Risk factors for admission and the role of respiratory syncytial virus-specific cytotoxic T-lymphocyte responses in children with acute bronchiolitis

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Background. Risk factors for admission of children with acute bronchiolitis have remained controversial. Technological advances in the measurements of cytotoxic T-lymphocyte (CTL) activity, enable respiratory syncytial virus (RSV)-specific CTLactivity to be studied in infants with bronchiolitis for the first time. We evaluated risk factors for admission of children with acute bronchiolitis and determined the role of CTL responses in those infected with RSV.

Method. Children between 3 and 24 months of age presenting with bronchiolitis to the paediatric outpatient department at King Edward VIII Hospital, Durban, over a 1-year period were enrolled. Management included clinical evaluation, nasopharyngeal aspiration, standard treatment and hospitalisation if indicated. Secretions were tested with monoclonal antibodies for RSV and pooled respiratory viruses; shell vial cultures were also established. Permission was requested from parents of RSV-infected subjects for blood draws for specific cytotoxic T-cell assays and CD4/CD8 cells on admission and repeat CTL on day 7.

Results. Viruses were identified in 55 of the 114 subjects studied (48.2%). RSV was seen in 41 cases (74.5%). Twenty-three infants (20.2%) required admission. Risk factors associated with inpatient admissions on univariate analysis included younger mean age (7.6 months v. 10.1 months), overcrowding ($p < 0.01$) and indoor exposure to products of combustion of cooking fuels ($p = 0.05$). Only the former two were significant on multivariate analysis. RSV-specific CTL responses were obtained in 21 children (51.2%). Responses were either very weak ($N = 7$) or negative ($N = 14$) on day 0 and did not alter significantly on day 7. The mean CD4/CD8 ratios in this group were 2.27:1. The highest frequency of CTL was directed against the proteins 'M4/5/6', with counts ranging from 100 to 400 spot forming cells (sfc)/million.

Conclusion. Measures to address risk factors identified in this study may decrease the need for hospitalisation from bronchiolitis. The lack of RSV-specific CTL responses in peripheral blood of immunocompetent RSV-infected children suggest an alternative method of induction of immunity or compartmentalisation of immune cells.

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Respiratory illnesses account for an estimated 4 million deaths each year, with a further 8 - 10 million children having related morbidity annually.[1] Respiratory syncytial virus (RSV) has been recognised as the commonest aetiological agent for acute lower respiratory tract infections (LRTI) in the developed world. In the USAalone it accounts for 50 - 80% of all cases of bronchiolitis, 91 000 paediatric hospitalisations and 4 500 deaths annually.[2] In developing countries there are scant data on the role of RSV in bronchiolitis. A study from the National Institute of Virology in South Africa between 1990 and 1996 revealed a hospital-based incidence of between 3% and 18%, mortality rates of between 12% and 43%, and morbidity rates of between 27% and 96%.[3] Prematurity with or without chronic lung disease and age less than 1 year have been associated with increased severity of disease.[4,5] Low socio-economic status, ethnicity and maternal RSV antibody levels have not consistently been shown to increase risk.[6] Hussey *et al.*[7] failed to identify any differences in the clinical presentation of children with or without RSV acute LRTI.

Recent evidence has confirmed the role of cytotoxic T-lymphocyte (CTL) responses to viral infections in animal models.[8] Mice with suppressed CTL responses had severe disease and poor outcomes when exposed to the influenza virus. Studies of CTL responses in adults infected with RSV have shown exaggerated responses in specific peptides.[9,10] Evidence suggests that T and B cells, cytokines (interleukin (IL)8 and IL2) and human neutrophil elastase play a significant role in the pathogenesis of bronchiolitis.[9-11] Elevated levels of CD23 + B cells, immunoglobulin E (IgE) and RSV-specific IL4 responses have been demonstrated, but treatment with an IL12 antagonist has not been beneficial.[10] RSV CTL responses have not been tested in children with acute bronchiolitis. We therefore conducted a study to determine the pathogenic role

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of RSV-specific cytotoxic T-cell responses in peripheral blood in these children. We evaluated factors that could be related to the need for hospitalisation.

Patient details

This study was conducted over a 1-year period between June 1999 and May 2000. Children aged 3 - 24 months with bronchiolitis who presented to the paediatric outpatient department, King Edward VIII Hospital, Durban, were recruited between 08h00 and 16h00 on weekdays. Informed consent was obtained from parents or guardians of subjects. Bronchiolitis was defined in accordance with international diagnostic criteria.[12] Children with asthma, chronic neurological deficits, severe malnutrition and other co-morbid illnesses or those in respiratory failure were excluded. Ethical approval for the study was obtained from the University of Natal.

A detailed questionnaire on patient demographics, birth details, feeding practices, home and family size, immunisations or family history of related illness, pollutant exposure and family order, was administered. Temperature, pulse oximetry, and respiratory and heart rate evaluations were conducted at two time points during physical examination.

Virus identification

Nasopharyngeal aspirates utilising an internationally accepted technique[13] were obtained and samples were transported immediately in viral transport medium to the virology laboratory at the University of Natal. For rapid viral diagnosis monoclonal antibodies against RSV (Davies Diagnostia, Calif.) and other pooled respiratory viruses, viz. influenzae A and B, adenovirus, parainfluenza 1, 2, 3 and mumps were used in an immunofluorescent assay. Shell vial cultures using A549 lung tissue carcinoma for RSV and adenovirus, and monkey kidney cell lines (vk) for pooled respiratory viruses were also performed. Permission was requested from parents / guardians of RSV monoclonal antibody-positive children to draw blood for RSV-specific cytotoxic T-cell assays and CD4 / CD8 cells on admission and for repeat CTLon day 7.

Lymphocyte isolation

The heparin-anticoagulated blood was diluted 1:1 with 0.1% fetal calf serum (FCS) RPMI, layered onto Ficoll Hypaque (Histopaque, Sigma-Chemicals, St. Louis, Mo, specific gravity 1.007 g / cc) and centrifuged at 400 rpm for 25 minutes at room temperature. The mononuclear cell layer was removed, washed twice with 0.1% FCS / RPMI 1640 (GIBCO-BRL, USA), counted, and tested for viability using trypan blue staining. The cells were then resuspended in complete RPMI (10% FCS, 10 mM L-glutamine, 10 mM herpes, penicillin / streptomycin 50 U).

RSV-specific CTL stimulation assay

Overlapping peptide sequences (10 - 20 mers) spanning the RSV proteins NP, M, SH, S and F were synthesised. These peptides have been shown previously to elicit RSV-specific CTLactivity as detected by the elispot assay.[13] Microtitre plates (Millipore) were pre-coated with anti-gamma-interferon for 24 hours and thereafter washed with 0.1% BSA / 0.5% FCS in phosphate-buffered saline (PBS). Fifty thousand peripheral blood mononuclear cells (PBMCs) per well were incubated with each peptide overnight. The plates were then washed with PBS and biotinylated anti-gamma interferon was then added for 100 minutes. This was followed by the addition of streptavidin / alkaline phosphatase conjugate for 30 minutes. The substrate bromochloroiodyl phosphate (BCIP) was then added and nitroblue tetrazolium chloride was used as the colour reagent. The presence of blue spots indicated gamma-interferon production and positive CTLactivity. The well with the highest frequency of CTLs indicated the responses to the immunodorminant peptide. Results were reported as spot forming cells (sfc) per million PBMC.

Management of enrolled subjects included the use of bronchodilators, humified oxygen, antibiotics, antipyretics and decongestants. Children with poor feeding, listlessness, falling oxygen saturation and increasing respiratory distress were considered to have inadequate clinical response and to need hospitalisation. Admissions had blood and nasal samples sent for bacterial isolation. Children were followed up on day 7.

Results

Of the 114 children enrolled in the study, 23 (20.2%) required hospitalisation. One hundred and four (91.2%) of all subjects studied were less than 1 year of age, with 14 of the 23 admissions (60.9%) being between 3 and 6 months of age. One hundred and nine children (95.6%) lived in urban areas ($p < 0.01$). The overall male-to-female ratio was 1.5:1, being similar for inpatient admissions and outpatients. Follow-up was achieved in 91 subjects (80%), with all children recovering.

The major findings of this study were a younger mean age (7.6 months v. 10.1 months), greater overcrowding ($p = 0.002$) and greater indoor exposure to products of combustion of cooking fuels ($p = 0.05$) in hospitalised children compared with those managed as outpatients (Table I). Multivariate analysis confirmed young age ($p = 0.01$) and overcrowding ($p = 0.02$) as risk factors for hospitalisation. A history of associated atopy or previous wheezing was significantly more common in children managed as outpatients compared with those who were hospitalised ($p = 0.04$ and $p = 0.05$ respectively).

The mode of delivery (normal vaginal or caesarean section, 2.29:1 v. 2.4:1), gestational age (term or pre-term, 4.66:1 v. 4.75:1) and passive cigarette smoke exposure (39.1% v. 47.3%)

Table I. Epidemiological risk factors associated with severity of bronchiolitis (range)

Category	Overall (*N* = 114 (%))	Admissions (*N* = 23 (%))	Outpatients (*N* = 91 (%))	*p*-value
Mean age (months)	9.1 (3 - 23)	7.6 (3 - 20)	10.1 (3 - 23)	
History of atopy	44 (38.6)	4 (17.4)	40 (44)	0.036
Overcrowding (> 2 per room)	69 (60.5)	23 (87.0)	46 (50.5)	0.002
Noxious cooking fuels*	36 (31.6)	11 (47.8)	25 (26.8)	0.050
Previous wheezing	51 (45.7)	6 (26.1)	45 (49.4)	0.045

* Kerosene, gas, wood.

were not significantly different between those children admitted and those managed as outpatients. Feeding practices, viz. exclusive breast-feeding, bottle-feeding, or mixed feeding, had no impact on the need to hospitalise patients. Although birth weight had no impact on the need to hospitalise, all but 4 cases were over 2.0 kg.

Cases of bronchiolitis occurred throughout the year, being highest between September and November (Fig. 1), with inpatient admissions occurring predominantly between December and March (*N* = 16). Viruses were identified in 55 cases (48.2%). RSV was identified in 41 cases (74.5%), parainfluenza 3 in 11 (20.0%), adenovirus in 2, and influenza B in a single case. The diagnostic yields for shell vial cultures and rapid monoclonal antibodies test for RSV were similar, with only 2 cases being positive on monoclonal antibodies and negative on culture due to delay in inoculation into culture.

Viral isolation was associated with increased risk of admission (26.7% v. 13.8%, $p = 0.08$). RSV was isolated throughout the year, but was associated with increased inpatient admission rates between December and March (Fig. 1) (the summer months). Parainfluenza 3 infections were predominantly identified between July and November (*N* = 11) (winter) and responsible for 3 admissions during that period.

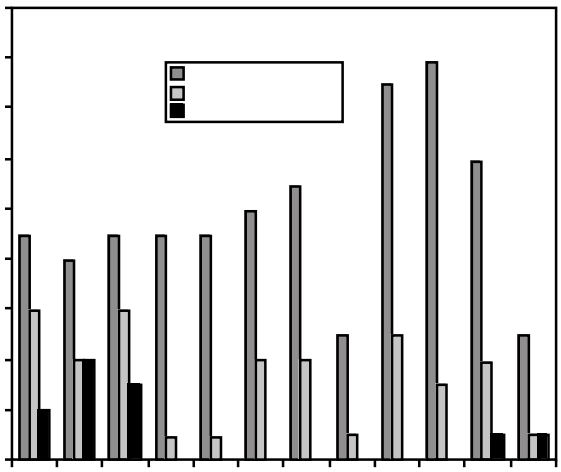

Fig. 1. Pattern of presentation of RSV-related bronchiolitis.

Blood cultures were positive in 3 of the 23 children admitted. *Staphylococcus epidermidis* (*N* = 2) and *Corynebacteria* species (*N* = 1) were identified. Nasopharyngeal swabs for bacteria revealed dual infections (*Streptococcus pneumoniae* and *Haemophilus influenzae*) in 4 cases, isolated *H. influenzae* and *S. pneumoniae* in 4 and 2 cases respectively, and no bacterial growth in 13 cases. Dual bacterial and viral infections were seen in 6 cases, 4 cases had bacteria only (*S. pneumoniae* and *H. influenzae*), 4 had viruses alone (RSV) and in 9 cases no pathogen was detected.

RSV-specific CTL responses and CD4/CD8 ratios

RSV-specific CTL responses were obtained in 21 cases. Permission to draw blood was refused in 17 cases, while the remaining 3 children had low total white cell counts that prohibited CTLanalysis. In 14 of the 21 cases (66.7%) no responses could be identified. In the remaining 7 subjects, the average spot colony forming units per millilitre of PBMC for the F, M matrix, SH, NS and the NPproteins were 233, 218, 180, 166 and 120 respectively. There were no changes in the CTL responses on day 7. The highest frequency of CTLwas directed against matrix protein 'M4/5/6', with a count of 400 sfc/million PBMC. This was the immunodorminant epitope response in 3 of the 4 patients who responded to the matrix proteins. The mean CD4/8 ratio for children where CTL responses were obtained was 2.27:1 (standard deviation (SD) 1.33), which is within the normal range for similarly aged children.

Clinical findings and management

Temperature fluctuations were recognised in 42 children (36.9%) but were not associated with increased risk of admission. The mean respiratory rates for all subjects were 56.9/min (37 - 98/min), being the same in those admitted and those treated as outpatients. The mean oxygen saturation was 94.5% (85 - 99.5%); all children with oxygen saturations below 92% (*N* = 13) required admission. Respiratory distress was seen in 112 children (98.1%), hyperinflation in 57 (50%) and

crackles in 46 (40%). No child had a decreased level of consciousness and all children received humidified oxygen. Nebulisations with an anticholinergic agent alone were utilised in 74 cases (64.9%), a β_2-agonist alone in 16 cases (14.0%), a combination of β_2-agonist and anticholinergic in 22 (19.3%), and adrenaline in 2 (1.8%). Antibiotics were given in 38 cases (33.3%), cough mixtures in 9 (7.9%) and antipyretics in 11 (9.6%) children. Corticosteroids were not utilised in this cohort.

Discussion

The most striking finding of this study was the weak or absent RSV-specific CTL responses in apparently immunocompetent children with RSV bronchiolitis. There is no clear reason why this should be the case as epitopes directed against the NP, M SH, NS2 and F proteins of RSV here have elicited CTL responses in adults.[14] This lack of response may be related to several factors, viz. the limited investigation of PBMCs, the redistribution of the T-lymphocyte subsets between the intravascular compartment and the lung parenchyma, or a different immunopathology mechanism of RSV injury.[15] Other possibilities include the use of peptides based on the wrong sequence of virus, or helper responses that are not so easily detectable using Elispot assays. Also, these low-frequency responses (< 400 sfc / million) may yet be significant in control of infection. The lack of substantial changes in CTL responses over the follow-up period suggest a more prolonged effect of these cells and may be important in future study design.

We have shown that young age, overcrowding, and exposure to products of fuel combustion during food preparation (kerosene) were associated with increased risk of hospitalisation. Alterations in these factors may reduce the need for hospitalisation. Birth-related factors, feeding practices and passive cigarette smoke exposure were not associated with the need for admission, which is unlike the findings of other studies where prematurity carried a 70 - 75% risk of hospital admission and a 25% risk of admission to an ICU.[5]

The higher RSV-related admissions between December and March have been seen in other tropical regions such as the Gambia.[16] Studies from Argentina and the USAhave confirmed a predominant winter peak, with the disease occurring mainly in infants under 1 year of age and carrying a mortality of 0.5 - 1.5%.[2,17] The isolation of RSV throughout the year may be explained by the concurrent HIV epidemic in this population. HIV infection has been recognised to prolong carriage of RSV.[18,19] Despite very high clinical suspicion of HIV disease in this population we were unable to detect any clinical markers suggestive of HIV infection, although HIV testing was not performed.

Pulse oximetry provided the best predictor of the need for hospitalisation. Temperature aberrations and respiratory rates were not predictive of the need for hospitalisation. This is unlike the findings of a UK study where fever (> 37.8°C) was associated with a more severe clinical course and more radiological changes and the need for hospitalisation.[20]

Several limitations regarding usefulness of this study remain. The small sample size, the evaluation of CTL response at just two time points and the lack of patients with high risk for severe RSV disease may have contributed to the low frequency of CTL responses. However, CTL evaluations and identification of the appropriate epitopes for RSV disease in its infancy are extremely expensive.

In conclusion, the primary pathogenic and protective mechanisms against RSV infection require further evaluation. This study provides knowledge on the risk factors associated with increased likelihood of admission for bronchiolitis. Avoidance of overcrowding and provision of adequate ventilation to areas of the home where food preparation occurs may reduce the burden of this disease on children in developing countries.

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