nagai, H. Yamazaki, and S. Chiba. 1991. Clinical characteristics of respiratory syncytial virus (RSV) subgroup infections in Japan. Scand. J. Infect. Dis. 23:671-674.
61. Vardas, E., D. Blaauw, and J. McAnerney. 1999. The epidemiology of respiratory syncytial virus (RSV) infections in South African children. S. Afr. Med. J. 89:1079-1084.
62. Walsh, E. E., K. M. McConnochie, C. E. Long, and C. B. Hall. 1997. Severity of respiratory syncytial virus infection is related to virus strain. J. Infect. Dis. 175:814-820.
63. Wang, E. E., B. J. Law, and D. Stephens. 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.
64. Waris, M. E., C. Tsou, D. D. Erdman, S. R. Zaki, and L. J. Anderson. 1996. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J. Virol. 70:2852-2860.
65. Wright, A. L., C. Holberg, F. D. Martinez, and L. M. Taussig. 1991. Relationship of parental smoking to wheezing and nonwheezing lower respiratory tract illnesses in infancy. Group Health Medical Associates. J. Pediatr. 118:207-214.

Curriculum Vitae

De schrijfster van dit proefschrift is geboren op 4 november 1964 te Bakhuizen, Friesland. Zij doorliep het VWO aan de Rijks Scholengemeenschap Magister Alvinus te Sneek van 1977 tot 1983. Aansluitend studeerde zij 2 jaar biologie aan de Rijksuniversiteit Utrecht. In 1985 stapte zij over naar de studie geneeskunde aan de Rijksuniversiteit Utrecht. Het doctoraal geneeskunde behaalde zij in december 1989 en het artsexamen in september 1992. Hierna begon zij met de opleiding tot arts-microbioloog, met als aandachtsgebied de virologie, in het Academisch Ziekenhuis Rotterdam. Na het voltooien van de opleiding tot arts-microbioloog in oktober 1996 is zij tot juli 1999 werkzaam geweest als staflid op de afdeling virologie in het Academisch Ziekenhuis Rotterdam. Tijdens de periode in Rotterdam werkte zij aan het onderzoek dat de basis vormt voor dit proefschrift. Thans is zij werkzaam als arts-microbioloog op het Laboratorium voor de Volksgezondheid in Friesland en is als staflid verbonden aan het ziekenhuis Nij Smellinghe te Drachten.

Publicaties

1. Oosterlaken, T. A., A. Brandenburg, P. Schielen, R. Fransen, C. A. Kraaijeveld, and H. Snippe. Efficient induction of Semliki Forest virus and mumps virus neutralizing anti-anti-idiotypic antibodies using Quil A as adjuvant. J. Immunol. Methods 1991;136:169-175.
2. Brandenburg, A. H., M. G. Smits, B. S. Voorbrood, A. M. Hemmes, and J. J. Ros. Submandibular salivary gland hypertrophy induced by phenytoin. Epilepsia 1993;34:151-152.
3. van Milaan, A. J., M. J. Sprenger, P. H. Rothbarth, A. H. Brandenburg, N. Masurel, and E. C. Claas. Detection of respiratory syncytial virus by RNA-polymerase chain reaction and differentiation of subgroups with oligonucleotide probes. J. Med. Virol. 1994;44:80-87.
4. Dellemijn, P. L., A. Brandenburg, H. G. Niesters, M. J. van den Bent, P. H. Rothbarth, and L. T. Vlasveld. Successful treatment with ganciclovir of presumed Epstein-Barr meningoencephalitis following bone marrow transplant. Bone Marrow Transplant. 1995;16:311-312.
5. Kneyber, M. C., A. H. Brandenburg, P. H. Rothbarth, R. de Groot, A. Ott, and H. A. Van Steensel-Moll. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch. Dis. Child 1996;75:137-140.
6. Langedijk, J. P. M., W. G. J. Middel, W. M. M. Schaaper, R. H. Meloen, J. A. Kramps, A. H. Brandenburg, and J. T. Van Oirschot. Type-specific serologic diagnosis of respiratory syncytial virus infection, based on a synthetic peptide of the attachment protein. J. Immunol. Methods 1996;193:157-166.
7. Brandenburg, A. H., A. van Belkum, C. van Pelt, H. A. Bruining, J. W. Mouton, and H. A. Verbrugh. Patient-to-patient spread of a single strain of Corynebacterium striatum causing infections in a surgical intensive care unit. J. Clin. Microbiol. 1996;34:2089-2094.

8. Brandenburg, A. H., J. Groen, H. A. Van Steensel-Moll, E. J. C. Claas, P. H. Rothbarth, H. J. Neijens, and A. D. M. E. Osterhaus. Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. J. Med. Virol. 1997;52:97-104.
9. Brandenburg, A. H., P. Y. Jeannet, H. A. Steensel-Moll, A. Ott, P. H. Rothbarth, W. Wunderli, S. Suter, H. J. Neijens, A. D. Osterhaus, and C. A. Siegrist. Local variability in respiratory syncytial virus disease severity. Arch. Dis Child 1997;77:410-414.
10. Langedijk, J. P., A. H. Brandenburg, W. G. Middel, A. Osterhaus, R. H. Meloen, and J. T. Van Oirschot. 1997. A subtype-specific peptide-based enzyme immunoassay for detection of antibodies to the G protein of human respiratory syncytial virus is more sensitive than routine serological tests. J. Clin. Microbiol. 35:1656-1660.
11. Hu, K. F., M. Elvander, M. Merza, L. Akerblom, A. Brandenburg, and B. Morein. The immunostimulating complex (ISCOM) is an efficient mucosal delivery system for respiratory syncytial virus (RSV) envelope antigens inducing high local and systemic antibody responses. Clin. Exp. Immunol. 1998;113:235-243.
12. Kneyber, M. C., A. H. Brandenburg, R. de Groot, K. F. Joosten, P. H. Rothbarth, A. Ott, and H. A. Moll. Risk factors for respiratory syncytial virus associated apnoea. Eur. J. pediatr. 1998;157:331-335.
13. Nur, Y. A., A.H. Brandenburg, H. G. Niesters, J. A. Wagenaar, A. D. Osterhaus, and J. Groen. Need to increase awareness among family doctors and medical specialists of rickettsioses as an import disease in non-endemic areas. Neth. J. Med. 2000;56:186-189.
14. Ang, C. W., B. C. Jacobs, A. H. Brandenburg, J. D. Laman, F. G. van der Meche, A. D. Osterhaus, and P. A. van Doorn. Cross-reactive antibodies against GM2 and CMV-infected fibroblasts in Guillain-Barre syndrome. Neurology 2000;54:1453-1458.
15. Brandenburg, A. H., A. Kleinjan, B. van het Land, H. A. Moll, H. H. Timmerman, R. L. de Swart, H. J. Neijens, W. Fokkens, and A. D. M. E. Osterhaus. A type 1 like immune response is found in children with RSV infection regardless of clinical severity. J. Med. Virol. 2000;62: (in press)
16. Brandenburg, A. H., L. de Waal, H. H. Timmerman, P. Hoogerhout, R. L. de Swart, and A. D. M. E. Osterhaus. HLA Class-I restricted cytotoxic T cell epitopes of the respiratory syncytial virus fusion Protein. J. virol. 2000;74(21): (in press)
17. Brandenburg, A. H., R. van Beek, H. A. Moll, A. D. M. E. Osterhaus, and E. C. J. Claas. G protein variation in respiratory syncytial virus group A: no correlation with clinical severity. J Clin Microbiol. 2000;38(10): (in press)

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