REGULAR ARTICLE

Respiratory viral infection in lower airways of asymptomatic children

S Thavagnanam[1,2], SN Christie[1,2], GM Doherty[1,2], PV Coyle[3], MD Shields[1,2], LG Heaney (l.heaney@qub.ac.uk)[1]

1.Respiratory Research Group, Centre for Infection and Immunity, Queen's University of Belfast, Belfast, UK
2.Royal Belfast Hospital for Sick Children, Belfast, UK
3.Regional Virus Laboratory, Royal Group of Hospitals, Belfast, UK

Abstract

Aim: The aim of this study was to determine if asthmatic children have viruses more commonly detected in lower airways during asymptomatic periods than normal children.
Methods: Fifty-five asymptomatic children attending elective surgical procedures (14 with stable asthma, 41 normal controls) underwent non-bronchoscopic bronchoalveolar lavage. Differential cell count and PCR for 13 common viruses were performed.
Results: Nineteen (35%) children were positive for at least one virus, with adenovirus being most common. No differences in the proportion of viruses detected were seen between asthmatic and normal 'control' children. Viruses other than adenovirus were associated with higher neutrophil counts, suggesting that they caused an inflammatory response in both asthmatics and controls (median BAL neutrophil count, 6.9% for virus detected vs. 1.5% for virus not detected, p = 0.03).

Conclusions: Over one-third of asymptomatic children have a detectable virus (most commonly adenovirus) in the lower airway; however, this was not more common in asthmatics. Viruses other than adenovirus were associated with elevated neutrophils suggesting that viral infection can be present during relatively asymptomatic periods in asthmatic children.

INTRODUCTION

Viral infections trigger acute wheezing in young children and asthma exacerbations in older children (1–4). Little information is known on the role of viruses in chronic persistent asthma in the absence of an obvious exacerbation. Children with asthma may be more susceptible to effects of viruses, and frequent recurrent respiratory viral infections could drive an ongoing inflammatory response. The incidence of rhinovirus infections is similar in asthmatic and normal controls but the asthmatics experience more severe symptoms (5). It is possible that viruses may persist as low-grade infections and potentiate inflammatory response in the airway of asthmatic children.

Maček et al. (6) observed an increased incidence of adenoviral carriage in children with chronic airways obstruction. They demonstrated that adenoviral capsid protein was present in either the bronchoalveolar lavage (BAL) or bronchial biopsies upon follow-up of these cases. Enterovirus and rhinovirus RNA may persist for several weeks in the nasal secretions of children following exacerbations of asthma (7).

Adenovirus has been infrequently found in asymptomatic asthmatic children but in one study in which adenovirus was only detected in one of 20 control samples, it was present in almost 80% of asthmatic nasal samples (8). This suggests that adenovirus infection is common in asthmatics during asymptomatic periods. Recently, Wos et al. (9) detected human rhinovirus more frequently in bronchial biopsies of asthmatic compared with non-asthmatic controls. Taken collectively, these data suggest a possible link between chronic wheezing and persistent viral carriage.

Following an acute infection, viral proteins are produced without replication of a complete virus. Latent infections can influence the inflammatory response to stimulus in both asthma and chronic obstructive pulmonary disease (10). RT-PCR can be used for the molecular diagnosis of latent viruses (11,12). Jartti et al. (13) found many studies describing asymptomatic subjects with high respiratory virus detection rates using PCR techniques. However, the persistence of PCR-positive respiratory viral detection was short lasting and infrequent (<5%).

We took the opportunity to obtain BAL samples and nasal swabs from asthmatic children who happened to be undergoing an elective surgical procedure. We report the prevalence of viruses detectable in the lower respiratory tract of asymptomatic children with and without asthma

Abbreviations

BAL, bronchoalveolar lavage; RT-PCR, real-time polymerase chain reaction; PCR, polymerase chain reaction; ET, endotracheal; HSV, Herpes Simplex virus; EBV, Epstein–Barr virus; IQR, interquartile range; SD, standard deviation; DNA, deoxyribonucleic acid.

attending for elective surgery and report the relationship with airways inflammation.

MATERIALS AND METHODS

Subjects

Children attending the Royal Belfast Hospital for Sick Children for elective surgery were recruited. An asthma and allergy questionnaire was administered (14), and children were classified into two groups; persistent current wheeze within the last year (asthma) and controls who had never wheezed. Parents were asked when the child last had symptoms of a cold, cough or wheeze. Consent and assent were obtained from parents and children, and local Research Ethics Committee approval was obtained. The procedures in this study were in accordance with the Helsinki Declaration of 1983.

Sample collection and processing

Once the child was anaesthetized and intubated, sampling was performed immediately.

Nasal swabs

Following pre-oxygenation, each nostril was swabbed and the swabs placed in lysis buffer (Qiagen Ltd., Crawley, England, UK).Samples were vortexed, and the lysate was frozen at −70°C for subsequent viral PCR analysis.

Non-bronchoscopic BAL

An 8 French gauge neonatal catheter (Portex Ltd., Hythe, Kent, UK) was inserted down the endotracheal (ET) tube until wedged in a distal airway. Sterile normal saline (20 ml) was instilled and immediately aspirated (15). BAL fluid was transferred on ice to the laboratory. A 250–500 μL aliquot of BAL was placed in an equal volume of 8 M lysis buffer and stored at −70°C for viral PCR.

Cellular analysis of BAL fluid

Coverslip cytospins were made and stained with DiffQuik (American Scientific Products, McGaw, IL, USA) (15). Differential cell counts in BAL fluid were expressed as percentage differential (%).

PCR for respiratory viruses

A validated molecular diagnostic protocol was used for the detection of common respiratory viruses on nasal and BAL samples using a combination of single and multiplex assays for the simultaneous detection of the following: parainfluenza virus types 1, 2, 3, influenza A, influenza B, coronavirus, rhinovirus, respiratory syncytial virus types A and B, human metapneumovirus and adenovirus (16). In addition, herpes simplex virus (HSV) and Epstein–Barr virus (EBV) were co-assayed in separate reactions as these viruses have previously been shown to be shed from the respiratory tract in adults (17,18).

All of the primers used have been previously validated (19–26) and were in routine diagnostic use during the period of the study. First- and second-round products were run on 2% agarose gel (SeaKem LE, Cambrex Corporation, East Rutherford, NJ, USA) and the products were visualized by staining with ethidium bromide; the gel was photographed [Polaroid Land Camera, ISO 3000 film, transilluminator wavelength 254 (UVC)] to allow the determination of product size. Positive and negative controls were included in each run.

Statistical analysis

Data are presented as median with interquartile range (IQR) or mean (SD) where appropriate. Comparison between groups was performed using the Kruskal–Wallis test with *post hoc* Dunn's analysis. Comparisons between proportions was made using Fisher's exact test.

RESULTS

Fifty-five children underwent non-bronchoscopic BAL sampling (38 males, median age 7.3 years, range: 1.0–14.2). Fourteen children were classified as having asthma and 10 of these had other clinical signs of atopy (eczema and/or allergic rhinitis), although this was not confirmed by objective testing. The remaining four wheezed episodically with head colds and had no other features in keeping with atopy. Seven children with asthma were reported taking inhaled corticosteroid [median dose 400 μg BDP (IQR: 200–800 μg)] and another four had been on inhaled steroids previously. All asthmatic children used short acting inhaled beta-agonists intermittently. All the children were well at the time of sampling. Fourteen control children and four children with asthma had recovered from an upper respiratory tract infection or asthma exacerbation in the last 4 weeks.

Viruses were detected in 19 (35%) of the 55 subjects [three rhinovirus, one metapneumovirus, nine adenovirus (2 with co-existing EBV), one EBV alone and five HSV]. In two children, adenovirus was detected in nasal but not the BAL sample. Asthmatics and controls had similar proportions of viruses (asthmatics 47% vs. controls 31%, p = 0.35) and were of similar age [virus detected: mean age 8.0 (SD: 3.5) years, no virus detected: mean age 6.7 (SD: 3.1) years]. Children with asthma were not more likely to have detectable adenovirus, the most prevalent virus, in their BAL fluid (p = 0.64). Overall eighteen children (asthmatics and controls) had reported recovering from an upper respiratory tract infection or asthma exacerbation within the last 4 weeks; however, no difference in prevalence of virus detection between asthmatic and control subjects (asthmatics 32% vs. controls 39%, p = 0.63) was seen.

Of the 19 children with a virus identified on BAL, only seven (37%) gave a reported history of recent respiratory tract symptoms in the preceding month. The seasonal distribution of samples with and without viruses detected is shown in Figure 1.

Differential cell count and relationship to virus detection

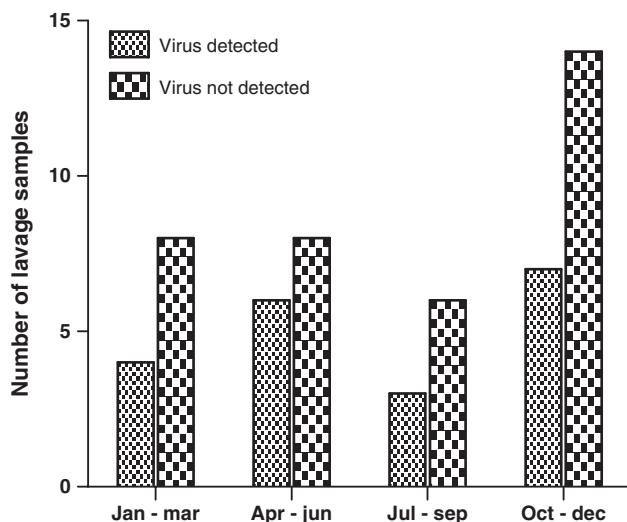Lavage volume return and cell counts for children with virus detected and not detected are presented in Table 1.

Figure 1 Seasonal distribution of lavage samples with and without detected viruses.

Table 1 Lavage volume returned and cell counts for children with virus detected and not detected

	Virus not detected		Virus detected		
	Median	IQR	Median	IQR	p-value
BAL volume return (%)	30	25.0–36.1	28.9	20.0–35.0	ns
Total cell count ($\times 10^5$/mL)	0.9	0.55–1.25	1.1	0.57–2.01	ns
Macrophages (%)	70	56.3–87.9	65.7	41.3–85.1	ns
Epithelial cells (%)	24.1	8.6–37.8	11.5	3.0–39.0	ns
Neutrophils (%)	1.5	0.6–4.5	3.2	1.2–22.5	0.012
Lymphocytes (%)	0.2	0.02–0.6	0.2	0–0.36	ns
Eosinophils (%)	0.2	0–0.37	0.2	0–0.4	ns

Bronchial alveolar lavage data are presented as median with interquartile range.

The median neutrophil count (%) was higher in children with a virus detected [virus detected: median 3.2, (IQR: 1.2–22.5), no virus detected: median 1.5, (IQR: 0.6–4.5), p = 0.012]. The median neutrophil count was similar between the asthmatics and controls [asthmatics: median 2.1, (IQR: 1.0–8.3) vs. controls: median 2.6, (IQR: 0.8–6.2), p = 0.73].

The median neutrophil count (%) was similar in asthmatics with and without virus detected [asthmatic with a virus detected: median 4.4, (IQR: 0.5–15.2) vs. asthmatic with no virus detected: median 2.1, (IQR: 1.15–5.7), p = 0.49]. However, the median neutrophil count (%) was significantly higher for controls with a virus detected [control with a virus detected: median 3.2, (IQR: 2.6–32) vs. controls with no virus detected: median 1.4, (IQR: 0.26–4.5), p = 0.007].

The median eosinophil count (%) was similar in both groups [with virus detected: median 0.18, (IQR: 0.0–0.4), without a virus detected: median 0.16, (IQR: 0.0–0.37), p = 0.65]. While the median eosinophils was similar in asthmatics [asthmatics: median 0.13, (IQR: 0.0–8.8) vs. controls: median 0.18, (IQR: 0.0–1.4), p = 0.92] the spread of values was much greater for asthmatics. No significant differences were detected in eosinophil counts for asthmatic children (p = 0.8) and control children with and without a virus detected (p = 0.86).

Children positive for adenovirus (%) had a median neutrophil count of 3.0 (IQR: 0.78–7.8) similar to children without viruses detected, median 1.5, (IQR: 0.6–4.5). This contrasted with the significantly higher median neutrophil count observed in children positive for any respiratory viruses other than adenovirus [median 6.9%, (IQR: 1.55–38.4), p = 0.03], (Fig. 2). Control children without viruses identified had a median neutrophil count of 1.32% [(IQR: 0–14.5) and upper 95th centile: 8.4]. There was a similar low neutrophil cell count in BAL of children with adenovirus detected only in nasal sample alone. In the six children with HSV or EBV detected, three samples had a neutrophil count >15%, consistent with a lower airways inflammatory response.

DISCUSSION

This study has shown that about one-third of children have a detectable virus in BAL. The children in our study were attending for elective surgical procedures and we believe that they are representation of the population in general. We identified a wide range of viruses, similar to those seen in children presenting acutely with respiratory symptoms. It is possible that some samples were obtained in the convalescent phase of viral infection as seven of the 20 children with positive PCR results gave a history of an upper respiratory tract infection (URTI) in the preceding 4 weeks.

While the children in our study appeared asymptomatic, our data suggest that detectable viruses may be associated with latent or persistent infection in the lower airways, because we observed a relatively increased BAL neutrophil
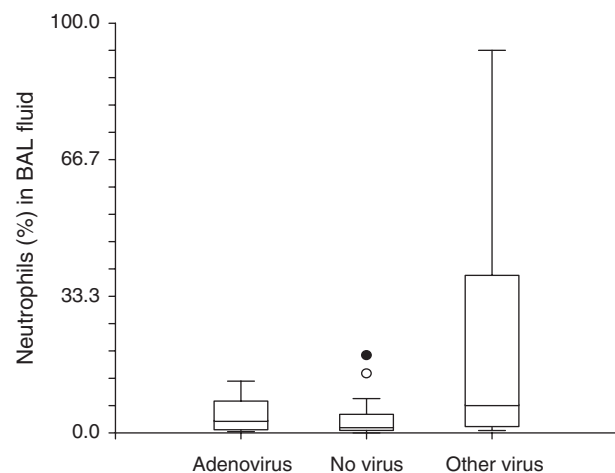

Figure 2 Box and whisker comparison plots of neutrophil (%) counts in children with adenovirus, no virus detected and all other viruses. Other viruses in bronchoalveolar lavage were associated with higher neutrophil counts compared with no virus or adenovirus (p = 0.03). Circles are outliers.

count compared with normals. This was observed for all viruses (rhinovirus, metapneumovirus, HSV and EBV) other than adenovirus suggesting that the viral detection is not because of contamination from upper airways. In a previous study of asthmatic and normal adult volunteers, viral shedding was still detectable in 54% of adult subjects 14 days after experimental inoculation with human rhinovirus-16 (27). However, the quantity of virus was low at this point, and cold symptoms were generally either gone or resolving. Another study showed that adenovirus was detected for 2 weeks in six (75%) of eight cases suggesting that this DNA virus can be latent (10,12). In addition, the normal reference values for neutrophil counts (%) from controls in this study, without a detectable virus in BAL (median neutrophil 1.32%, 95th centile 8.4%, n = 27), are lower than previously reported (median neutrophil 3.2%, 95th centile 29.8%) (28). Concurrent asymptomatic viral infection may have artificially inflated our previously published range and this suggests that the presence of a virus in BAL should be taken into account when interpreting BAL neutrophil counts.

We identified six samples with herpes viruses (HSV and EBV), which are known to be shed on the respiratory tract in adults, during physiological stress or disease (17,18). While shedding into respiratory tract may be the explanation in these children, rather than acute infection, it is notable that in four of the five cases (where symptom data were available), parents recorded recent respiratory tract symptoms and in three of these samples, there was a significant BAL neutrophilia. We cannot exclude the possibility that these represented acute *de novo* infection but we believe it is more probable that a prior stress, such as an acute respiratory tract infection (in one sample there was EBV and adenovirus present), caused acute airways inflammation and subsequent HSV/EBV shedding. However, given the persisting neutrophilia, it is possible that HSV/EBV shedding in the respiratory tract is associated with an ongoing neutrophilic acute inflammatory response in the lower respiratory tract and could potentially lead to more protracted symptoms in some children.

The clinical significance of these findings remains unknown. None of the children with viruses detected in BAL had significant peri- or post-operative respiratory infective complication or asthma exacerbation. Our data suggest that viruses can be present in BAL even in the absence of a reported respiratory infection in the previous 4 weeks, but with associated airway neutrophilia.

The lack of airways neutrophilia for adenovirus is significant because this virus has been associated with latent infection in both adults and children (3,29,30). In children, persistent wheeze and lack of response to therapy have been reported to be associated with persistent adenovirus isolation (3). Our data raise the possibility that adenovirus can be present without the typical inflammatory response in the lower airways, which could potentially explain the failure to eradicate this virus.

Asthmatic children were as likely as non-asthmatic children to be virus positive, which is perhaps a little surprising because asthma exacerbations have been strongly associated with viral infection (3). This may be due to the small number of asthmatic children is this study. Notably, our data suggest that in some asthmatic children, viral infection is associated with an airway inflammatory response with no evidence of asthma exacerbation. This could be due to the children having relatively mild disease or being stable on anti-inflammatory therapy at the time of the infection. The children with asthma recruited into this study had lower eosinophil counts than we previously reported (28). Indeed, in this study, the asthmatic eosinophil counts were not statistically significantly higher than the controls.

If we were to undertake this study now, we would use quantitative real-time assays for detection and quantification. However, as the lower respiratory tract is a nominally sterile environment, our data are still relevant, and the use of a very sensitive, qualitative assay is appropriate to detect low titre virus infections. As higher titres are associated with acute clinical infections, and these patients were asymptomatic, it is likely that the copy numbers would be very low.

One factor which may potentially explain the presence of viruses is the parental reporting of the time elapsed since the last URTI. This may be inaccurate, particularly if the symptoms were mild and parents did not want the elective surgical procedure to be postponed.

In conclusion, we have shown in an unselected population of children attending for elective surgery, a high prevalence of virus detectable in BAL with equal prevalence in both asthmatic and normal control children. There was an associated airway neutrophilia consistent with airway inflammation, but this was not seen with adenovirus detection, where airways neutrophilia was not identified.

References

1. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by thirteen years. *Lancet* 1999; 354: 541–5.
2. Pattemore PK, Johnston SL, Bardin PG. Viruses as precipitants of asthma symptoms. I. Epidemiology. *Clin Exp Allergy* 1992; 22: 325–36.
3. Johnston SL, Pattermore PK, Sanderson G, Smith S, Lawpe F, Josephs L, et al. Community Study of the Role of Viral Infections in Exacerbations of Asthma in 9-11 Year Old Children. *BMJ* 1995; 310: 1225–9.

4. Wark PAB, Johnston SL, Moric I, Simpson JL, Hensley MJ, Gibson PG. Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. *Eur Respir J* 2001; 19: 68–75.

5. Corne JM, Marshall C, Smith S, Schreiber J, Sanderson G, Holgate ST, et al. Frequency, Severity and Duration of Rhinovirus Infections in Asthmatic and Non Asthmatic Individuals; A Longitudinal Cohort Study. *Lancet* 2002; 359: 831–4.

6. Maček V, Šorli J, Kopriva S, Marin J. Persistent adenoviral infection and chronic airway obstruction in children. *Am J Respir Crit Care Med* 1994; 150: 7–10.

7. Jartti T, Lehtinen P, Vuorinen T, Koskenvuo M, Ruuskanen O. Persistence of rhinovirus and enterovirus RNA after acute respiratory illness in children. *J Med Virol* 2004; 72: 695–9.

8. Marin J, Jeler-Kacar D, Levstek V, Maček V. Persistence of viruses in upper respiratory tract of children with asthma. *J Infect* 2000; 41: 69–72.

9. Wos M, Sanak M, Soja J, Olechnowicz H, Busse WW, Szczeklik A. The Presence of Rhinovirus in Lower Airways of Patients with Bronchial Asthma. *Am J Respir Crit Care Med* 2008; 177: 1082–9.

10. Hogg JC. Role of latent viral infections in chronic obstructive pulmonary disease and asthma. *Am J Respir Crit Care Med* 2001; 164: S71–5.

11. Hyypiä T, Puhakka T, Ruuskanen O, Mäkelä M, Arola A, Arstilla P. Molecular diagnosis of human rhinovirus infections: comparison with virus isolation. *J Clin Microbiol* 1998; 36: 2081–3.

12. Jartti T, Lee WM, Pappas T, Evans M, Lemanske RF Jr, Gern JE. Serial viral infections in infants with recurrent respiratory illnesses. *Eur Respir J* 2008; 32: 249–51.

13. Jartti T, Jartti L, Peltola V, Waris M, Ruuskanen O. Identification of respiratory viruses in asymptomatic subjects. *Pediatr Infect Dis J* 2008; 27: 1103–7.

14. Stevenson EC, Turner G, Heaney LG, Schock BC, Taylor R, Gallagher T, et al. Bronchoalveolar lavage findings suggest two different forms of childhood asthma. *Clin Exp Allergy* 1997; 27: 1027–35.

15. Laviolette M, Carreau M, Coulombe R. Bronchoalveolar lavage cell differential on microscope glass cover. A simple and accurate technique. *Am Rev Respir Dis* 1988; 138: 451–7.

16. Coyle PV, Ong GM, O'Neill HJ, McCaughey C, De Ornellas D, Mitchell F, et al. A touchdown nucleic acid amplification protocol as an alternative to culture backup for immunofluorescence in the routine diagnosis of acute viral respiratory tract infections. *BMC Microbiol* 2004; 4: 41–3.

17. Ong GM, Lowry K, Mahajan S, Wyatt DE, Simpson C, O'Neill HJ, et al. Herpes simplex type 1 shedding is associated with reduced hospital survival in patients receiving assisted ventilation in a tertiary referral intensive care unit. *J Med Virol* 2004; 72: 121–5.

18. Lung ML, Lam WK, So SY, Lam WP, Chan KH, Ng MH. Evidence that respiratory tract is major reservoir for Epstein-Barr virus. *Lancet* 1985; 1: 889–92.

19. Ong GM, Wyatt DE, O'Neill HJ, McCaughey C, Coyle PV. A comparison of nested polymerase chain reaction and immunofluorescence for the diagnosis of respiratory infections in children with bronchiolitis, and the implications for a cohorting strategy. *J Hosp Infect* 2001; 49: 122–8.

20. Stockton J, Ellis JS, Saville M, Clewley JP, Zambon MC. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. *J Clin Microbiol* 1998; 36: 2990–5.

21. Santti J, Hyypiä T, Halonen P. Comparison of PCR primer pairs in the detection of human rhinoviruses in nasopharyngeal aspirates. *J Virol Methods* 1997; 66: 139–47.

22. Myint S, Johnston S, Sanderson G, Simpson H. Evaluation of nested polymerase chain methods for the detection of human coronaviruses 229E and OC43. *Mol Cell Probes* 1994; 8: 357–64.

23. Mitchell S, O'Neill HJ, Ong GM, Christie S, Duprex P, Wyatt DE, et al. Clinical assessment of a generic DNA amplification assay for the identification of respiratory adenovirus infections. *J Clin Virol* 2003; 26: 331–8.

24. Coyle PV, Desai A, Wyatt D, McCaughey C, O'Neill HJ. A comparison of virus isolation, indirect immunofluorescence and nested multiplex polymerase chain reaction for the diagnosis of primary and recurrent herpes simplex type 1 and type 2 infections. *J Virol Methods* 1999; 83: 75–82.

25. Stockton J, Stephenson I, Fleming D, Zambon M. Human Metapneumovirus as a cause of community-acquired respiratory illness. *Emerg Infect Dis* 2002; 8: 897–901.

26. Niesters HGM, van Esser J, Fries E, Wolthers KC, Cornelissen J, Osterhaus ADME. Development of a real-time quantitative assay for detection of Epstein–Barr virus. *J Clin Microbiol* 2000; 38: 712–5.

27. Mosser AG, Vrtis R, Burchell L, Lee WM, Dick CR, Weisshaar E. Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. *Am J Respir Crit Care Med* 2005; 171: 645–51.

28. Heaney LG, Stevenson EC, Turner G, Cadden IS, Taylor R, Shields MD, et al. Investigating paediatric airways by non-bronchoscopic lavage: normal cellular data. *Clin Exp Allergy* 1996; 26: 799–806.

29. Retamales I, Elliott M, Meshi B, Coxson HD, Pare PD, Sciurba FC, et al. Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am J Respir Crit Care Med* 2001; 164: 469–73.

30. Matsuse T, Hayashi S, Kuwano K, Keunecke H, Jefferies WA, Hogg JC. Latent adenoviral infection in the pathogenesis of chronic airways obstruction. *Am Rev Respir Dis* 1992; 146: 177–84.