PULMONARY EXACERBATIONS OF CYSTIC FIBROSIS: DEFINITION, INFLAMMATORY MARKERS, AND THE ROLE OF ATYPICAL BACTERIA AND RESPIRATORY VIRUSES

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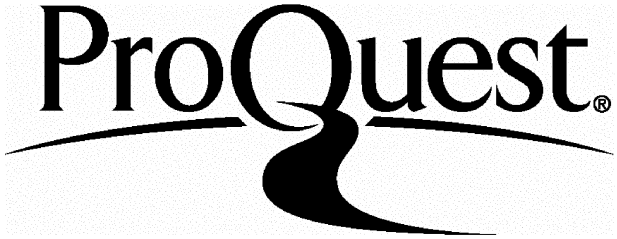

Abstract of Thesis

The underlying hypothesis was that co-infection with respiratory viruses and/or "atypical" bacteria in young adult patients with cystic fibrosis, who already experience chronic pulmonary bacterial colonisation, contributes to pulmonary morbidity. The most likely mechanism would be the precipitation of acute pulmonary exacerbations.

Peripheral markers of inflammation were first assessed as end-points. White cell count, plasma viscosity, and c-reactive protein increased in a quadratic fashion with declining pulmonary function. White cell count and c-reactive protein, but not plasma viscosity, demonstrated a fall with intravenous antibiotic treatment which could not be accounted for by improvement in pulmonary function. All three markers were frequently normal at the outset of treatment. In contrast neutrophil elastase was elevated in all patients tested both at the outset and completion of treatment.

To define respiratory exacerbation, four approaches were compared. Inflammatory markers were too insensitive. The coefficient of variation of quantitative sputum bacteriology was too high. A combination of change from baseline FEV1 or FVC of 10% or more, or an increase in two or more lower respiratory symptoms, was found to be optimal. With this definition, no permanent loss of lung function was attributable to exacerbations.

With conventional culture and serological methods, respiratory viruses were temporally associated with 11 of 373

exacerbations (3%). There was one asymptomatic seroconversion. All diagnoses were made with serology. No atypical bacteria were identified. The rate of seroconversion in the 60 patients who were followed for the full two year period was 0.083/patient/year.

Because of concern about the insensitivity of the culture methods, a multiplex PCR method was developed. With combined serology and PCR, 12 of 82 (15%) exacerbations in an 8 month period were associated with viral infection. Infections were no more common in patients with CF than in healthy controls, controls with asthma, and controls with non-CF bronchiectasis.

Patients who had viral infections had worse pulmonary function throughout the two year study. Plasma viscosity and CRP, but not white cell count, were higher in exacerbations with viral co-infection, and there was a greater decline in pulmonary function from pre-exacerbation baseline. FEV1 remained depressed following treatment of the exacerbation, but in most patients gradually improved subsequently. Viral infection was associated with a rise in anti-pseudomonal IgG ELISA score, which may predate deterioration in pulmonary function.

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Statement of Originality

The spirometric values analysed in this thesis were obtained by members of the physiotherapy department, and in particular by Anita Watson. In-patient clinical samples were obtained in part by the author, and in part by nursing and medical staff, according to protocols defined by the author. In-patient clinical histories were obtained in part by the author and in part by junior doctors, using a questionnaire defined by the author. Clinical samples and histories from the community were all obtained personally by the author.

Inflammatory markers were assayed routinely by the relevant laboratories, with the exception of neutrophil elastase, which was assayed by Julie Heald in collaboration with the author. Viral isolation and serology was undertaken by the department of virology, Leeds Public Health Laboratory. Anti-*Pseudomonas aeruginosa* IgG was assayed by Ian Carr.

Parainfluenza and coronavirus oligonucleotides were selected by Dr A Easton, in collaboration with the author. All other PCR development and diagnostic work was undertaken by the author.

The hypotheses tested in this thesis were all formulated by the author. All data was collated, subjected to statistical analysis, and interpreted by the author.

Chapter I Genetics, the basic defect (CFTR), and pulmonary pathophysiology in Cystic Fibrosis, and scope of the thesis

I.1 Genetics

Cystic fibrosis is the commonest inherited disorder leading to significant morbidity and mortality in Caucasian populations. Inheritance is autosomal recessive. In the UK white population the frequency of the abnormal allele is 1/25, leading to an incidence of affected pregnancies of 1/2500 [Dodge 1993]. One postulated mechanism for the very high prevalence of the abnormal allele is a heterozygote advantage that protects against excessive fluid loss in cholera. Cystic fibrosis is much less common in non-white populations, eg in the USA allele frequency is 1/30 in the white population, 1/60 in the black population and 1/80 in the Asian population [FitzSimmons 1993]. It should be noted that the exact incidence is unknown in all these ethnic groups.

The CF gene was localised to the long arm of chromosome 7 in 1985 [Wainwright 1985], and cloned in 1989 [Riordan 1989; Rommens 1989; Kerem 1989]. The gene was subsequently found to code for a 1480 amino acid, 168,138 kDa, protein, CFTR (cystic fibrosis transmembrane regulator), which forms a membrane chloride channel. CFTR has two units, each with a membrane-spanning domain and a hydrophobic nucleotide-binding domain. The two units are joined by a highly charged

regulatory, or "R" domain, which contains potential sites for phosphorylation by protein kinases A and C.

In excess of 300 CFTR mutations have now been identified, the commonest being the dF508 mutation, a triple base deletion leading to loss of a phenylalanine residue at position 508 of the CFTR protein. This position corresponds to the first of the two hydrophobic nucleotide-binding domains. This deletion is thought to have arisen in a single ancestor some three to five thousand years ago. dF508 accounts for 32% (Turkey) to 82% (Denmark) of mutations in white populations.

I.2 The basic defect in cystic fibrosis

Cultured CF pancreatic and airway cells show a chloride permeability defect, which can be corrected by insertion of CFTR into the cell membrane [Rich 1990]. Insertion of CFTR into a lipid bilayer confirmed its function as a chloride channel [Bear 1992]. The basic defect in cystic fibrosis is therefore defective transmembrane chloride transport. This results in increased transepithelial potential difference [Knowles 1981], which provides a convenient way of monitoring CFTR activity both in vitro and in nasal epithelium in vivo.

CFTR and CFTR mRNA have been localised to ciliated epithelium and submucous glands in the airways, pancreatic ductal epithelium, basal cells of intestinal crypts, bile duct epithelium, sweat gland reabsorptive duct epithelium,

and renal collecting tubules. Lower levels of CFTR occur in other sites [Crawford 1991]. This distribution matches the organ systems which are primarily involved in cystic fibrosis, with the exception of the kidneys, which are rarely involved.

At the cellular level, CFTR function in cystic fibrosis depends on the particular mutation. The dF508 mutation primarily results in defective intracellular processing of the protein, with accumulation in the Golgi apparatus and reduced amounts reaching the cell membrane [Cheng 1993]. In addition, CFTR in patients with the dF508 mutation functions inefficiently as a chloride channel. Genotype - phenotype correlations do not however extend to the tissue level. Several studies have now shown no difference in mortality, pulmonary function, or age of onset of chronic bacterial colonisation between dF508 homozygotes and other patients not homozygous for the dF508 deletion [Hamosh 1992; Anonymous 1994]. A higher incidence of pancreatic insufficiency is the only difference that has been found consistently in dF508 homozygotes [Kristidis 1992].

I.3 Pathophysiology of pulmonary disease in cystic fibrosis

Although cystic fibrosis is a multisystem disorder the main morbidity and mortality are primarily due to pulmonary involvement. Disorders of organs other than the lungs in cystic fibrosis are beyond the scope of this thesis, and will

not be considered further.

The pathophysiological link between defective CFTR and pulmonary damage is unknown. Defective transmembrane chloride transport from the intracellular to extracellular space is accompanied by sodium influx into the cell. In the lung epithelium this probably results in relatively dehydrated periciliary fluid. It is thought that airway clearance by the mucociliary escalator could then be impaired as a result of this abnormal fluid. Submucosal gland hypertrophy, duct obstruction and mucus cell hyperplasia can be seen in the first few days of life. This predates overt respiratory tract infection [Oppenheimer 1978].

Subsequent lung damage is thought to arise as a result of bacterial colonisation. The initial organism is nearly always *Staphylococcus aureus* [Hoiby 1982], with or without non-encapsulated *Haemophilus influenzae* [Hoiby 1976]. Colonisation with these organisms is in some cases associated with subsequent deterioration of lung function [Hoiby 1982]. At some later stage, most often during the second decade of life, 90% of patients become colonised with *Pseudomonas aeruginosa* [Hoiby 1975; Kulczycki 1978]. The rate of loss of pulmonary function accelerates following acquisition of *pseudomonas* [Hoiby 1990], but the rate of decline and the time of onset following acquisition varies widely between patients [Kerem 1990].

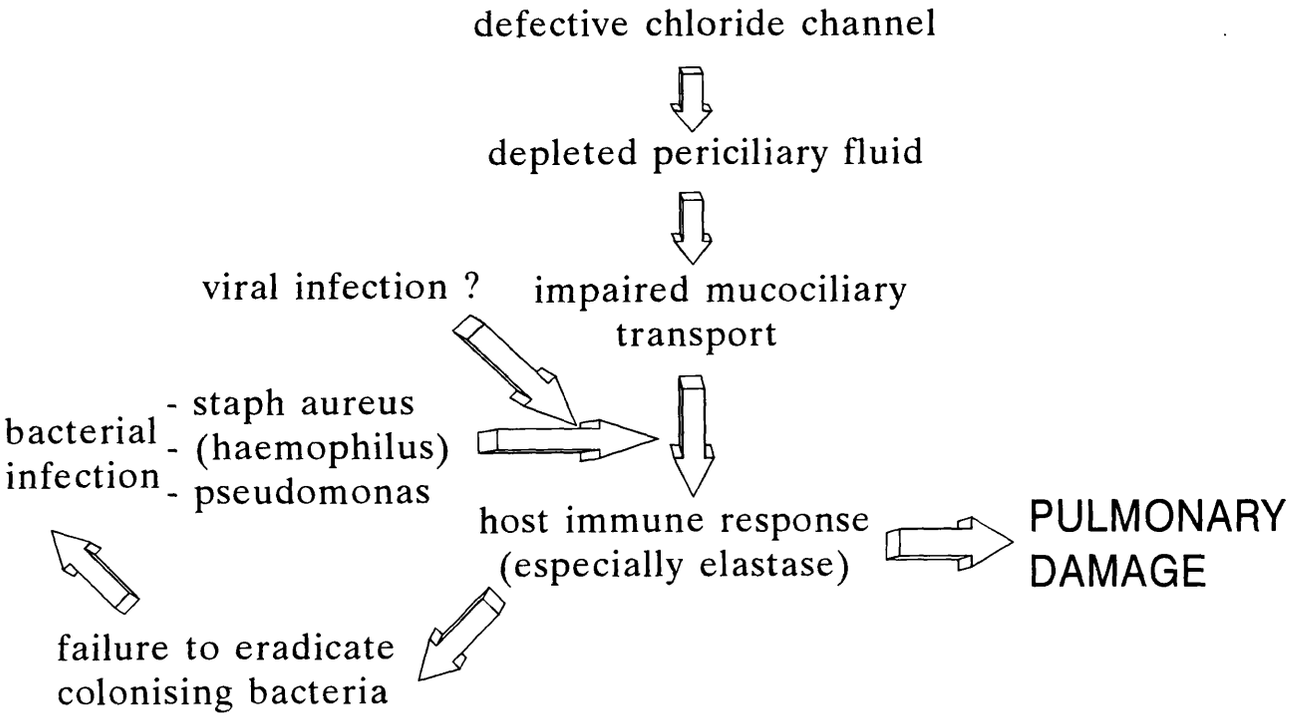
The link between defective CFTR activity and bacterial colonisation is not known, but the most popular hypothesis is that the dehydrated periciliary fluid on the epithelial

surface leads to impaired clearance of inhaled microorganisms. There is no evidence either for or against this mechanism, and the physical properties of periciliary fluid in CF have not been determined. There is in vitro evidence for an alternative explanation of the propensity of CF lung for bacterial colonisation [Bryan 1994]. Decreased CFTR activity correlates with increased endosomal pH. This in turn leads to reduced sialyltransferase activity, resulting in a greater availability of asialylated sugars on the cell surface, to which bacteria, and particularly *P aeruginosa*, can bind.

Once chronic colonisation is established, patients with cystic fibrosis go on to develop tubular, and then saccular, bronchiectasis. Bronchiectasis presumably arises in accordance with Cole's "vicious circle" hypothesis, of continued inflammation following the inability to clear infecting organisms [Cole 1984]. The postulated mechanism is summarised in figure 1. The predictable progression from staphylococcal through to pseudomonal colonisation, a pattern not observed in non-CF bronchiectasis, and the excessive intensity of the pulmonary inflammation and lung destruction seen in cystic fibrosis compared to non-CF bronchiectasis, require further explanation.

Staphylococcal colonisation almost always predates pseudomonal colonisation, and consequently is assumed to somehow "pave the way" for the later pseudomonal colonisation. The direct contribution of staphylococcal colonisation to pulmonary damage is less clear cut, and seems

Figure 1 - Pulmonary pathophysiology - the vicious circle hypothesis in cystic fibrosis



to be quite variable between patients [Hoiby 1982], but radiographic changes do correlate with the titre of IgG antibody to staphylococcal teichoic acid [Ericsson 1986].

Since the major deterioration in pulmonary function in cystic fibrosis follows acquisition of *P aeruginosa*, most attention has been focused on the pseudomonal-host relationship. Many "virulence" factors produced by *P aeruginosa* have been identified. While some of these may directly injure lung tissue, more important roles are probably in establishing chronic colonisation, and in stimulating chronic intrapulmonary inflammation. The most important virulence factor is alginate. This is a polysaccharide produced by *P aeruginosa* only when it colonises the airways of patients with cystic fibrosis.

Production of alginate results in the establishment of pseudomonal microcolonies within the airways, each one surrounded by a layer of alginate which forms a barrier, inhibiting the access of inflammatory cells, which may be the reason for the development of the intense intrapulmonary chronic inflammation. Alginate also inhibits the penetration of most antibiotics into microcolonies [Bolister 1991; Allison 1992].

The bulk of the pulmonary injury is thought to be due to release of host proteases, particularly neutrophil elastase, in response to chronic pseudomonal colonisation. Neutrophil elastase concentration in BAL correlates with percent predicted FEV1 [Meyer 1993]. Host-derived oxidants, cytokines and lipid products are also mediators of pulmonary damage

[Meyer 1993; Witko 1995; Konstan 1993].

Following acquisition of *P aeruginosa*, percent predicted FEV1 deteriorates progressively [Kerem 1990], but the pattern of deterioration is very variable, both within and between patients, who frequently show prolonged periods of improvement in pulmonary function before relapsing once again.

The long-term trends in pulmonary function are punctuated by "acute exacerbations", ie increases in lower respiratory tract symptoms and/or sharp deteriorations in pulmonary function. Exacerbations usually require intensive treatment with intravenous antibiotics, physiotherapy, and nutritional supplementation, to restore pulmonary function and abolish symptoms. Exacerbations generally have been assumed to be detrimental to long-term outcome. Hitherto the term "exacerbation" has not been defined. In the absence of such a definition, there are no data concerning the prognostic significance of exacerbations. Likewise the precipitating causes of exacerbations, if indeed there are any, are unknown. In the absence of any other obvious candidates, co-infection with respiratory viruses and/or atypical bacteria has been invoked.

I.4 Scope of the thesis

The main question to be examined was whether co-infection with viruses and "atypical" bacteria is a significant cause of morbidity in patients with cystic fibrosis of young adult age, almost all of whom are chronically colonised with *Pseudomonas aeruginosa*. The underlying hypothesis to be tested was that such co-infections occur in a significant number of acute pulmonary exacerbations, and lead to acute, and possibly chronic, deterioration in clinical status. The literature considering such infections in relation to cystic fibrosis is reviewed in chapter II, and that in relation to other respiratory disorders in chapter III. The literature concerning mechanisms of viral pathogenesis is reviewed in chapter IV. Treatment strategies in adult cystic fibrosis, and the treatment protocol adopted at the investigator's institution are detailed in chapter V.

Peripheral markers of inflammation may be useful clinical end-points, and it was intended to employ them as markers of severity of exacerbation. They could also have a role in defining exacerbations. However, very little is known about how such inflammatory markers behave in CF populations. The behaviour of four such markers is examined in chapter VI.

To determine whether a significant association exists between co-infection and pulmonary exacerbation, it was first necessary to arrive at a satisfactory definition of exacerbation. Four methods of defining exacerbation are

therefore considered in chapter VII, and the effects of exacerbation on pulmonary function examined.

The association between exacerbation and co-infection with viruses and atypical bacteria, diagnosed by conventional culture and serological methods, is examined in chapter VIII. Concern about the sensitivity of culture methods for the detection of respiratory viruses led to the adoption of a polymerase chain reaction diagnostic method. The development of this method is covered in chapter IX. The association between exacerbation and co-infection with respiratory viruses diagnosed by serology and PCR methods is re-examined in chapter X. The short-term and long-term clinical effects of such infections are examined in chapter XI.

Viral-bacterial interaction, determined by anti-pseudomonal IgG ELISA, is investigated in chapter XII.

Chapter II Studies relating to viral and "non-bacterial" infection in cystic fibrosis populations

Thirteen studies and two case reports have specifically addressed the issue of the incidence and impact of viral and/or atypical bacterial infection in patients with cystic fibrosis, and one study has evaluated influenza vaccination in cystic fibrosis.

II.1 Study 1

Wright et al [1976] initially set out to evaluate the safety of amantadine in respiratory viral infections in children with cystic fibrosis. In 1972-1973 a variant of Hong Kong influenza virus, influenza A/England/42, caused epidemic disease in parts of the United States, and was predicted to spread to New England. In expectation of its arrival a placebo-controlled double blind parallel group trial of amantadine was carried out in 153 patients with cystic fibrosis (age range 15 months to 32 years) for 60 days from mid-January to mid-March 1973. Subjects were reviewed one month and then two months after the initiation of drug or placebo therapy. Subjects were also asked to report if they developed a respiratory exacerbation, defined on the basis of a symptom-derived score. Paired serology was obtained for influenza A and B, RSV, parainfluenza virus types 1,2,3 and 4, adenovirus and *Mycoplasma pneumoniae*.

There were 48 respiratory exacerbations. Serology was positive for respiratory viruses in 12 (25%). Three influenza seroconversions were asymptomatic. There was no difference in incidence or severity of exacerbations between the amantadine and placebo groups, but the expected epidemic did not materialise, and it was not possible to draw any conclusions as to the efficacy of amantadine.

This study provided the first suggestion of a temporal association between respiratory viral infection and respiratory exacerbation in patients with cystic fibrosis. The study was limited by its short duration, the absence of serological testing in asymptomatic subjects, and its reliance on serology.

II.2 Study 2

DeForest et al [1976] reported viral seroconversions in a series of 126 episodes "showing some clinical evidence of deterioration or increased severity of pulmonary disease" in 76 patients with cystic fibrosis in the course of one year. A fourfold increase in antibody titre occurred in 42 (33%) episodes. All seroconversions were reported to be associated with lower respiratory tract exacerbations, one patient with influenza A dying. The authors concluded that the incidence of viral infection was similar in a cystic fibrosis population to a non-cystic fibrosis population but that the clinical effects were more serious in cystic fibrosis.

Unfortunately, the study only appeared in abstract form, in which the control data was not included.

The study was limited by its short cross-sectional nature, the absence of criteria to define respiratory exacerbation, and the reliance on serology. The rate of respiratory exacerbation seems very low, at less than one per patient per year.

II.3 Study 3

Petersen et al [1981] investigated the relationship of "non-bacterial" infection ("NBI", ie respiratory viral, *mycoplasma*, or *chlamydia*) to pseudomonal colonisation status in 116 patients with cystic fibrosis, age 6 months to 29 years, from October to May (8 months). Paired serology was obtained in the event of increasing pulmonary symptoms or a deterioration in clinical findings, lung function or chest Xray.

The patient population was divided into three groups: 1) 57 patients chronically colonised with *Pseudomonas aeruginosa* during the whole of the study, 2) 7 patients who became chronically colonised with *P aeruginosa* during the study, 3) 52 patients who were either not colonised or intermittently colonised with *P aeruginosa*. Chronic colonisation was determined by the presence of ≥ 2 precipitin bands vs P aeruginosa on crossed immunoelectrophoresis together with positive sputum culture.

Three hundred and thirty two acute exacerbations were recorded in 108 patients, 8 remaining symptom free for the whole eight month period. There was no difference in rate of exacerbation between patients of different age groups, but there were more exacerbations in the chronically colonised group.

Seventy-six NBI were serologically proven in 57 (49%) patients. The incidence of NBI decreased from 0.88 per patient in the 5-9 year old group to 0.30 per patient in the 20-24 year old group. Eight (11%) NBI were unrelated to exacerbations. NBI were thought to have contributed to 20% of respiratory exacerbations. RSV was the most frequently identified agent (n = 31) and was found at higher frequency in the transitional group than in the non-colonised group. Otherwise there were no differences between the three groups in the specific aetiology of NBI. Forty-seven percent of RSV infections were associated with a significant but sometimes temporary increase in serum precipitins to *P aeruginosa*. The authors concluded that NBI, particularly RSV, may act synergistically with *P aeruginosa* in the aetiology of respiratory exacerbations.

This study went further than most in pre-defining respiratory exacerbation, but the criteria were still vague. This could have resulted in an overestimate of the frequency of exacerbation. In the eight month period of the study there were 2.9 exacerbations per patient, which seems high. The serological criteria were loose, with a titre of ≥ 64 considered sufficient for a positive diagnosis of viral and

chlamydial infection, although a fourfold rise in titre was required to diagnose mycoplasma infection. This might have lead to an overestimate of the frequency of proven NBI. Again, the only diagnostic method used was serology. The frequency with which NBI contributed to respiratory exacerbation may therefore have been underestimated or overestimated.

This study was the first to suggest in vivo synergism between a respiratory virus and *P aeruginosa* in patients already colonised with pseudomonas. However no criteria were given to define a significant increase in precipitins and no statistical analysis of the association of RSV with an increase in serum precipitins vs *P aeruginosa* was offered. Although there were significantly more RSV-associated exacerbations in the transition group compared with the non-colonised group this may well have represented a chance finding, as there were only seven patients in the transition group.

II.4 Study 4

The first study of non-bacterial infection in a young adult population with cystic fibrosis classified 46 patients, mean age 22.2 years (range 16-41) as either "deteriorated" or "stable" [Efthimiou 1984]. The "deteriorated" group (n = 24) had reported an increase in cough, sputum production or breathlessness associated with a decrease in FEV1 to a value

at least 15% lower than obtained at a preliminary visit one month previously. A control group of thirty healthy hospital volunteers was also included. Nose and throat swabs, sputum and serology for respiratory viruses, *M pneumoniae, chlamydia, C Burnetii* and *L pneumophila*) were collected at baseline. Serology was repeated one month and one year after the initial visit. Viral isolation with three culture systems was attempted, although the timing of the cultures was not specified.

In the course of the year serology was positive in 7 of the 24 patients in the "deteriorated" group and in 1 of 22 patients in the "stable" group, but in none of the healthy control group. Because patients in the deteriorated group had poorer baseline pulmonary function the authors suggested that patients' poor lung function per se was associated with non-bacterial infection. All the documented infections were accompanied by acute exacerbations of lower respiratory tract disease.

Eight patients were considered to have serological evidence of co-infection with *L pneumophila*, associated with a clinical deterioration which responded to erythromycin. However, seven of these diagnoses relied on a single moderately raised titre. A fourfold increase in antibody titre was only found in one case, and it is probable that this was due to cross-reaction with antibodies to *P aeruginosa* [Collins 1984].

This study was the first to suggest that *L pneumophila* might be a significant pathogen in cystic fibrosis, and the

first to suggest that more severely affected patients with cystic fibrosis might be more prone to NBI. Because of the problems with *legionella* serology in cystic fibrosis, neither of these findings can be considered definite.

II.5 Study 5

The first prospective case-control study was published in 1984 [Wang]. Patients with cystic fibrosis and their unaffected siblings were followed for two years. Both cases and controls were reviewed at three monthly intervals when blood for serology, sputum for bacterial isolation, and nasopharyngeal secretions for isolation were obtained. Pulmonary function was only performed routinely twice per year. Chest radiography was performed and Shwachman-Kulczyki scores were derived annually. In addition to routine monitoring, respiratory illness was identified with a telephone questionnaire, administered weekly. Respiratory illness was classified as upper respiratory, lower respiratory, flu-like illness or pneumonia based on the pattern of symptoms. At the time of respiratory illness further serology, sputum and nasopharyngeal secretions were obtained, and pulmonary function was retested. Virus isolation was attempted for adenovirus, influenza A and B, RSV and rhinovirus.

Forty-nine patients but only 19 sibling controls were enrolled. Thus the study was not truly case-control and a

substantial proportion of the patient group may have been exposed to viral infection to which the control group would not have been. Mean age of the subjects was 13.7 years, of the controls 14 years. Reported respiratory illnesses were more common in the patients than in the controls (3.7/patient/year vs 1.7/patient/year, $p < 0.001$). One hundred and five viral infections were identified in the patients (mean 1.07/patient/year), all with serology. There were no positive cultures, despite a total of 1046 nasopharyngeal aspirates being submitted for examination, 322 of these being obtained at the time of respiratory illness. Forty-two seroconversions were asymptomatic. The rate of viral seroconversion was reported to be similar in the patients and controls, but no data were presented for the control group.

This study was the first to attempt to determine the clinical impact of respiratory viral infection on patients with cystic fibrosis. Linear regression was performed for each patient to determine the yearly rate of decline of lung function (FEV1, FVC and FEF 25-75), percent predicted weight for height and Shwachman-Kulczyki score. Strong positive correlations were found between these indices and rate of viral seroconversion, and also between frequency and length of hospitalisations and rate of viral seroconversion.

The most revealing aspect of this study was the failure to isolate a single virus, despite apparently adequate sample collection and culture techniques. The authors postulated that there may be an unidentified inhibitor of in vitro viral

replication present in the secretions of patients with cystic fibrosis, but it should be noted that no virus was isolated from the controls either.

II.6 Study 6

Stroobant [1986] included previously unpublished data on 38 children with cystic fibrosis, age 5-16 years, in a review of the subject. In the course of one year, 21 viral infections were identified in 17 children colonised with *P aeruginosa*, but only 3 in 13 non-colonised children, using serology and isolation. The were 3 viral isolates.

In all cases where a viral infection was shown "pulmonary deterioration" occurred. The increased frequency of viral infection was associated with greater clinical deterioration in the colonised children, again suggesting a viral-bacterial (viral-pseudomonal) interaction. Unfortunately no clinical or pulmonary function data were presented to substantiate this interpretation.

II.7 Study 7

A second prospective case-control study set out to determine whether respiratory viral and *mycoplasma* infection is more common in patients with cystic fibrosis than in healthy controls [Ramsey 1989]. Fifteen sibling pairs

consisting of one patient and one unaffected sibling were followed for a period of two years. Sibling pairs were seen routinely every two months and were also encouraged to report acute respiratory illnesses, defined with standardised criteria. Oropharyngeal swabs for viral isolation and paired baseline serum were collected within 48 hours of the onset of a presumed infection. Subjects were contacted once between clinic visits to encourage compliance with the study. Serology was performed for respiratory viruses and *M pneumoniae*.

At routine visits and at the time of acute respiratory illness spirometry and plethysmographic volumes were measured. In addition height, weight and triceps skinfold thickness were measured and a Shwachman-Kulczyki score was derived.

Fifteen pairs completed the study. Mean age at enrolment was 13.4 +/- 5.0 years in the patients and 13.4 +/- 3.4 years in the siblings. There was no difference in seroconversion or viral isolation between patients and controls. Only one third of viral infections were identified at the time of acute respiratory illness. Thirty-seven percent of all the study participants seroconverted to *M pneumoniae* in the two year study period. This very high rate of seroconversion was not commented on, and the point in the usual four year cycle of *mycoplasma* infection at which the study took place was not described.

Age correlated inversely with infection rate ($r = -0.5$), but infection rate did not correlate with Shwachman-Kulczyki

score or FVC. In contrast to Stroobant's findings, patients colonised with *P aeruginosa* were less likely to develop viral or *Mycoplasma* infections, and this difference remained significant after controlling for age. Patients colonised predominantly with *S Aureus* had similar infection rates to the siblings; patients colonised with *P aeruginosa* had a lower infection rate.

In patients with cystic fibrosis, respiratory rate was greater, percent predicted FEF 25-75 lower, predicted FVC lower, and RV/TLC ratio higher, in the presence of both confirmed viral infection and symptoms. Curiously the best values were recorded in the presence of viral infection but the absence of symptoms.

Patients with higher infection rates had better pulmonary function at the time of enrolment, and were predominantly colonised with *S Aureus*. Conversely those with lower infection rates were predominantly colonised with *P Aeruginosa*. The rate of decline of FVC, RV/TLC ratio, and triceps fat fold thickness was significantly slower in the high infection rate group.

II.8 Study 8

Abman et al [1988] investigated respiratory viral infection in infants with cystic fibrosis. Forty-eight infants were followed prospectively following diagnosis at birth. Nasopharyngeal aspirates for viral isolation and sera

for RSV only were obtained at birth, 6 months age, 12 months age, and then yearly, and also during hospital admission for "respiratory distress". No attempt was made to collect paired sera.

At mean age 28.8 months 18 (38%) infants had been hospitalised thirty times for acute respiratory distress. Viral pathogens were identified in 12 of these admissions. RSV was found in 7. Seven of 79 culture specimens (9%) were positive for other respiratory viruses. RSV infection was commonly associated with a requirement for mechanical ventilation, a need for continuous home oxygen and poorer chest Xray scores after discharge.

II.9 Study 9

Hordvik et al [1989] followed 10 patients with cystic fibrosis (age range 5-32) for 20 months. Subjects kept a symptom diary and recorded PEFR at home. Following an increase in two or more upper respiratory symptoms nasal discharge was transported by post before attempted viral isolation, and paired serology obtained. "Acute respiratory illness" (ARI) was defined as viral isolation, seroconversion, or symptoms occurring in a household member within 3 days of onset in a patient. Thirty five ARIs were observed, with an annual rate of 1.75/patient/year. Only 43% of ARI were confirmed in the laboratory (57% defined symptomatically).

PEFR declined in 22 episodes of ARI. PEFR did return to baseline values but the mean number of days for PEFR to recover was greater in those with initially more severe disease (22 days vs 15 days).

II.10 Study 10

Pribble et al [1990] set out to test the hypothesis that if nonbacterial infections are an incidental finding, exacerbations of cystic fibrosis associated with viral or mycoplasmal co-infection would be indistinguishable from exacerbations not associated with such infections. Pulmonary exacerbations, defined on symptomatic grounds, were studied between September and April. Specimens for viral isolation were obtained within 48 hours of "enrolment in the study", with paired serology.

Eighty exacerbations in 54 patients were included. Mean age was 15.4 years (range 10 months to 32 years). Forty-four exacerbations (55%) required hospital treatment. A total of 29% of exacerbations was associated with viral or mycoplasma infection.

CRP was elevated in 67% of exacerbations with co-infection compared with 47% of exacerbations with no co-infection (not significant). All six influenza-associated exacerbations demonstrated elevated CRP levels. Pulmonary function testing was only performed in 65% of exacerbations. The change from baseline of FEV1 and FEF25-75 did not differ

between the two groups, but was greater in those with influenza than in NBI other than influenza and the exacerbations with no NBI.

In general, NBI-associated exacerbations were clinically similar to non-NBI exacerbations, but those associated with influenza appeared to have a worse outcome. However, no attempt was made to allow for interindividual patient differences. In addition both the collection of specimens for NBI identification and of the outcome data was far from complete. It is possible that the non-NBI group contained undiagnosed NBI.

II.11 Study 11

Ong et al [1989] studied 36 adults with cystic fibrosis prospectively over one year, who initially presented with influenza-like symptoms. They were then encouraged to report suspected respiratory exacerbations. Nasopharyngeal aspirates and throat swabs were collected for viral isolation and antigen detection. Paired sera for respiratory viruses and *C psittaci*, *C burnetii*, *M pneumoniae* and *L pneumophila* were obtained. Unfortunately the timing of sample collections was not described and no information on the completeness of sample collection given.

Patients were divided into two groups on the basis of presence or absence of recent clinical deterioration at the time of enrolment. Seroconversion occurred in 5 of the

deteriorated and 6 of the stable group. No seroconversion to *L pneumophila* was documented. One throat swab grew coxsackie B5 virus.

The authors stated that infections with respiratory viruses and atypical bacteria were similar in patients with cystic fibrosis with and without an acute exacerbation of respiratory symptoms. However this is based on symptoms at the time of enrolment, and it is implied that the study continued to look at exacerbations over a period of one year. No information about subsequent symptoms was given, and it is not possible to validate this statement from the data presented. The study underlines once again the paucity of viral isolates in adult patients with cystic fibrosis, possibly related to the difficulty of viral isolation in older subjects [Cooney 1972]. The absence of seroconversion to *L pneumophila*, and the similarity between deteriorated and stable groups contrasts with Efthimiou's study.

II.12 Study 12

Johansen and Hoiby [1992] investigated the relationship between seasonal variation and the onset of "initial and chronic *pseudomonas* infection" in 300 Danish patients with cystic fibrosis. Intermittent pseudomonal colonisation was defined as presence in the respiratory tract of less than 6 months duration, and a peripheral antibody response of less than two pseudomonal precipitins. Chronic infection was

defined as repeatedly positive sputum culture for 6 months with two or more precipitins. Records of all patients attending the centre from 1965 to 1990 (26 complete years) were examined retrospectively and the dates of initial and chronic colonisation/infection determined. Sixty-six percent of initial colonisation occurred during the winter months, defined as October to March, with the lowest incidence in July and the highest in October. Similarly, 68% of chronic infections began during the winter months, with the lowest incidence in July and the highest in November. The authors postulated that this seasonal variation might be explained by viral-bacterial synergism, as respiratory viral infection rates peak in the winter months.

II.13 Study 13

Smyth et al [1995] followed one hundred and eight patients, median age 7.9 years, range 2 months to 20 years, for a one year period. Patients were asked to report at the time of coryzal symptoms, or when they had increased cough, at which time naso-pharyngeal aspirates were obtained. Direct immunofluorescence was performed on the aspirates for parainfluenza types 1-3, RSV, adenovirus, and influenza A and B. In addition, aspirates were subjected to semi-nested reverse transcriptase PCR for rhinovirus. Paired serology for respiratory viruses was also performed. Forty-four viral infections were diagnosed, nineteen with serology, and

twenty-five rhinoviral infections with PCR. Only one aspirate was positive on immunofluorescence testing, for influenza (type unspecified). Patients with viral infection (n = 33) were then compared with those without an infection (n = 75). There were no differences in baseline characteristics between the two groups. Shwachman-Kulczyki score, FEV1, FVC, weight, and Chrispin-Norman score were compared in all patients at the start, and at the end, of the study. There was a significant difference between the two groups in the magnitude of deterioration in Shwachman-Kulczyki score, but no differences in the deterioration in the other parameters. Subgroup analysis showed that patients with rhinoviral infection required more days of intravenous antibiotics than those with other viral infection. Comparison of the "other virus" group separately with the non-infected group showed a significantly greater decline in FEV1 in the virally-infected group.

The authors concluded that although rhinoviral infection gave rise to symptoms which prompted treatment with intravenous antibiotics, there were no long-term effects of infection. In contrast, they concluded that other viral infection, which de facto was seroconversion to other viruses, resulted in lower FEV1 at one year. The findings must be viewed with caution for several reasons. Firstly, given the extreme short-term intra-individual variation in cystic fibrosis, the comparison of end-points based on only two measurements at the start and end of a year, together with the measurement of multiple end-points, is likely to

result in type 1 errors. Secondly, in the virally-infected group, all the end points were measured at the same time. Therefore, the putative effects of viral infection were being assessed anywhere between 0 and 365 days after viral infection. Thirdly, the conclusion that viruses other than rhinoviruses cause significant long-term morbidity was based on a sub-group analysis in a small study, without being explicitly stated as a prior hypothesis. Finally, it is difficult to envisage a virally-induced mechanism for a significant deterioration in Shwachman-Kulczycki score in the absence of significant changes in pulmonary function.

This study is important in its identification of rhinovirus at the time of clinical illness in a paediatric age group, although in the absence of specimens collected when the patients were well a specific association with respiratory illness cannot be confirmed. Also of note was the insensitivity of immunofluorescence in viral diagnosis, even in a primarily paediatric age group. This insensitivity is unlikely to represent late presentation, given the 25 specimens that were positive with PCR.

II.14 Case reports

In addition to the above prospective studies two papers describing case reports of viral infection have been published. MacDonald et al [1987] published a series of three children with cystic fibrosis who developed varicella

infection. All three patients were male, with ages 7, 8 and 13 years. All had stable pulmonary disease without any previous hospital admissions for treatment of pulmonary exacerbations. Each patient had moderate varicella with 50 to 100 skin lesions. Each patient developed cough and dyspnoea 2 to 5 days after the onset of the rash. All three patients showed a deterioration in FVC which returned towards baseline at about 6 months after the initial infection. Xray appearances were not typical of varicella pneumonitis.

Conway, Simmons and Littlewood reported acute severe deterioration in 3 patients with cystic fibrosis following serologically proven influenza A infection [Conway 1992]. In two cases diagnosis was on the basis of acute titres of 512, which then fell to 32 or less in one month. All three pulmonary exacerbations were associated with a decline in FEV1 and FVC of at least 10%, which recovered to baseline values following intravenous antipseudomonal antibiotic treatment. C-reactive protein was grossly elevated in one case, normal in one, and not measured in the third case.

II.15 Summary of studies of viral and atypical bacterial infection in cystic fibrosis

A temporal association between influenza A infection and exacerbations of cystic fibrosis was first suggested in 1976 [Wright]. Most of the following studies looked at subject groups with a paediatric age bias. Four of these suggested a

temporal link between viral infection and exacerbation [DeForest 1976; Petersen 1981; Stroobant 1986; Pribble 1990], but as none included adequate sampling during periods of respiratory health the link remains unproven. Two prospective studies which did employ routine specimen collection found no difference in the rate of positive viral diagnosis in the presence or absence of symptomatic respiratory illness [Wang 1984, Ramsey 1989]. These last two studies were also case-controlled, and found no difference in the rate of positive viral diagnosis between patients and healthy sibling controls. Two studies have been limited to an adult age range. In the first, [Efthimiou 1984], more seroconversions were observed in a group with "deteriorated" respiratory function compared to a "stable" group, but the difference was largely due to seroconversion to legionella, which almost certainly represented cross-reactivity with *P aeruginosa*. In contrast, in a similar study [Ong 1989] seroconversion rates were similar between "deteriorated" and "stable" groups.

The association of viral and atypical bacterial infection with respiratory exacerbation in the twelve prospective studies is summarised in table 1. Despite inconsistencies in the definition of exacerbation, the proportion of associated infections in paediatric age groups is reasonably consistent at 20-50%. No figures for adults have hitherto been published.

Six studies have addressed the clinical impact of respiratory viral infection in patients with cystic fibrosis. Wang et al [1984] found positive correlations between rate of

Table 1 - Association of respiratory viral and atypical bacterial infection with exacerbations of cystic fibrosis

Study	Age group	Diagnostic methods	Proportion of exacerbations with positive viral diagnosis
Wright 1976	paediatric	serology	25%
DeForest 1976	paediatric	serology	33%
Petersen 1981	paediatric	serology	20%
Efthimiou 1984	adult	isolation + serology	no data
Wang 1984	paediatric	isolation + serology	no data
Stroobant 1986	paediatric	isolation + serology	20% - 50%
Abman 1988	infants	isolation + serology	40%
Ramsey 1989	paediatric	isolation + serology	no data
Hordvik 1989	paediatric	isolation + serology	no data
Ong 1989	adult	isolation + serology	no data
Pribble 1990	paediatric	isolation + serology	29%
Smyth 1995	paediatric	isolation + serology + antigen detection + PCR	no data

increase of measures of illness severity with rate of viral seroconversion. Conversely, Ramsey et al [1989] found lower rates of increase of similar markers in patients with the highest rates of viral infection. Hordvik [1989] found an acute drop in peak expiratory flow rate in association with loosely defined upper respiratory tract infection, but values returned to baseline. Pribble [1986] found no difference in CRP or change from baseline in pulmonary function between exacerbations with and without viral co-infection, but there was a suggestion that influenza-associated exacerbations were more severe. Three case reports [Conway 1992] have implicated influenza infection with severe acute deteriorations in clinical status. Smyth et al [1995] found no difference in the rate of increase of various markers of illness severity between patients with and without viral co-infection, unless those with PCR-diagnosed rhinovirus infections were excluded, when a greater decline in FEV1 in the virally-infected group occurred.

The two Danish studies, [Petersen 1981; Johansen 1992], postulated a role for respiratory viruses in the acquisition of *P aeruginosa*. In the first of these, an increased rate of RSV infection was observed in a group of patients in transition to chronic pseudomonal colonisation. In addition, transient rises in anti-pseudomonal precipitins were observed in association with RSV infection in patients with established chronic colonisation. In the second, retrospective, study, 68% of patients with chronic colonisation acquired *pseudomonas* during the winter months,

defined as October to March.

Several general criticisms apply to all of the above studies. None has employed a rigorous definition of the term "exacerbation". None has evaluated serial pulmonary function data, and only two (Pribble 1990; Hordvik 1989), have acknowledged the short-term intra-individual variation in pulmonary function seen in cystic fibrosis, and used change in pulmonary function as an end-point. Only one study (Smyth 1995) has employed PCR techniques in an attempt to overcome the lack of sensitivity of conventional diagnostic methods, and then only for rhinoviruses. No study has used intraindividual comparisons when assessing the clinical impact of non-bacterial infection. Without such a comparison, type 2 statistical errors are very likely, because of the added inter-individual variation.

Chapter III Epidemiology of respiratory viral and atypical bacterial infection in conditions other than cystic fibrosis

III.1. Introduction

Because of the limited number of studies of infection with respiratory viruses and atypical bacteria in cystic fibrosis, especially of studies in young adults, it is important to review studies of such infections in other patient groups. In addition to a small number of epidemiological studies in healthy individuals, attention has focused on four populations: patients with asthma, patients with chronic obstructive airways disease, the institutionalised elderly, and children with bronchiolitis. Several studies have investigated the effect of experimental respiratory viral infection on pulmonary function.

III.2. Epidemiology of respiratory viral and atypical bacterial infection in healthy individuals

Anderson et al [1990] found increased mortality in the winter months, coincident with peaks in isolation rates of RSV and influenza A, in children under five. RSV infection is cyclical, group A and B strains of RSV alternately predominating in two yearly cycles [Waris 1990]. Strain A may be associated with more severe infection, as judged by the

need for ventilation in children with bronchiolitis [McConnochie 1990].

Foy et al [1979] reported an age-standardised annual rate of *M pneumoniae* pneumonia of 1.8/1000 per year. Infection was not seasonal, but there were two clear epidemics. During the 1974 epidemic year seroconversion occurred in 35% of the 10-21 year old cohort. One of the other cohorts included 15-19 year olds - seroconversion occurred in 6% of this group in one of the non-epidemic years.

Viruses were identified in 979 (30%) of a series of 3300 admissions for respiratory symptoms or febrile convulsions, reviewed retrospectively [Carlsen 1983]. RSV was found in 58% of confirmed infections, and peaked in November, December and January in 5 of the 7 years studied, and in March and April in the other 2 years. Adenovirus was the second commonest isolate (17% of confirmed infections), with no seasonal distribution. Influenza A and B were found in 5% and 2% of confirmed infections respectively, and only in late winter or early spring. Parainfluenza isolation was sporadic. Rhinovirus was found in 6% of confirmed infections, mostly in spring and autumn. Dual infection was identified in 20 cases, of which 19 involved RSV as one of the isolates.

Roughly 50% of the population reviewed was aged one year or less. RSV and parainfluenza 3 were found predominantly in the youngest age group; rhinovirus, influenza A, adenovirus serotypes 1,2 and 5 and parainfluenza 1 and 2 in the intermediate age group; and influenza B and adenovirus

serotype 7 in the older age group.

Lower respiratory tract infections were diagnosed in 87% of RSV infections, in 50% of adenovirus serotype 7 infections and in 45% of parainfluenza 3 infections. Ninety-one percent of cases of bronchiolitis were associated with RSV infection. Twelve patients had a pneumonia associated with adenovirus serotype 7. Upper respiratory tract infections were diagnosed in 62% of cases of adenovirus serotypes 1,2 and 5, and 8% of RSV.

A second study retrospectively reviewed the clinical characteristics of all 873 positive viral diagnoses (based on isolation, immunofluorescence, or serology) in children admitted to hospital in Oslo from November 1972 to December 1979 [Carlsen 1984]. RSV infection predominated, being identified 10 times as often as rhinovirus. This is of course dependent on the relative sensitivity of diagnostic techniques as much as actual prevalence rates. "Broncho-pulmonary obstruction" was more common in those infected with RSV than in other viral infection.

Kimball et al [1983] routinely collected sputum specimens for viral isolation from 100 adults hospitalised with pneumonia, diagnosed on the basis of new radiological pulmonary infiltrates. Six influenza A, 2 RSV, 3 rhinoviruses (11% of total) were isolated.

Kellner et al [1988] obtained specimens for isolation and immunofluorescence from 519 children admitted to hospital for treatment of respiratory tract infections, from September 1984 to May 1986. Pathogens were identified in 42.4% of

samples. RSV was the most commonly isolated agent (23.1% of samples), followed by rhinovirus (11.6%). RSV infection clustered in winter and spring but rhinoviral infection occurred throughout the year with slight peaks in autumn and spring. Adenovirus, enteroviruses, parainfluenza, and influenza A and B viruses each constituted less than 2.5% of isolates.

Thom et al [1990] reported the results of serology for *C pneumoniae*, *C trachomatis*, *M pneumoniae*, influenza A and B, adenovirus and RSV, and throat swab culture for *C pneumoniae* in 667 patients presenting to a university student health clinic with respiratory tract symptoms. Pneumonia was diagnosed clinically in 149 cases and confirmed by radiograph in 59. This is the only data concerning respiratory infection in otherwise healthy individuals of a similar age to adult patients with cystic fibrosis. The data are therefore summarised in detail in table 2.

McCarthy et al [1978] examined the CRP response in 156 children admitted consecutively with radiological pulmonary infiltrates. Serology was obtained in 106. Positive viral titres were found in 7.7% of cases with a raised CRP, compared with 38.9% in those with a normal CRP. The data were not analysed to present the frequency with which a positive viral diagnosis was associated with elevated CRP. This is the only study to have attempted to document CRP response to respiratory viral infection. It should be noted again that the population was a paediatric one.

Table 2 - Incidence of viruses and atypical bacteria in lower respiratory tract infections in normal adults (Thom 1990)

associated infection	percent cases of pneumonia	percent cases of all respiratory illness
C pneumoniae	9	3
M pneumoniae	11	3
influenza A	7	3
influenza B	3	2
RSV	1	1
adenovirus	1	1
total	32	13

III.3 Respiratory viruses and atypical bacteria in patients with asthma

III.3.i Studies in asthma employing conventional diagnostic techniques (serology, isolation, and antigen detection)

Respiratory viruses were first implicated in the precipitation of exacerbations of asthma in 1970 [Berkovich]. Positive respiratory viral serology was demonstrated in 27 of 84 children (32%) with an acute episode of wheezing.

Several cohort studies in the 1970's and 1980's attempted to determine the frequency of viral infection in the aetiology of asthma. In the first of these [Minor 1974], 23 viral isolates were considered to have caused "severe respiratory illnesses", and 21 of these were considered to be associated with an episode of asthma. Data from the same study published separately [Minor 1974] suggested a greater frequency of viral isolation in the asthmatic patients compared with their siblings (5.1 vs 3.8 per subject). In a similar study [Minor 1976] viruses were isolated in 17 (13%) of 128 symptom episodes. Rhinoviruses were the most common isolate. There were only 3 positive cultures in the adult group.

Mitchell et al [1978] isolated viruses in 13 (10%) of 127 episodes of wheezing in 16 children with asthma. Rhinovirus (5) was the most common isolate. More than half the isolates were obtained in the months August, September and October. Only one of 120 cultures obtained in the absence

of symptoms was positive.

In a primary care based study in London of 163 children [Horn 1979] 554 four episodes of "wheezy bronchitis" in 163 children were investigated. A virus was isolated in 146 (26%) of 554 episodes of wheezy bronchitis, compared with 1 (3%) of 31 swabs taken in the absence of symptoms ($p < 0.05$). Rhinoviruses accounted for 46% of isolations. In a related study the same authors recovered rhinovirus from sputum in 35 (49%) of 72 attacks of "wheezy bronchitis" in twenty two children aged 5 to 15 years [Horn 1979].

Carlsen et al [1984] noted an increased frequency of attacks of asthma in the spring and autumn. A positive viral diagnosis was made in 73 (29%) of 252 attacks. Rhinovirus was identified most frequently (33), in keeping with the seasonal trend, followed by RSV (14). The disparity between suggestive symptoms and positive viral diagnoses suggested that the sensitivity of conventional diagnostic techniques for respiratory viruses is limited.

Kava et al [1987] adopted a purely symptom-based approach. Sixty-three (25%) of 253 exacerbations (25%) in 68 patients were associated with a "symptomatic respiratory tract infection" (SRI). In total there were 141 episodes of SRI, 63 of which (45%) were associated with an exacerbation of asthma. Exacerbations associated with SRI lasted 11.4 days, compared with 8.9 days in the absence of SRI ($p < 0.01$).

Mertsola et al [1991] found respiratory viruses or *mycoplasma* in 45% of episodes of upper or lower respiratory

tract symptoms in 54 patients aged 1 to 6 years with a previous history of wheezy bronchitis during a 3 month surveillance period from October to December. Sixty-five percent of documented infections were due to rhinovirus and coronavirus.

Beasley et al [1988] were the first investigators to look at an adult population. Thirty-one patients with atopic asthma were followed for 11 months. Twenty three viruses were identified, and 10% of asthma exacerbations were associated with viral isolation. The commonest isolate was RSV (9), followed by rhinovirus (6).

Following serologically-proven influenza infection, FEV1 decreased from baseline by ≥ 20% in 15 of 20 patients who had already been admitted to a residential centre in order to achieve control of difficult asthma [Kondo 1991]. The nadir (30% below baseline) occurred 2 days after the onset of symptoms. FEV1 recovered to within 10% of baseline by day 10.

III.3.ii Studies in asthma employing the polymerase chain reaction for viral detection

The sensitivity of conventional diagnostic techniques in the detection of respiratory viruses is probably limited, although in the absence of a definitive "gold standard" it is impossible to be sure. Viruses are not identified in many symptom episodes which are highly suggestive of viral infection. Diagnostic methods have been evaluated most extensively in the case of RSV, for which serology, isolation

and antigen detection are all feasible. None of these methods is clearly more sensitive than the others, but antigen detection is favoured in clinical practice because of its rapidity in confirming RSV infection in children with bronchiolitis. Unfortunately, many clinical episodes of bronchiolitis are negative for RSV by one method while positive by another. Therefore even seroconversion cannot be regarded as a gold standard, and may be relatively insensitive. The situation is exacerbated in the cases of rhinovirus, for which serology is impracticable, and coronavirus, for which serology is not widely available, and further exacerbated in adults, as the rate of viral isolation decreases with increasing age [Carlsen 1984].

Two studies have used PCR techniques to detect respiratory viruses in exacerbations of asthma. In the first of these Johnston [1995] et al undertook a thirteen month prospective community-based study in 108 children aged 9-11 years identified by questionnaire as having wheeze and/or troublesome cough. Subjects kept a diary card of daily upper and lower respiratory tract symptom scores and twice daily PEFR recordings. Children were visited at home within 48 hours of reporting an episode (based on symptoms or fall in PEFR $\geq$ 50 l/min) and nasal aspirate and paired sera were obtained. Viruses were identified in 228 of 292 (78%) episodes. Standard culture techniques were employed and PCR was also carried out for rhinovirus and coronavirus. The rate of isolation was similar with upper respiratory tract symptoms, lower respiratory tract symptoms or episodes of

wheeze. Picornaviruses were found in 50% of episodes (66% of these were thought to be rhinoviruses), coronavirus in 13%, influenza in 7%, parainfluenza in 7%, and RSV in 4%.

Viral culture was positive for rhinovirus in 14 (8%) of 181 nose and throat swabs obtained during acute infection. No rhinovirus was identified in 101 swabs obtained during convalescence or at baseline. In contrast 76 (33%) of 230 acute swabs tested positive for rhinovirus with RT-PCR, including all 14 swabs positive on cell culture. Because of the risk of obtaining false positive results with PCR 29 convalescent and 61 baseline swabs were also tested, with 3 positives from convalescent swabs and none from baseline swabs.

As a corollary to the previous study, the same workers compared the fortnightly viral isolation rates from their community-based paediatric study with fortnightly hospital admission rates for exacerbations of asthma [Johnston 1993]. There were significant correlations with admissions for children, $r = 0.55$, $p = 0.003$ and for adults, $r = 0.46$, $p = 0.013$. The authors concluded that it was likely that hospital admissions were precipitated by viral infection.

Because PCR-based methods are potentially extremely sensitive there can be problems with false positive results. This will be expanded upon in the chapter on PCR methodology in this thesis. This problem can be overcome by using secondary PCR, in which the initial DNA fragment isolated from the clinical sample is subjected to a further cycle of polymerisation reactions with fresh oligonucleotides.

Nicholson et al [1993] used this technique to explore the role of rhinoviruses in exacerbations of asthma. One hundred and thirty eight adults with asthma, mean age 32.7, range 19 - 46, were studied. Subjects recorded PEFR twice daily, and were asked to report any new respiratory symptoms. These were classified as doubtful cold, cold, subjective exacerbation of asthma, cold with asthma, and doubtful cold plus asthma. Nose and throat swabs and paired sera (separated by 21 days) were collected in the event of symptoms. Serology included an ELISA for coronavirus. Nasal and throat swabs were subjected to standard culture methods, and rhinoviruses were also identified by a semi-nested reverse transcriptase PCR, with identification by band size (202 base pairs).

Three hundred and fifteen symptomatic episodes were identified in 112 subjects. There was objective evidence of exacerbation of asthma (reduction in PEFR of ≥ 50 l/min) in 84 episodes.

Coronavirus infection was identified in 36 (16%) of paired serum samples (15 229E, 21 OC43). A single pathogen was identified in 114 of 229 (50%) clinical samples, and dual infection in 5, with a total of 52% of samples positive. Rhinoviruses were responsible for 64% of confirmed infections, and coronaviruses for a further 30%. Laboratory confirmation accorded to clinical classification, being more common in episodes classified as colds than in episodes classed as doubtful colds and exacerbations of asthma without colds.

Symptoms of asthma exacerbation were present in roughly

70% of laboratory confirmed infection, and a reduction in PEFR of ≥ 50 l/min throughout the 7 days after the onset of symptoms in 24%. Of the 84 episodes with a drop in PEFR of ≥ 50 l/min, 71% were associated with symptoms of cold. Diagnostic specimens were available for 61 of these episodes, and infection was confirmed in 44% of these 61.

III.3.iii Summary of studies of respiratory viruses in asthma

The great majority of these studies have looked at paediatric populations. This is presumably because symptoms of respiratory viral infection tend to be more common in children, and it is generally felt that viral isolation is easier in this age group. One of the two PCR-based studies looked at a paediatric population, the other at an adult population. The proportion of exacerbations of asthma associated with confirmed viral infection is summarised in table 3. Culture-based studies have suggested that 10-30% of asthmatic exacerbations are associated with respiratory viral infection, while PCR-based studies suggest a figure closer to 50%.

In only three studies [Horn 1969; Nicholson 1993; Johnston 1995], has the design allowed an estimate of the statistical significance of the association between respiratory viral infection and exacerbation of asthma to be made. All three showed positive associations.

Rhinovirus, coronavirus and RSV have been the most

frequently implicated viruses in both age groups. In adults, Beasley identified RSV more frequently than rhinovirus, but with PCR Nicholson found the reverse.

There is little data relating to the short-term and long-term effects of viral infections in asthma - one retrospective study only showing a temporary decrease in FEV1 following serologically-proven influenza infection [Kondo 1991].

Table 3 - Association of respiratory viral infection with exacerbations of asthma

Study	Age group	Diagnostic methods	Proportion of exacerbations with positive viral diagnosis
Berkovich 1970	paediatric	serology	32%
Minor 1974	paediatric	isolation	no data given
Minor 1976	paediatric + adult	isolation	13%
Mitchell 1978	paediatric	isolation	10%
Horn 1979	paediatric	isolation	26%
Carlsen 1983	paediatric	isolation + serology + antigen detection	29%
Beasley 1988	adult	isolation + serology	10%
Mertsola 1991	paediatric	isolation	45%
Johnston 1993	paediatric	isolation + PCR	78%
Nicholson 1993	adult	isolation + serology + PCR	44%

III.4 Respiratory viruses in patients with COAD

In a prospective study of 15 patients with COAD (follow up 1 to 2.5 years) 12 rhinoviruses and 1 para-influenza 1 virus (total 13, 17%) were isolated in 75 exacerbations of chronic bronchitis [Eadie 1966].

Stenhouse et al [1967] looked specifically for rhinoviruses in 34 subjects with chronic bronchitis and 19 controls for 9 months from August to May. Rhinoviruses were isolated in 14.3% of exacerbations, but in only 2.1% of periods of quiescence.

McNamara et al [1969] studied twenty-nine patients with "chronic lung disease", age range 19 - 75 and FEV1/FVC ratios < 50%, for varying lengths of time over an 18 month period. Forty-two symptomatic exacerbations were identified. Twenty-three rhinovirus, 6 RSV and 5 *M pneumoniae* infections were identified. No other viruses were identified. Eighty percent of viral isolations was associated with an exacerbation.

Lamy et al [1973] studied 111 patients with chronic bronchitis for 1 to 9 months. Viral infection was confirmed in 63.3% of exacerbations. Paired sera were also obtained for 436 one month intervals. Exacerbations were identified in 36 of these intervals, seroconversion occurring in 21 (58.3%), compared with 15 seroconversions (3.7%) during intervals without exacerbation.

Buscho et al [1978] followed 49 adult men with chronic bronchitis prospectively over a period of 5 years for

infection with respiratory viruses and mycoplasma. Similar rates of viral and *mycoplasma* infection were found during exacerbations and periods of "remission", 30.1% vs 27.5%. Influenza A, parainfluenza virus type 3 and coronavirus OC43 accounted for two thirds of all the documented infections. Serology was positive for *mycoplasma* in only 4 exacerbations and no remissions.

Using serology for the six rhinoviruses isolated locally with the greatest frequency, Monto et al [1978] found a statistically higher rate of seroconversion in 51 male subjects compared with an equal number of male healthy controls (31% vs 15%), but not in females (48% vs 34%). The authors concluded that male patients with chronic bronchitis were more susceptible to rhinoviral infection than controls.

Smith et al [1980] monitored one hundred and fifty subjects and controls for evidence of acute respiratory illness and viral or mycoplasma infection over a 7 year period. Thirteen subjects were normal. The remaining 137 were considered to have COAD, although 13 of these had normal spirometry.

Viral or *mycoplasma* infection was associated with 186 (18.1%) of 1030 respiratory illnesses. Eighty six infections were identified in 1398 (6.2%) of illness free routine visits. Rhinoviruses were the most commonly isolated infectious agent (56, 4.8% of respiratory illnesses). Nasal washes were only slightly more commonly culture positive for rhinovirus than throat swabs. Overall, the rate of viral and *mycoplasma* infection was similar in the COAD and the control

groups.

In a parallel study Smith et al [1980] reported the results of the only prospective study of the association between natural viral infection and changes in pulmonary function in patients with COPD. Ninety-two subjects were observed for a mean of 4.4 years. FEV1 and FVC were compared in the six months before and the three months after a documented infection. The statistical method used compared (mean pre-infection FEV1 {or FVC}) - (mean post-infection FEV1 {or FVC}) with a similar value derived from computer-derived "simulated" infective episodes, in an attempt to define whether specific infections were associated with real changes in pulmonary function. Significant differences were found between real and simulated changes in FEV1 but not FVC for influenza viral infection on 17 occasions in 14 individuals. Twenty-nine rhinoviral and 11 parainfluenza infections were identified during the study, but no significant difference between real and simulated differences was found. Few infections with adenovirus, coronavirus, RSV and *M pneumoniae* were identified. The use of simulated, "non-viral" infections to compare with real viral infections was a novel way of attempting to identify the specific pathogenic role of respiratory viral infection, but unfortunately the parameters with which the simulated infections were generated were not listed, and conclusions are therefore hard to draw.

III.4.i Summary of studies of respiratory viruses in COAD

The proportion of exacerbations of COAD associated with confirmed viral infection is summarised in table 4. Despite methodological differences, the proportions (10% - 30%) are similar in patients with asthma, COAD and cystic fibrosis. The study of Lamy et al is exceptional, with a figure of 63%, and the discrepancy is not readily explicable. Four studies have attempted to demonstrate a statistically significant association. Three [Stenhouse 1967; Lamy 1973; Smith 1980] found such an association, while one [Buscho 1978] did not. Monto found viral infection to be more common in patients with COAD than in controls, but only in males. The only study to examine the clinical effects of respiratory viral infection [Smith 1980] found no detectable effects on pulmonary function three months following infection.

Table 4 - Association of respiratory viral infection with exacerbations of COAD

Study	Diagnostic methods	Proportion of exacerbations with positive viral diagnosis
Eadie 1966	isolation	17%
Stenhouse 1967	isolation	14%
McNamara 1969	isolation + serology	no data
Lamy 1973	isolation + serology	63%
Bushco 1978	isolation + serology	no data
Monto 1978	serology	no data
Smith 1980	isolation + serology	18%

III.5 Respiratory viruses in the institutionalised elderly

This population is of questionable relevance to a young adult cystic fibrosis population, but the literature is reviewed briefly for completion.

The subject was reviewed by Falsey [1991]. There have been several reported outbreaks of RSV pneumonia in the institutionalised elderly [Hart 1984; Sorvillo 1984; Garvie 1990], parainfluenza infection [PHLS 1983], and influenza A [Gross 1988]. Data on rhinoviral infection in the elderly are limited, but in 14 culture-proven cases self-limiting coryzal illnesses only were found [Falsey, unpublished data, 1990]. Nicholson [1990] described 11 cases of rhinoviral infection in institutionalised elderly patients, with only one developing lower respiratory tract symptoms.

Arroyo et al [1988] confirmed viral infection with serology in 21 (55%) of 38 respiratory infections in a twelve-month prospective study. Falsey et al demonstrated a viral aetiology in 62 (42%) of 148 respiratory illnesses, a rate of viral infection for the whole institution of 10.5%. Visual analogue symptom scores were significantly higher during RSV infection than during rhinoviral infection.

III.6 Studies specifically relating to paediatric RSV infection

Most of these studies concern RSV bronchiolitis/croup in infancy, and hence are not really comparable with an adult CF population. They are relevant in that they comprise most of the literature relating to sequelae of naturally acquired wild type respiratory viral infection.

RV/TLC ratio was raised in 9 (41%), and arterial oxygen tension lowered in 18 (78%), of 23 symptom-free children 10 years after a clinical diagnosis of bronchiolitis [Kattan 1977]. PEFR was lower and exercise-induced bronchial lability greater in 35 eight year old children known to have had RSV bronchiolitis in infancy when compared with an equal number of controls [Simms 1978]. Small airways obstruction, as judged by FEF 25-75, persisted for 8 years in 29 children who had been hospitalised for RSV lower respiratory tract infection in infancy [Hall 1984]. PEFR was reduced, and bronchial lability increased three-fold in 105 children 10 years after RSV lower respiratory tract infection compared with controls [Pullan 1982].

Bronchial hyper-reactivity was found in 14 (52%) of 27 children with a past history of croup compared with 1 (10%) of 10 normal controls [Loughlin 1979]. Three further, uncontrolled, studies demonstrated a high degree of bronchial hyper-reactivity in children nine years after a clinical diagnosis of croup (35%) [Gurwitz 1980], nine years after a clinical diagnosis of bronchiolitis (57%) [Gurwitz 1981], and

5 years after confirmed RSV bronchiolitis (50%) [Sly 1989]. Despite these findings, an uncontrolled questionnaire study found a normal prevalence of asthma (8%) in 37 patients who had documented RSV infection when aged 4 years or less ten years earlier [Twiggs 1981].

One study of childhood RSV infection is particularly interesting for its suggestion of viral/bacterial synergy [Korppi 1989]. The study group comprised children aged less than 15 years with admissions to hospital with "middle" or "lower" respiratory tract infection. Seroconversion to *S pneumoniae, H influenzae, B catarrhalis, Chlamydia spp*, and *M pneumoniae* were significantly more frequent in 90 children with RSV infection (39%), diagnosed by seroconversion and/or direct antigen detection, than in 99 children without RSV infection (24%).

In summary, there are several (largely uncontrolled) studies documenting an increased frequency of bronchial hyper-reactivity, but not asthma, in children several years following RSV infection. There is some evidence for long-term reduction in pulmonary function.

III.7 Studies of pulmonary pathophysiology following respiratory viral inoculation

Five studies have demonstrated changes in airway reactivity following inoculation with respiratory viruses. In an uncontrolled study, increased airway reactivity was demonstrated in beagles following infection with influenza C virus [Inoue 1986]. Arterial pO_2 decreased and total lung resistance increased in rats infected with Sendai virus compared to controls [Sorkness 1991]. Arterial pCO_2, dynamic lung compliance, and vital capacity were unaffected. Infected rats were more sensitive to methacholine than controls, although both virus and control groups became less sensitive with age. In six healthy human subjects bronchial reactivity was increased following experimental infection with live attenuated influenza virus, despite the absence of symptoms at the time of infection [Laitinen 1991]. There was no change in reactivity in eight control subjects. In nine patients who developed a fourfold rise in HI titre following influenza vaccination an increase in histamine sensitivity was observed over 48 hours, mean PD_{20} falling from 3.27 mg/ml to 1.47 mg/ml [Banks 1985]. No changes were observed in the 10 patients who did not develop a significant antibody response. Eight of ten patients with ragweed allergy demonstrated a late asthmatic reaction following inoculation with rhinovirus illness compared to one at baseline [Lemanske 1989]. Airway reactivity to both histamine and ragweed antigen increased during the acute infection.

Three studies have failed to show deleterious effects following viral inoculation. No change in bronchial reactivity or spirometry was observed in ten COAD patients and three normal subjects successfully infected with influenza A [Lowenberg 1986]. No differences in PEFR or symptom score were found one week after administration of killed influenza vaccine or placebo in a double blind study [Stenius-Aarniala 1986]. No differences in symptom score were observed during a further 8 months of follow up. No difference in specific airways conductance could be demonstrated between asthmatic subjects given influenza vaccine and asthmatic controls [Kava 1987].

III.8 Summary of studies of respiratory viral and atypical bacterial infection in conditions other than cystic fibrosis

In a cohort of patients of similar age to adults with cystic fibrosis, seroconversion to *M pneumoniae* occurred in 35% in an epidemic year, and 6% in a non-epidemic year [Foy 1979]. Influenza, adenovirus and RSV have been isolated most commonly in this age group. In a student population seroconversion to *M pneumoniae* and *C pneumoniae* occurred with equal frequency to seroconversion to these viruses [Thom 1990].

Respiratory viruses have been linked with 10-30% of exacerbations of asthma and COAD by culture and serology based studies, and with roughly 50% of exacerbations of asthma by PCR based studies.

Studies in the institutionalised elderly have suggested pathogenic roles for influenza and RSV, but rhinovirus infection appeared to be innocuous.

RSV infection in children appears to give rise to bronchial hyper-reactivity, and possibly minor changes in pulmonary function, several years following the initial infection. One report has suggested in vivo synergism between RSV and respiratory bacterial infection.

Equivocal changes in bronchial hyper-reactivity, but no changes in pulmonary function, have been shown following inoculation with respiratory viruses.

Chapter IV - Pathophysiology of respiratory viral infection

IV.1. Patho-physiology of uncomplicated respiratory viral infection

The histopathology of severe influenza infection was reviewed by Loosli [1973]. The lungs of fatal cases of otherwise uncomplicated influenza infection show extreme destruction and desquamation of tracheo-bronchial epithelium. Over 3 to 5 days the normal epithelium is replaced by a single layer of basal epithelial cells. Type I and type II pneumocytes lining the alveoli slough with hyaline membrane formation. Secondary bacterial pneumonia is a recognised sequelum, with *S pneumoniae, S aureus, H influenzae and K pneumoniae* frequently implicated. Further mucosal ulceration and necrosis then occurs in addition to that caused by influenza itself. Following recovery regenerating membranes at first consist of stratified squamous epithelium which is gradually replaced by ciliated and mucus secreting cells.

In more mildly infected individuals, Soderberg et al collected sixty three bronchoscopic bronchial mucosal biopsies from 12 patients recovering from respiratory infections with influenza virus (n = 8) and Mycoplasma (n = 4) 1-6 weeks after the onset of symptoms, and from healthy controls [Soderberg 1990]. In the influenza patients the epithelial height was greater compared to that of the healthy subjects and subepithelial lymphocytosis was noted. Areas with damaged epithelium, epithelial shedding, and

occasionally a thickened basement membrane, considered typical for asthma, occurred in both infected groups, and in the biopsies of the healthy subjects.

In epithelial monolayer cultures inoculated with rhinovirus and coronavirus 229E no detectable damage or cytopathic effect developed [Winter 1990]. In contrast, infection of epithelial monolayers with influenza A or adenovirus resulted in CPE and destruction of the monolayer. If these in vitro findings are reflected in vivo, the pathogenesis of symptom production during rhinovirus and coronavirus infections must be by mechanisms other than destruction of the lining of the respiratory tract. However nasal clearance was prolonged in six of nine symptomatic volunteers infected with rhinovirus or influenza B [Wilson 1987]. In six of the seven volunteers who were biopsied less than 50% of the epithelium was ciliated. Ciliary beat frequency was not reduced and ultrastructural ciliary abnormalities were not increased.

Panuska [1990] showed that human alveolar macrophages exposed to RSV demonstrated expression of RSV fusion gene, which increased in a time-dependent manner and correlated with RSV protein expression. RSV-exposed alveolar macrophages produced and released infectious virus into supernatants for at least 25 days after infection. This capability of alveolar macrophages to support prolonged RSV replication may have a role in the pulmonary response to RSV infection.

IV.2. Specific immunological mechanisms in respiratory viral infection

IV.2.i Mast cells, IgE, and histamine release

Castleman et al [1990] showed that bronchiolar mast cell density was higher in Sendai virus-inoculated rats than in sham-inoculated rats at 30, 60, and 90 days after inoculation (P less than 0.01), but not at 7 or 15 days after inoculation. PC_{50} was increased threefold at 42 days post-inoculation compared to baseline.

Leibowitz et al [1988] subjected mice to intranasal inoculation with RSV or sham inoculation. After three days the mice were exposed by inhalation to ragweed antigen for 5 consecutive days and rechallenged with ragweed on day 31. The IgG and IgE antibody responses to ragweed developed at significantly higher levels and the rise in serum IgE occurred earlier in the infected group.

In vitro, respiratory viruses are capable of enhancing IgE-mediated histamine release in the presence or absence of interferon from leukocytes of healthy individuals [Chonmaitree 1988]. Histamine release from peripheral blood leukocytes in vitro was enhanced by the addition of leukocytes to a supernatant of monocytes which had previously been exposed to influenza or RSV in vitro, suggesting that these viruses may cause the release of a cytokine which stimulates histamine release [Chonmaitree 1991].

In vivo, histamine release from guinea pig basophils

following antigen challenge was enhanced in animals infected with parainfluenza 3 virus compared to controls [Graziano 1989]. In a study of 27 rhinovirus-inoculated healthy subjects, kinins increased more than 10-fold in the fifteen subjects who became ill, but did not increase in those who remained well or in non-infected controls. However histamine levels were unchanged [Naclerio 1988]. Enhanced histamine release in vivo may therefore be dependent on antigen challenge.

The studies by Clementsen et al [1989, 1989, 1990] showed that influenza A is incapable of inducing histamine release itself, but may potentiate bacterially-induced histamine release (see below).

IV.2.ii Leukotrienes

Leukotriene C4 (LTC4) was detected in nasopharyngeal secretions obtained in the acute phase of RSV illness from 67% of infants with bronchiolitis and from 33% of infants with upper respiratory illness alone ($p < 0.025$), with concentrations of LTC4 5-fold higher in the bronchiolitis group [Volovitz 1988]. In children with episodes of acute wheezing, LTC4 concentration in those who shed respiratory viruses was found to be consistently elevated compared with values in wheezing children without evidence of viral infection. Little LTC4 activity was detected in healthy children [Volovitz 1988].

IV.2.iii Peptides

Jacoby et al [1988] showed that infection of ferret airway tissues with influenza virus increased the contractile response of airway smooth muscle to substance P. The effect was due to decreased enkephalinase activity in infected tissues.

Borson et al [1989] showed greater neurogenic inflammatory responses in rats with previous respiratory infections than in healthy control rats, in association with a lower activity of tracheal neutral endopeptidase.

Drusser et al [1989] found a greater bronchoconstrictor response to substance P and capsaicin in guinea pigs infected with Sendai virus than in non-infected controls. The addition of phosphoramidon, a neutral endopeptidase inhibitor, enhanced bronchoconstriction in uninfected animals, but not in the infected animals, who were presumably already maximally bronchoconstricted. Nonadrenergic noncholinergic (NANC) bronchial smooth muscle responses to electrical field stimulation in vitro were also increased in tissues from infected animals compared with noninfected animals. The addition of phosphoramidon again increased bronchoconstriction of tissues from noninfected animals greatly but not from infected animals. Responses to acetylcholine were unaffected by viral infection. Neutral endopeptidase activity was decreased by 40% in the tracheal epithelial layer of the infected animals. Sendai virus infection thus caused enhanced airway responsiveness to

tachykinins, probably mediated by NANC pathways, and probably by decreasing neutral endopeptidase-like activity in the airway epithelium.

In conclusion, there is evidence that some respiratory viruses can enhance peptide-induced bronchoconstriction by reducing the activity of epithelial endopeptidases.

IV.2.iv Interleukins

Turner et al [1988] showed that rhinovirus infection of human embryonic lung fibroblasts induces the production of a chemoattractant for polymorphonuclear leukocytes.

Becker et al [1991] demonstrated increased mRNA levels for TNF, IL-6 and IL-8 in human alveolar macrophages, within 1 hour of in vitro incubation with RSV. Peak expression occurred at 3-6 hours. Inactivated virus was almost as effective as live virus in inducing IL-6 and IL-8 mRNA, but live infectious RSV was necessary for inducing TNF mRNA expression. The addition of influenza A to human airway epithelial cell culture more than doubled IL-8 production [Choi 1992].

Noah et al [1994] showed that IL-8 was induced at 4 h after RSV infection (during the eclipse phase of RSV infection) in bronchial epithelial cells in vitro. Induction of IL-6 and granulocyte macrophage colony-stimulating factor in supernatants were only detected at 96 h after infection, during the RSV replicative phase.

IV.2.v Interferons

Interferon-alpha and -gamma transcripts were not detected in bronchial epithelial cell culture following RSV infection [Noah 1994]. Taylor et al postulated that defective interferon alpha production may underlie the development of RSV bronchiolitis, but found no correlation between infection and the level of interferons in the peripheral circulation.

IV.2.vi Intra-cellular adhesion molecule 1 (ICAM 1)

Maximal cytotoxic injury by polymorph neutrophils is dependent on tight cell-cell adhesion. Wegner et al [1990] showed that ICAM-1 partially mediated eosinophil adhesion to endothelium in vitro and was upregulated on inflamed bronchial endothelium in vivo. ICAM-1 expression was also upregulated on inflamed airway epithelium in vitro and in vivo. In a primate model of asthma, a monoclonal antibody to ICAM-1 attenuated airway eosinophilia and hyper-responsiveness. Upregulation of ICAM-1 expression is therefore a potential mechanism by which respiratory viruses could cause airway inflammation and asthma.

Salkind et al [1991] showed enhanced expression of ICAM-1 and its ligand, LFA-1, together with cell clustering, after exposure of human monocytes to influenza in vitro. In contrast, exposure of human monocytes to RSV resulted in suppressed expression of both ICAM-1 and LFA-1 and minimal detectable cell clustering.

Tosi et al [1992] found greatly increased in vitro neutrophil adhesion to tracheal epithelial cells infected with parainfluenza virus 2 (89% +/- 7%) compared with uninfected epithelium (< 5%). Surface ICAM-1 expression on epithelial cells doubled 24 h after infection and tripled after 48 h. Although ICAM-1 expression increased in association with increased neutrophil adhesion, treatment of virus infected epithelial cells with a monoclonal antibody directed against ICAM-1 had no significant effect on PMN adhesion. The significance of the increased ICAM-1 expression is thus unclear.

There is no clear evidence for a pathogenetic role for upregulation of ICAM-1 in respiratory viral infection from these two in vitro studies.

IV.2.vii Eosinophils, neutrophils and superoxide

Concentrations of eosinophil cationic protein recovered from naso-pharyngeal washes in RSV-infected children were elevated in bronchiolitis, compared to lower respiratory tract disease without wheezing, and upper respiratory tract illness only [Garofalo 1992]. After incubation for 2 h, 30% of peripheral blood eosinophils demonstrated specific binding of RSV to the cell membrane [Kimpen 1992]. Variable virus-induced superoxide generation was found among different subjects. In vitro incubation with influenza virus primes human polymorphonuclear leukocyte generation of superoxide [Busse 1991].

IV.3 Viral-bacterial interaction

IV.3.i Viral infection as a precursor of bacterial infection

As early as 1946 Harford showed that following sublethal influenza infection mice were more susceptible than controls to subsequent infection with *S pneumoniae*, as judged by colony counts of inocula of minced lung tissue. Two years later he showed that the pulmonary clearance of *S pneumoniae* was not inhibited by the replication of influenza virus alone, but required the development of pneumonia.

As the third part of their investigation of viral infection in COPD (see chapter II), Smith et al [1976] examined the association between viral and bacterial infection in 120 patients with COPD and 30 healthy controls who were monitored over a seven year period. Bacterial cultures (throat swab or sputum) were positive for *S pneumoniae* in 9.2%, *H influenzae* in 12.8% and *H parainfluenzae* in 41.9% of cases. Viral infection was found in 273 cases with culture or serology. One hundred and two viral infections (37%) were associated with a positive bacterial culture, but it is not possible to conclude that there is a statistical association from the data.

Kournikakis et al [1987] compared the effects of wild type and attenuated murine cytomegalovirus on the mortality of mice infected with *P aeruginosa*. Challenge with 5×10^5 pfu murine CMV intraperitoneally followed by 5×10^6 cfu intranasally resulted in 80% mortality at 48 hours. By

contrast, infection with attenuated virus was not associated with any mortality, or morbidity.

Finally, Saito et al [1988] showed that the incidence of infection with *S pneumoniae* in healthy guinea pigs cage-mated with infected animals rose from 2/30 to 25/30 in the presence of co-infection with Sendai virus in the index cases.

IV.3.ii Mechanisms of viral-bacterial interaction

Taylor et al [1974] showed that alveolar macrophages harvested from guinea pigs three days after the animals had been exposed to aerosolised influenza A1 showed enhanced bacterial ingestion but reduced intracellular killing, following incubation with a suspension of *K pneumoniae*, as compared to control animals not exposed to the virus.

Jakab et al [1976] challenged mice with aerosolised *S Aureus* 7 days after infection with a sublethal dose of Sendai virus. At 0 hours following challenge with *S Aureus*, 50% of bacteria were located intracellularly. With increasing time following staphylococcal exposure relatively more intracellular organisms were observed in focal areas of virus-infected lung compared with non-virus infected control lungs. It was concluded that Sendai virus interferes with intracellular bacterial processing mechanisms.

Clementsen et al [1989] explored the interaction between influenza A virus and bacteria in releasing histamine in three studies. In the first [Clementsen 1989a], influenza A virus enhanced *S. aureus*-induced histamine release in human

leukocyte suspensions from normal individuals and patients with intrinsic asthma, but the virus alone did not release histamine. The effect was thought to be mediated through neuraminidase, as a similar effect was observed with purified neuraminidase from *Vibrio cholerae*, and the potentiation in both cases was abolished by neuraminidase inhibitor.

In the second study BAL was obtained from 13 healthy non-atopic individuals [Clementsen 1989b]. After incubation of BAL cells with *S. Aureus*, histamine was released in 7 of 13 individuals, leukotriene B4 in 7 of 11, but leukotriene C4 in none of 4. After incubation of BAL cells with influenza A virus, histamine was released in 3 of 5 subjects, but leukotriene B4 in none of 7 subjects.

In the third study influenza A virus was found to enhance basophil histamine release induced by *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus sanguis*, but again the virus itself was not found to induce histamine release [Clementsen 1989]. Histamine release induced by other immunological and non-immunological stimuli, such as anti-IgE, calcium ionophore or agarose beads was enhanced in the presence of the virus.

Nain et al [1990] showed that exposure of human monocytes, rat alveolar macrophages, and murine macrophages, to influenza A virus resulted in TNF-alpha mRNA accumulation, TNF-alpha release and subsequent cell death. TNF-alpha production was dependent on exposure to live virus, in contrast to interferon release that was also induced by

UV-inactivated virus. Low amounts of lipopolysaccharide (1 to 10 ng/ml) from *Escherichia coli* or *Haemophilus influenzae* were capable of strongly potentiating TNF-alpha production from virus-infected macrophages. TNF-alpha production might therefore be implicated in the clinical effects of combined influenza A virus and bacterial infection.

Chapter V Treatment strategies for pulmonary disease

V.1 Gene therapy

The ultimate solution to cystic fibrosis would be replacement of the defective gene, resulting in normal CFTR in the critically affected tissues. Normalisation of transmembrane voltage has been achieved in vitro in cell monolayers following transfection with an adenoviral vector containing copies of DNA coding for normal CFTR [Rosenfeld 1994]. Approximately 10% of cells required infection for the voltage to normalise. Targeting vectors in vivo is more difficult, and systemic administration of DNA has not yet been contemplated. Since the main morbidity and mortality in cystic fibrosis arises through pulmonary damage, attention has been focused on the administration of correct copies of DNA directly to the airways. This would have to be achieved with a compressor and nebuliser system. Trials in human volunteers have so far been limited to topical application, initially to the nasal epithelium and subsequently to lower respiratory tract epithelium via a fibre-optic bronchoscope. The results have been variable. Hay et al [1995] demonstrated changes in nasal potential difference following transfection with adenoviral vector, but in a similar study Knowles et al [1995] were unable to show any change. Repeated administration has led to significant anti-viral IgG formation [Yang 1995; Yei 1994] with a loss of efficacy. Theoretically, liposomal vectors should be less immunogenic,

but transfection is less efficient than with viral vectors. Until these problems can be overcome gene therapy will not be practicable. It should also be noted that this whole approach assumes that airway epithelium is the only target for DNA delivery. It may be that delivery is also required to submucosal glands, which would not be accessible with a conventional nebuliser delivery system.

V.2 Anti-bacterial strategies

As yet there is no effective treatment for pulmonary disease in cystic fibrosis before the occurrence of bacterial colonisation. Most current treatment strategies are therefore directed at preventing initial bacterial colonisation, reducing bacterial load once colonisation has occurred, and treating airways obstruction secondary to the bronchiectatic state.

Since the response to colonisation with *S aureus* is so variable, there is no consensus on the optimal treatment regimen. The approach ranges from no treatment, through flucloxacillin prophylaxis following acquisition of the organism, to prophylaxis with low or high doses (1G qds) of flucloxacillin from the time of diagnosis. There is some evidence to support the use of regular prophylaxis [Weaver 1994].

Since acquisition of *P aeruginosa* is usually followed by progressive deterioration in lung function, attention has

been focused on strategies to minimise the resultant lung damage. Attempts at producing an effective pseudomonal vaccine continue, but have so far not met with success. The most recent vaccine has shown some promise [Lang 1995], but has not been evaluated in a controlled study. It is generally recognised that in the absence of an effective vaccine and without effective gene therapy, acquisition of *pseudomonas* is almost inevitable. Once the organism is grown from sputum, chronic colonisation usually follows. There is evidence that the onset of chronic colonisation can be delayed using a regime of nebulised colistin and/or oral ciprofloxacin at the time of the first pseudomonas-positive sputum culture [Valerius 1991], but this regime cannot prevent chronic colonisation occurring ultimately. Once patients are chronically colonised with *pseudomonas* there is now good evidence that use of regular nebulised anti-pseudomonal antibiotics can slow down the rate of loss of pulmonary function [Mukhopadhyay 1996], although the optimal antibiotic regimen has not yet been worked out. The efficacy of nebulised antibiotics is critically dependent on using appropriate compressors and nebulisation systems.

Systemic anti-pseudomonal antibiotics are used either solely to treat acute pulmonary exacerbations, or additionally in a prophylactic manner at a predetermined frequency in the absence of exacerbation, in patients colonised with *pseudomonas*. The latter practice follows the Danish experience of Szaff and Hoiby [1983], who instituted regular three-monthly intravenous anti-pseudomonal antibiotic

therapy, irrespective of the clinical condition of the patient. Following the institution of this regimen the authors recorded improved survival, but there was no prospective controlled study. Since survival of patients with cystic fibrosis was improving in other centres during the same period, it is difficult to know whether such a strategy is genuinely effective in preserving lung function.

Anti-pseudomonal antibiotics are assumed to have a beneficial effect by reducing the numbers of colonising bacteria, with clinical improvement following from the subsequent decline in the inflammatory response. Reductions in bacterial sputum density and peripheral inflammatory markers have been demonstrated [Norman 1991], but it is possible that intravenous antibiotics could be exerting at least part of their effect through a direct down-regulation of the inflammatory response. There is no evidence that routinely matching antibiotic selection to the in vitro sensitivity of pseudomonal strains is beneficial, but there is insufficient data to draw firm conclusions.

V.3 Physiotherapy

Cystic fibrosis sputum is extremely viscous. Consequently chest physiotherapy forms an integral part of treatment, both in long-term prophylaxis, and in the treatment of acute exacerbations. In the short-term physiotherapy is as effective as vigorous exercise in terms

of the volume of sputum expectorated, but physiotherapy and exercise together have an additive effect [Bilton 1992]. There is no data relating physiotherapy to survival or long-term change in pulmonary function. There is little comparative data between the various chest physiotherapy techniques. The active cycle of breathing technique with postural drainage is the method used in most UK cystic fibrosis centres, after Pryor [1979], but recent work has suggested that autogenic drainage may have slight advantages [Miller 1995].

V.4 DNase

Another method of aiding clearance of viscous secretions is to lower sputum viscosity pharmacologically. The excessive viscosity of cystic fibrosis sputum is due to its content of high molecular weight DNA, derived largely from white cells [Shak 1990]. A reduction in size of the DNA molecule within sputum leads to a greatly reduced viscosity. Bovine DNAse was first used in the late 1950's. Although highly effective in lowering viscosity, its use was associated with adverse effects, of presumed allergic aetiology, and it was withdrawn from use. The advent of a recombinant DNase (rHDNase) has stimulated interest in this approach again. Short and medium term studies have demonstrated sustained improvements in pulmonary function with daily administration, but the size of the mean improvement was small, and as yet there are no long-term data [Hubbard 1992; Ranasinha 1993; Fuchs 1994].

V.5 Bronchodilators

Bronchial obstruction occurs as part of the bronchiectatic process. Asthma can coexist with cystic fibrosis, but the incidence of asthma and bronchial hyperreactivity is probably no higher in cystic fibrosis than in the general population. In patients with moderate and advanced pulmonary disease bronchodilators form an integral part of treatment. In patients already using prophylactic nebulised anti-pseudomonal antibiotics, a nebulised bronchodilator may be administered first. There is evidence that the autonomic nervous system is deranged in cystic fibrosis [Davis 1980], with increased sensitivity to α-adrenergic and cholinergic agonists, and reduced sensitivity to ß-adrenergic antagonists. Some patients are more responsive to cholinergic bronchodilators than ß2 agonists [Sanchez 1993].

V.6 Anti-inflammatory treatments

Because most of the pulmonary damage in cystic fibrosis is thought to arise through the host inflammatory response, there should intuitively be a role for anti-inflammatory treatment. The only large trial of corticosteroid therapy to have been conducted showed a reduced rate of deterioration of pulmonary function in the treatment group, but there was an unacceptably high rate of side effects, notably of cataract

[Essen 1995]. Consequently there is no role at present for oral corticosteroids. Inhaled corticosteroids have a role in those thought to have coexistent asthma, but they have not been studied in the context of pulmonary inflammation in cystic fibrosis.

Encouraging results have recently been reported with ibuprofen [Konstan 1995], but only in mildly affected patients aged less than thirteen years. The data are preliminary, and a larger trial is required before non-steroidal therapy can be contemplated as a routine treatment.

V.7 Nutrition

Even when well, patients with cystic fibrosis require 120% of the calorific intake of age-matched controls [Vaisman 1987]. This target became attainable with the advent of microsphere pancreatic enzyme preparations in the 1980s, which enabled a switch from low calorie, low fat diets to high calorie, high fat, diets. Energy expenditure, and therefore energy requirements, increase with advancing pulmonary disease [Fried 1991], and at the time of acute pulmonary exacerbations, presumably due to the increased work of breathing and energy losses associated with the excess pulmonary inflammation. Energy supplementation, initially orally, and subsequently enterally, has an important role in maintaining body weight in these circumstances. Treatment of pulmonary exacerbations with intravenous antibiotics,

physiotherapy and calorie supplementation results in significant weight gain [data on file]. In malnourished patients calorie supplementation with an exercise programme results in increased maximal oxygen uptake [Heijerman 1992].

V.8 Treatment strategies adopted at the Leeds adult cystic fibrosis unit

The interindividual variation in disease severity and the long-term nature of pulmonary morbidity are both factors which make controlled studies of the efficacy of the various interventions difficult. Consequently good evidence for clinical benefit, particularly in the long-term, is lacking for most of these. Survival of cystic fibrosis patients is clearly increasing [Elborn 1991] but it is not possible to say which of the interventions employed have been primarily responsible for the improved prognosis. The practical clinical solution is to strike a balance between the available interventions, and the disruption to the patients' lifestyle consequent upon these interventions. Although this is very much a case of tailoring a treatment strategy to the individual patient, most patients in the Leeds adult unit would have followed the protocol below during the two year period of data collection, from May 1st 1991 to April 30th 1993.

V.8.i Prophylaxis

All patients entering the adult unit from the Leeds paediatric unit had been taking oral flucloxacillin as anti-staphylococcal prophylaxis from the time of diagnosis of cystic fibrosis. Patients entering the adult unit from other sources were started on regular flucloxacillin if not already receiving it. Flucloxacillin was administered irrespective of the presence of *S Aureus* on sputum culture.

Most patients would already be colonised with *P aeruginosa* by the time they came to the adult unit. In those who were not colonised, a short course of nebulised colistin and oral ciprofloxacin was instituted to attempt to delay the onset of chronic colonisation as soon as sputum cultures positive for *pseudomonas* were obtained. Prophylactic twice daily nebulised colistin or tobramycin (in the case of colistin-induced bronchospasm) was instituted in patients chronically colonised with *P aeruginosa* who were felt to have significant pulmonary disease. Most patients would be started on nebulised bronchodilators at this point, after formal assessment of reversibility.

In addition to nebulised antibiotic prophylaxis, a modified Danish approach to prophylactic systemic anti-pseudomonal antibiotics was used. Patients with relatively normal pulmonary function (predicted FEV1 > 80%) received no systemic antibiotics prophylactically. Patients with moderately impaired pulmonary function (predicted FEV1 50-80%) received systemic antibiotics at least twice yearly, and

patients with severely impaired pulmonary function (predicted FEV1 < 50%) were encouraged to receive systemic antibiotics at least four times per year.

All patients were encouraged to perform chest physiotherapy twice per day (active cycle of breathing technique with postural drainage). All patients were also informally encouraged to exercise. DNase was not available at the time of this study.

Corticosteroids and non-steroidal anti-inflammatory agents were not used prophylactically.

All patients received regular dietary supervision. Dietary intake was adjusted to keep percent predicted weight for height as close as possible to 100%.

V.8.ii Protocol for treatment of exacerbations and prophylactic courses of intravenous antibiotics

In patients chronically colonised with *pseudomonas*, minor increases in lower respiratory tract symptoms were treated with oral ciprofloxacin, irrespective of sputum sensitivities. More severe symptomatic exacerbations, or unexplained loss of pulmonary function, was treated with intravenous antibiotics, either in hospital or at home. The outcome of such treatment is not dependent on whether it takes place in hospital or at home [Pond 1994]. The protocol for intravenous antibiotic treatment was the same for treatment of exacerbations and for routine prophylactic

treatment. Nebulised antibiotics were discontinued, and two intravenous antibiotics were chosen, using the last recorded sputum sensitivities as a guide. Sputum was recultured during treatment, and antibiotics changed if necessary according to the updated sensitivities. Patients received supervised chest physiotherapy twice daily when treated in hospital, and were encouraged to perform physiotherapy twice daily when treated at home. In both cases further attention was given to dietary intake during the course of antibiotic treatment. All hospital-treated patients were screened for nocturnal oxygen desaturation with overnight oximetry and supplemental oxygen was administered if SpO_2 was less than 90% for more than 10% of the recording.

The response to therapy was monitored with serial recordings of pulmonary function, weight, and a "clinical score", derived from clinical signs [Conway 1985], which was recorded daily. Treatment of exacerbations was discontinued when there was no further improvement in these parameters. Prophylactic treatments were scheduled to last ten to fourteen days.

Chapter VI Peripheral markers of inflammation

VI.1 General Introduction

White cell count (WCC), plasma viscosity (PV), and c-reactive protein (CRP) are well-established peripheral markers of inflammation in disorders other than cystic fibrosis and are routinely available in most laboratories. Although statistically significant suppression of these markers, and of serum tumour necrosis factor alpha and serum neutrophil elastase α-1-antitrypsin complex, has been demonstrated in small numbers of patients with cystic fibrosis [Norman 1991; Elborn 1993; Elborn 1993; Watkin 1994], their utility in the systematic monitoring of a cystic fibrosis population has not been evaluated.

The utility of measuring peripheral inflammatory markers depends on the answers to the following questions.

1. Do they provide clinical information additional to that derived from other clinical measures eg pulmonary function ?

2. Can they contribute to our understanding of the pathogenesis and the clinical impact of pulmonary exacerbations?

3. Do all inflammatory markers behave in a similar fashion or are there inter-marker differences ?

4. Are peripheral inflammatory markers useful in the clinical situation, and can they be used in the definition of pulmonary exacerbation?

VI.2 Commonly measured inflammatory markers: white cell count, plasma viscosity, and c-reactive protein

Methods

All three markers were measured routinely at the start and end of courses of intravenous antibiotic therapy during the full two years of the study (May 1st 1991 to April 30th 1993). White cell count was determined using a standard Coulter counter, and plasma viscosity with a viscometer. C reactive protein was measured nephelometrically. All treatments, whether undertaken in hospital or at home, were included, as it has been shown that the response to treatment is equivalent wherever it is undertaken [Pond 1994]. Each marker was compared with percent predicted FEV1 and percent predicted FVC separately for the pre-treatment and post-treatment cases. Spirometric values were selected for the abscissa as these are direct measures of pulmonary function and are the most frequently used measures of disease severity. Percent predicted FEV1 and FVC are used in this way throughout the remainder of this thesis. In all cases FEV1 and FVC were recorded using one Vitallograph Compact spirometer. Patients were asked to repeat forced manoeuvres until the highest two readings differed by no more than 5%. FEV1 and FVC obtained from the expiratory effort with the greatest FEV1 were then recorded and expressed by the vitallograph as percent predicted based on the patient's sex, age and height.

Only those treatments with complete pulmonary function and inflammatory marker data were included in the analysis. C-reactive protein values less than 10 mg/dl were reported as such by the laboratory, and not given specific numerical values. For the purposes of the analysis such results were assigned an arbitrary value of 5 mg/dl. The crude data were distributed normally in all cases except for pre- and post-treatment CRP. Because the numbers of treatments varied between patients, pre- and post- treatment means for each parameter were found for each patient (except for CRP, where the median was found). Regression of each inflammatory marker was then performed in turn against pre-treatment FEV1, post-treatment FEV1, pre-treatment FVC and post-treatment FVC, with each point in the regression weighted for the number of treatments for which it represented the mean (or median). The final pre- and post- treatment regression curves were then compared for each of the three inflammatory markers.

Statistical analyses were performed manually, or with the Minitab programme, throughout the thesis.

Results

Data were complete for 294 of the total 459 (64%) treatments. Data were incomplete for 10 patients who each had only one treatment. In the remaining 79 patients the median number of treatments was 3, range 1 to 13. All five parameters (2 pulmonary function, 3 inflammatory) improved significantly with treatment (see table 5).

Regression results

Inspection of the scatter plots in all twelve cases (3 inflammatory markers × 2 spirometric measures × 2 occasions per treatment) suggested non-linearity, which was confirmed statistically with the exploratory lack of fit model. Again in all twelve cases, there appeared to be one statistical "peak" to the data, and a polynomial quadratic regression curve was therefore fitted in each case. All the regression curves were highly statistically significant. Figures 2 to 13 show the scatter plots with the fitted regression curves. Table 6 shows the correlation coefficients.

Figure 2 - Scatterplot, regression and 95% confidence intervals, white cell count vs percent predicted FEV1, pre-treatment

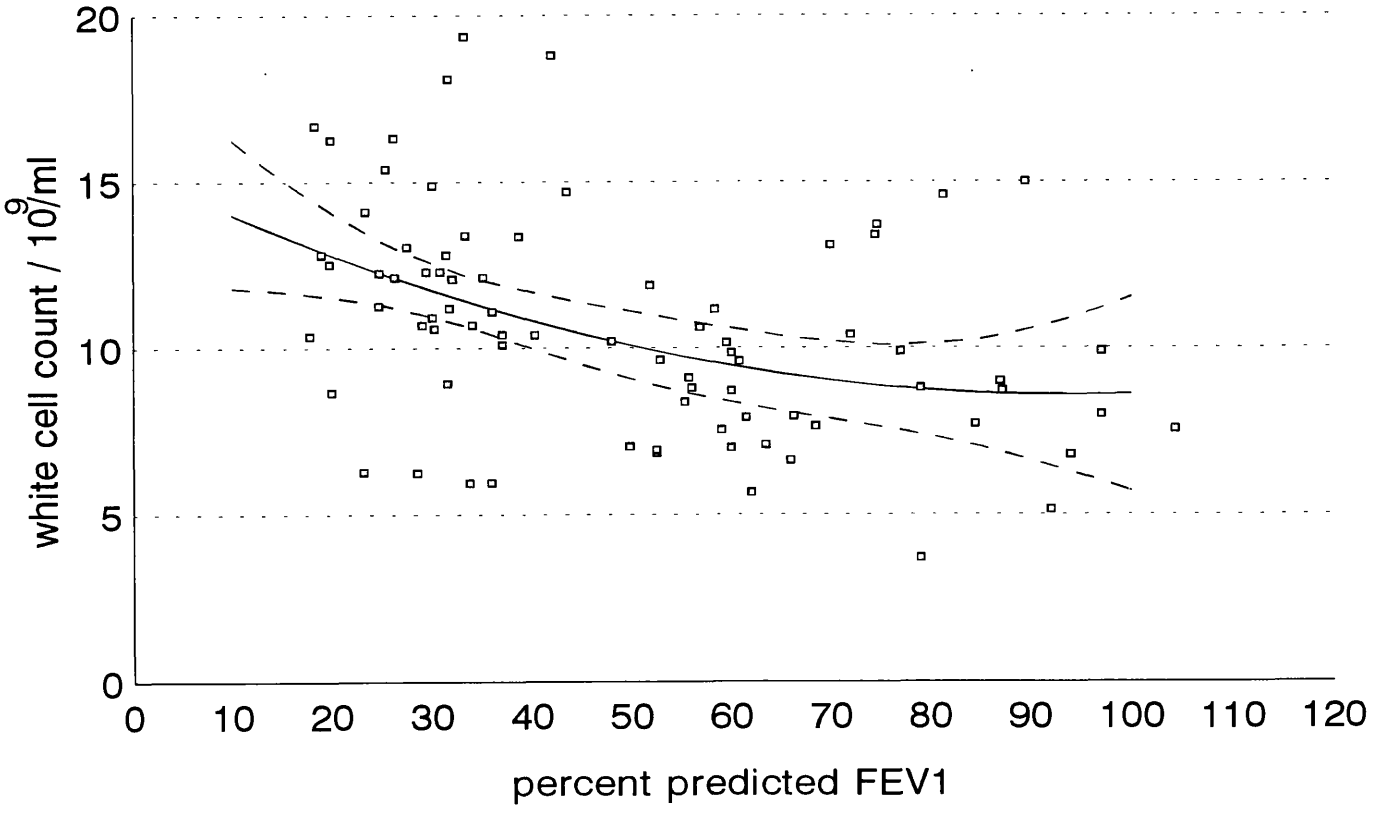


Figure 3 - Scatterplot, regression and 95% confidence intervals, white cell count vs percent predicted FEV1, post-treatment

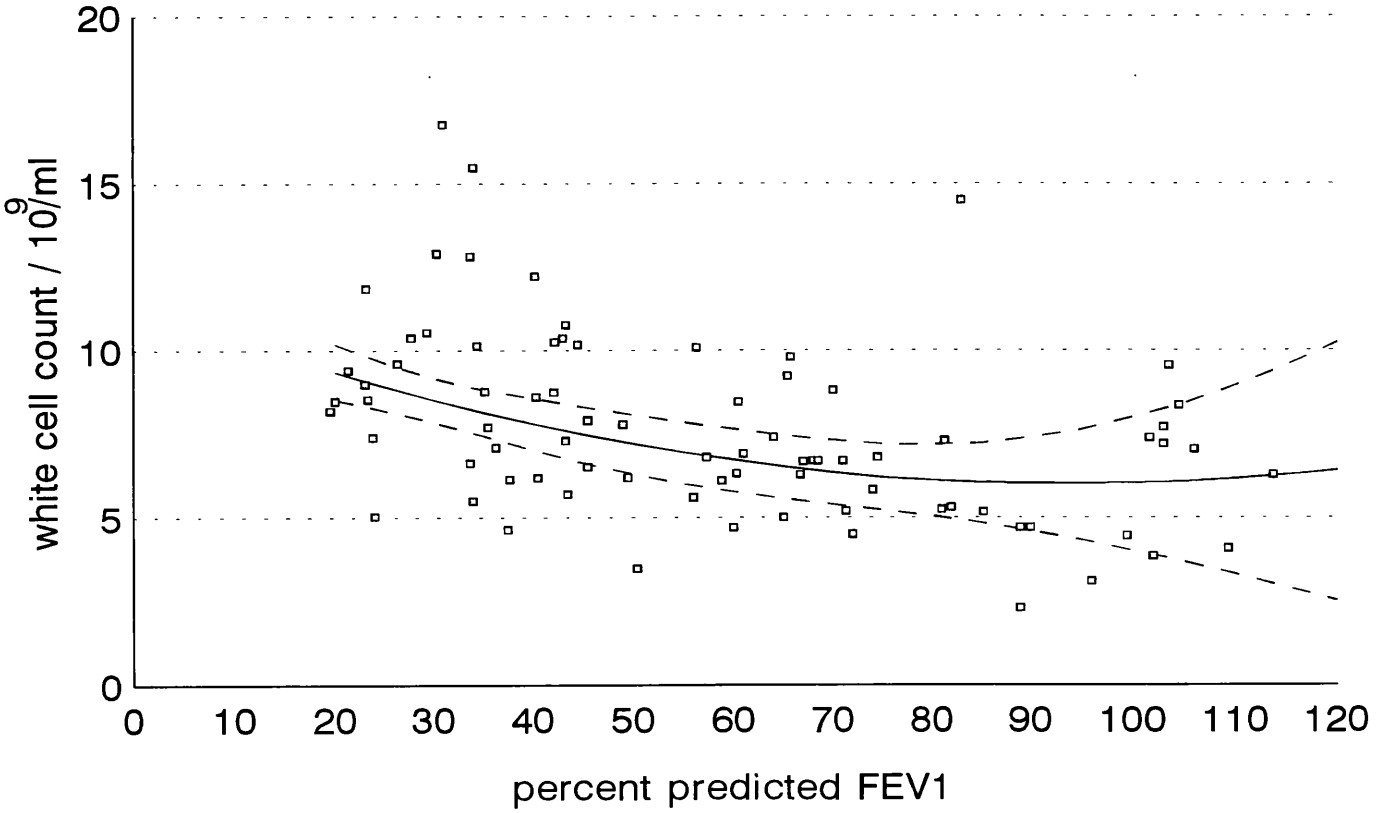


Figure 4 - Scatterplot, regression and 95% confidence intervals, white cell count vs percent predicted FVC, pre-treatment

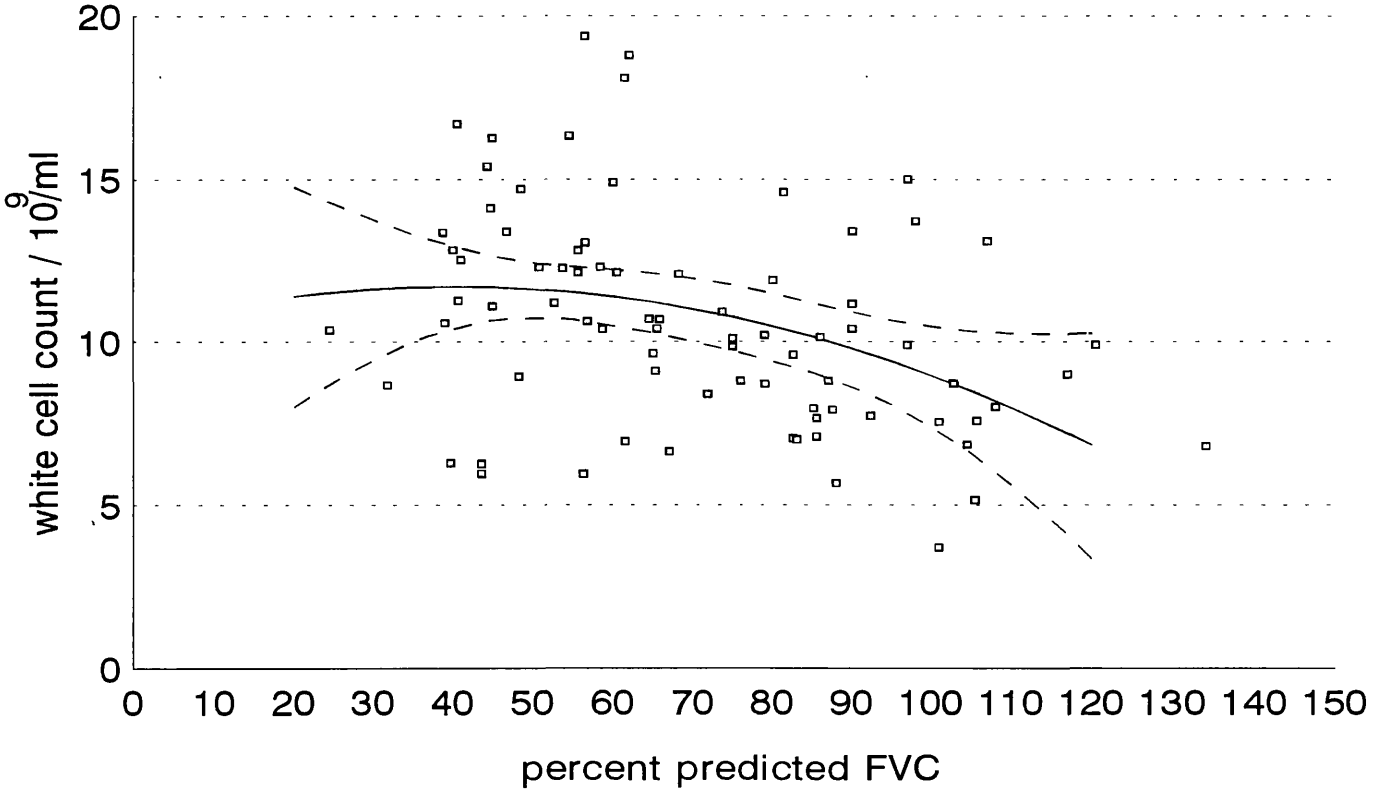


Figure 5 - Scatterplot, regression and 95% confidence intervals, white cell count vs percent predicted FVC, post-treatment

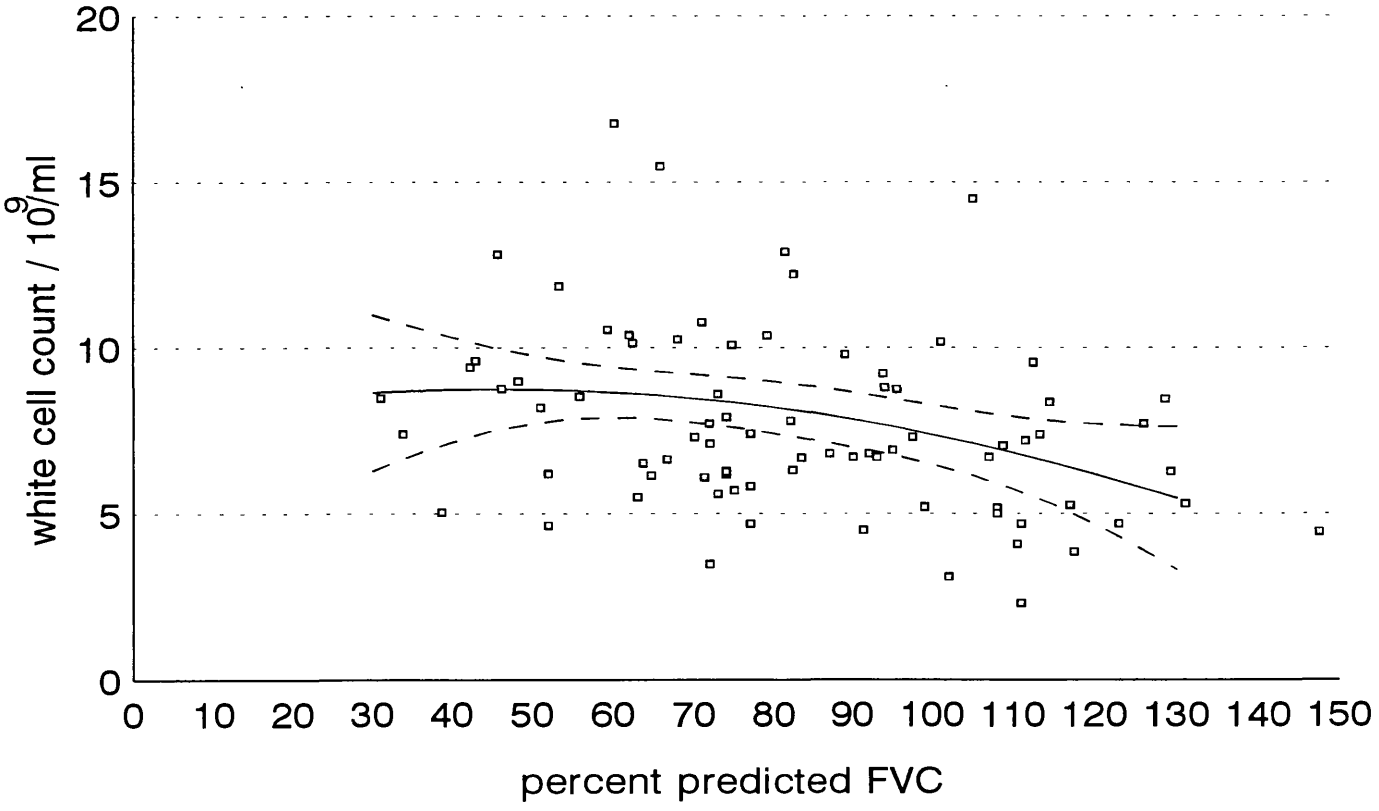


Figure 6 - Scatterplot, regression and 95% confidence intervals, plasma viscosity vs percent predicted FEV1, pre-treatment

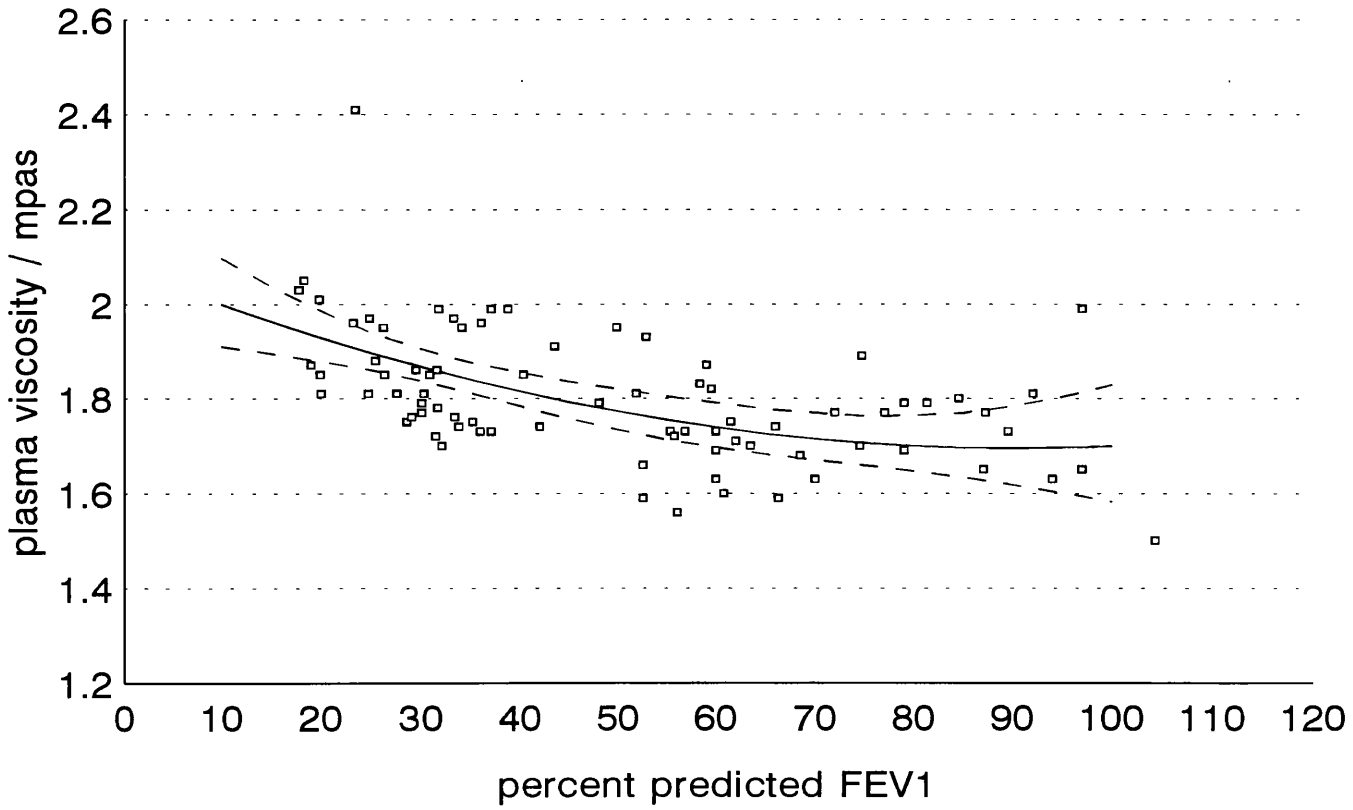


Figure 7 - Scatterplot, regression and 95% confidence interval, plasma viscosity vs percent predicted FEV1, post-treatment

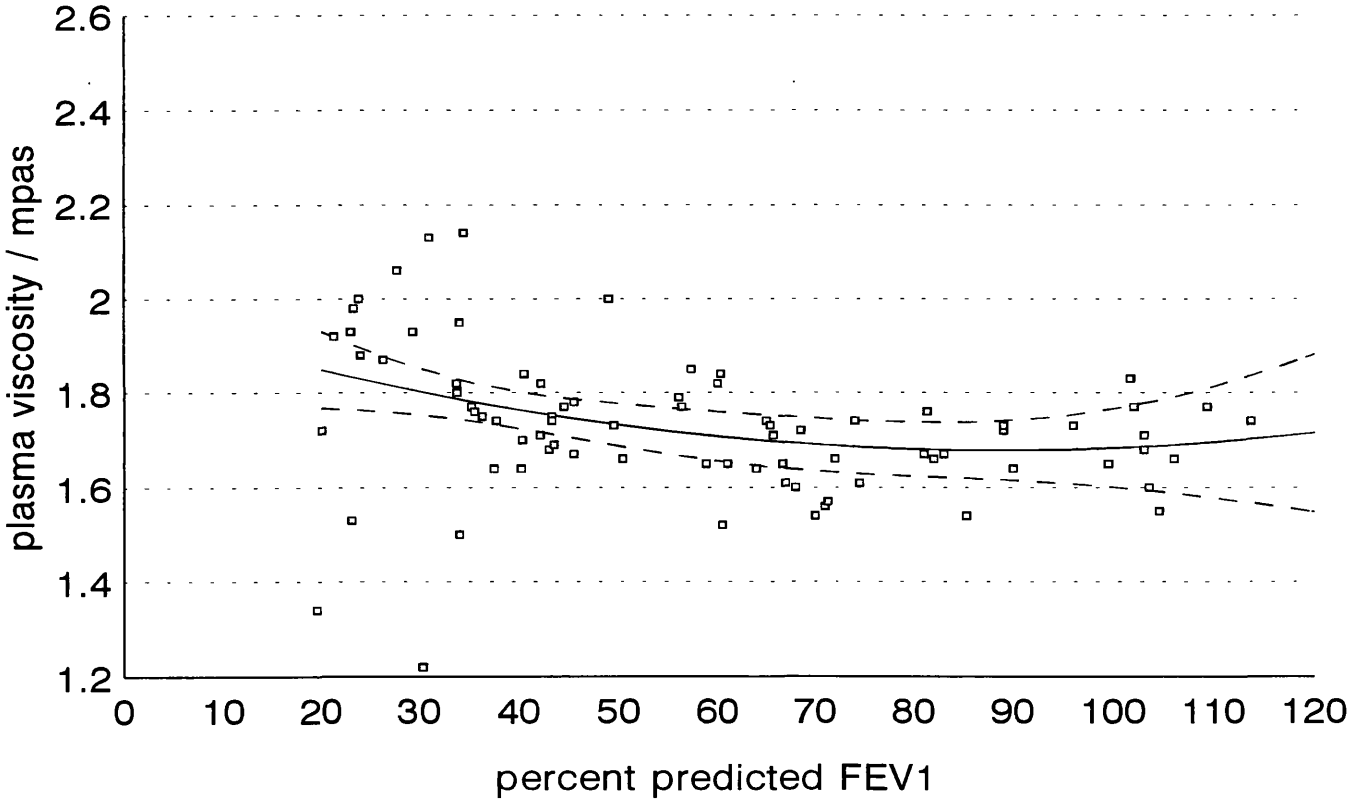


Figure 8 - Scatterplot, regression and 95% confidence intervals, plasma viscosity vs percent predicted FVC, pre-treatment

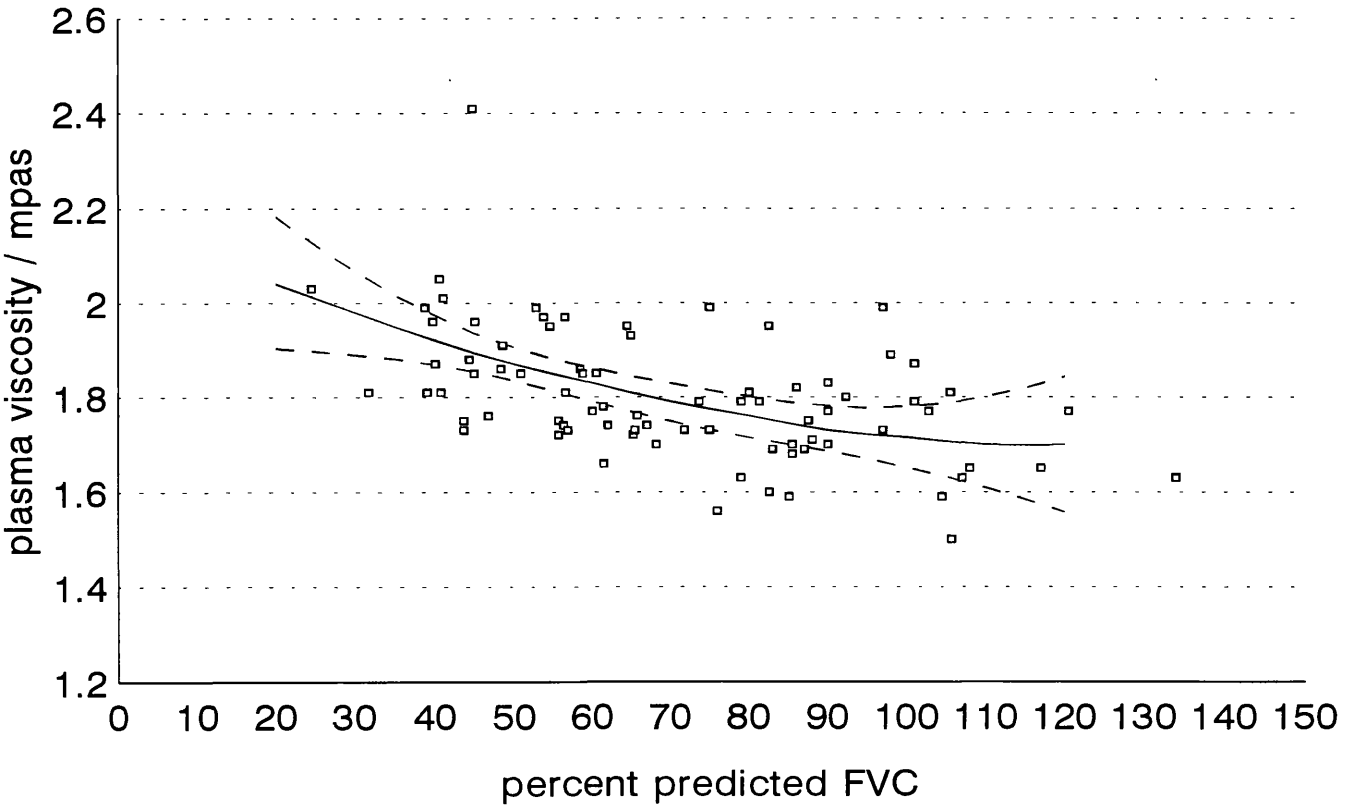


Figure 9 - Scatterplot, regression and 95% confidence intervals, plasma viscosity vs percent predicted FVC, post-treatment

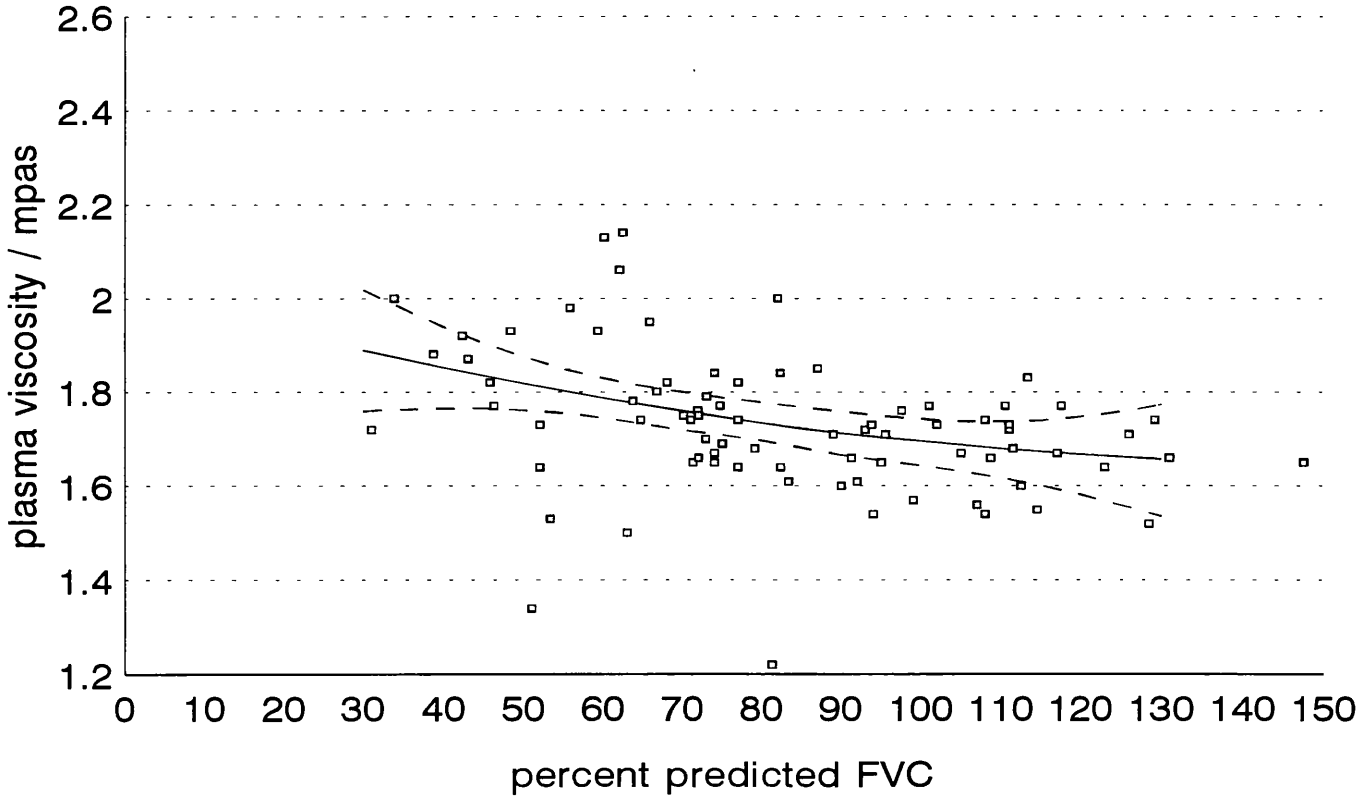


Figure 10 - Scatterplot, regression and 95% confidence intervals, CRP vs percent predicted FEV1, pre-treatment

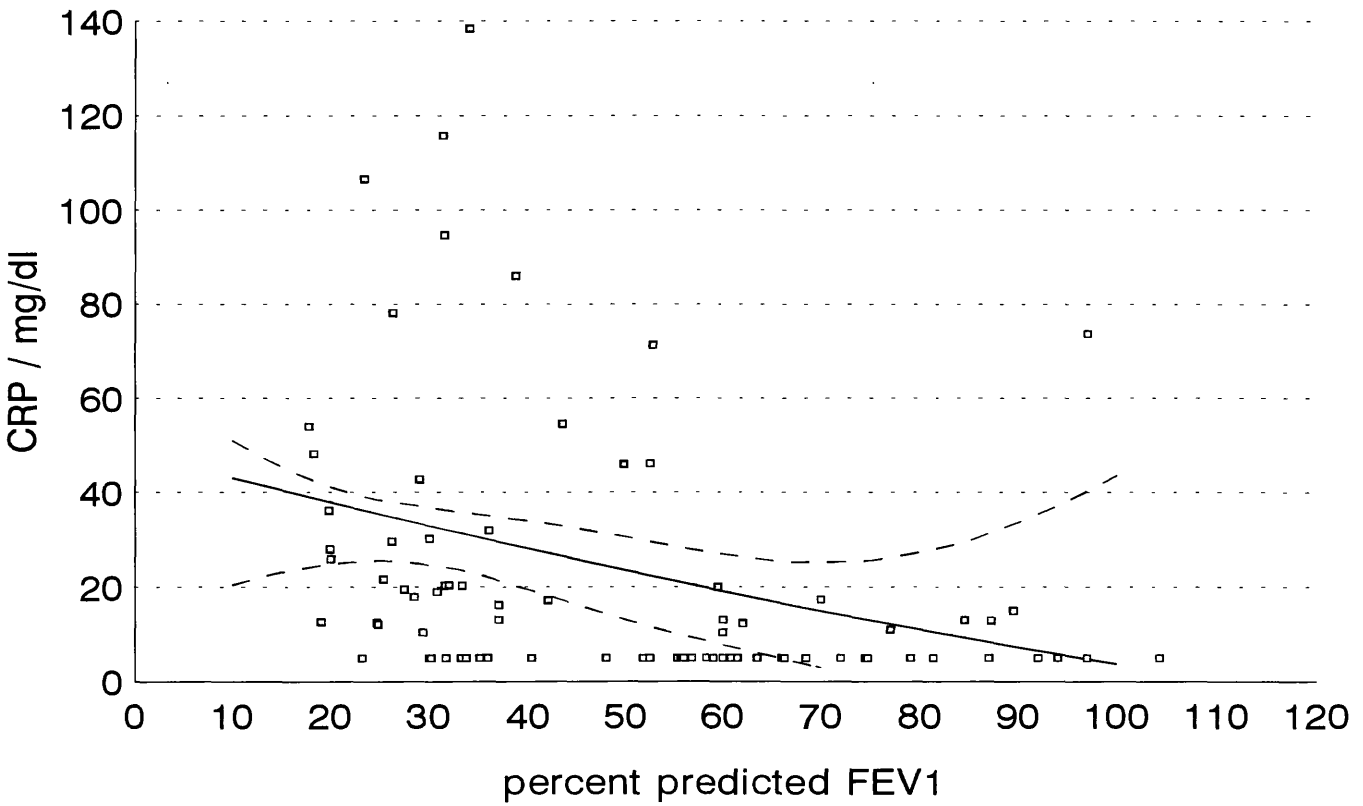


Figure 11 - Scatterploot, regression and 95% confidence intervals, CRP vs percent predicted FEV1, post-treatment

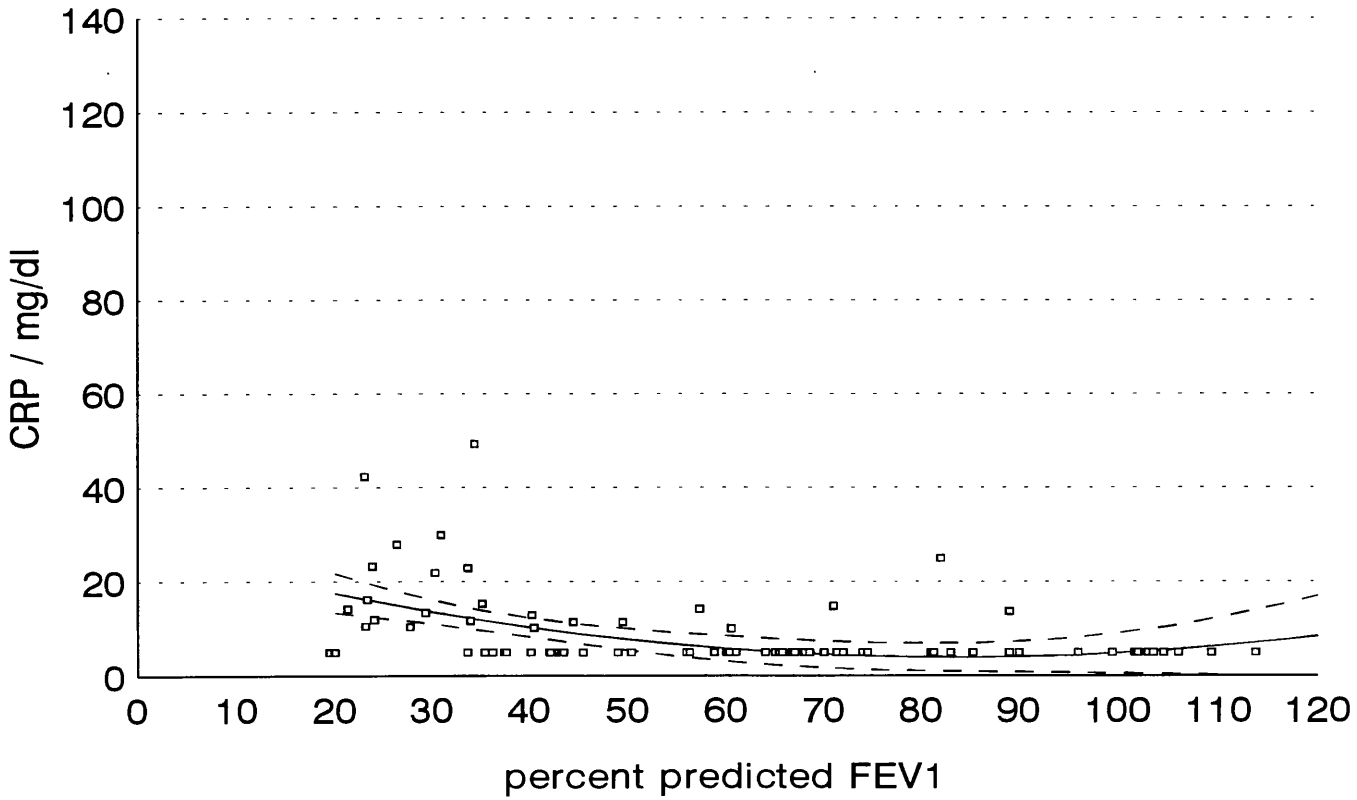


Figure 12 - Scatterplot, regression and 95% confidence intervals, CRP vs percent predicted FVC, pre-treatment

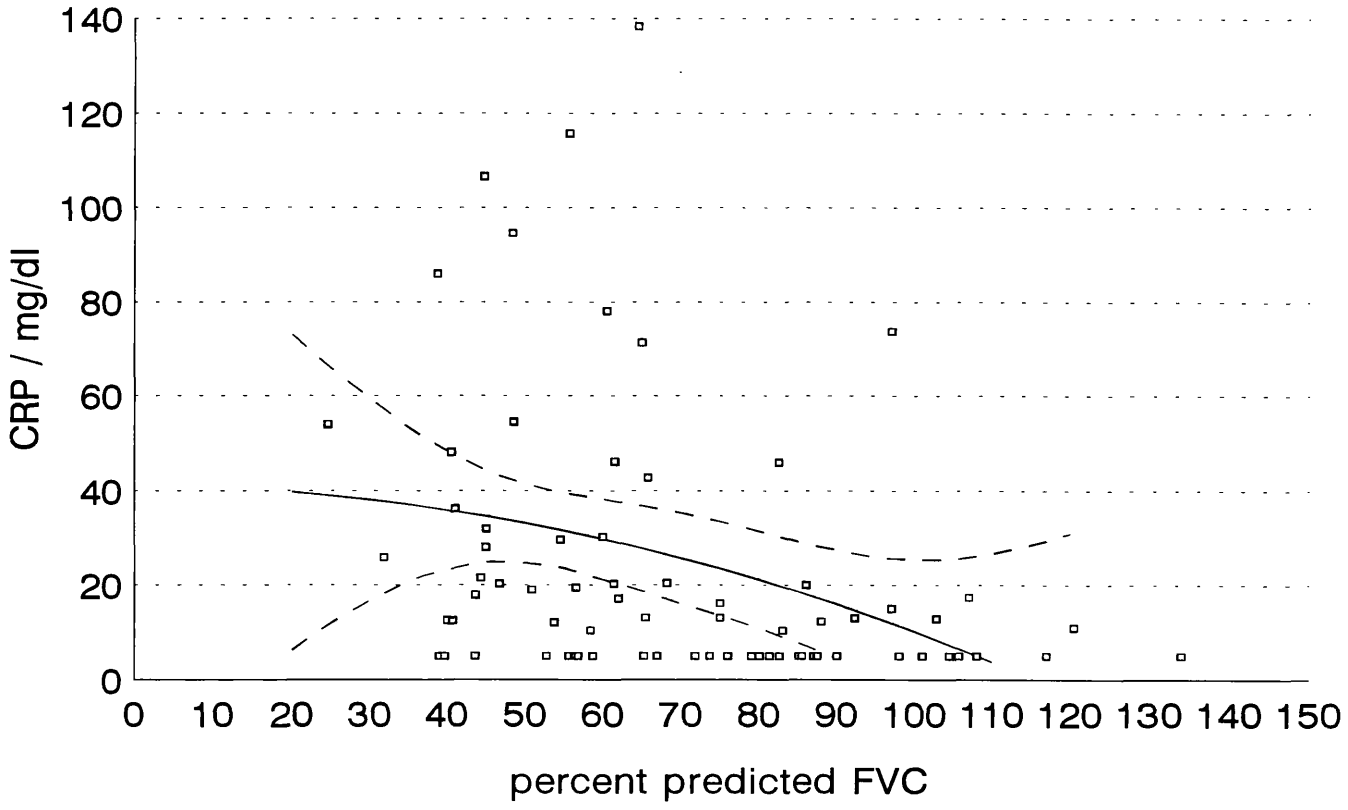


Figure 13 - Scatterplot, regression and 95% confidence intervals, CRP vs percent predicted FVC, post-treatment

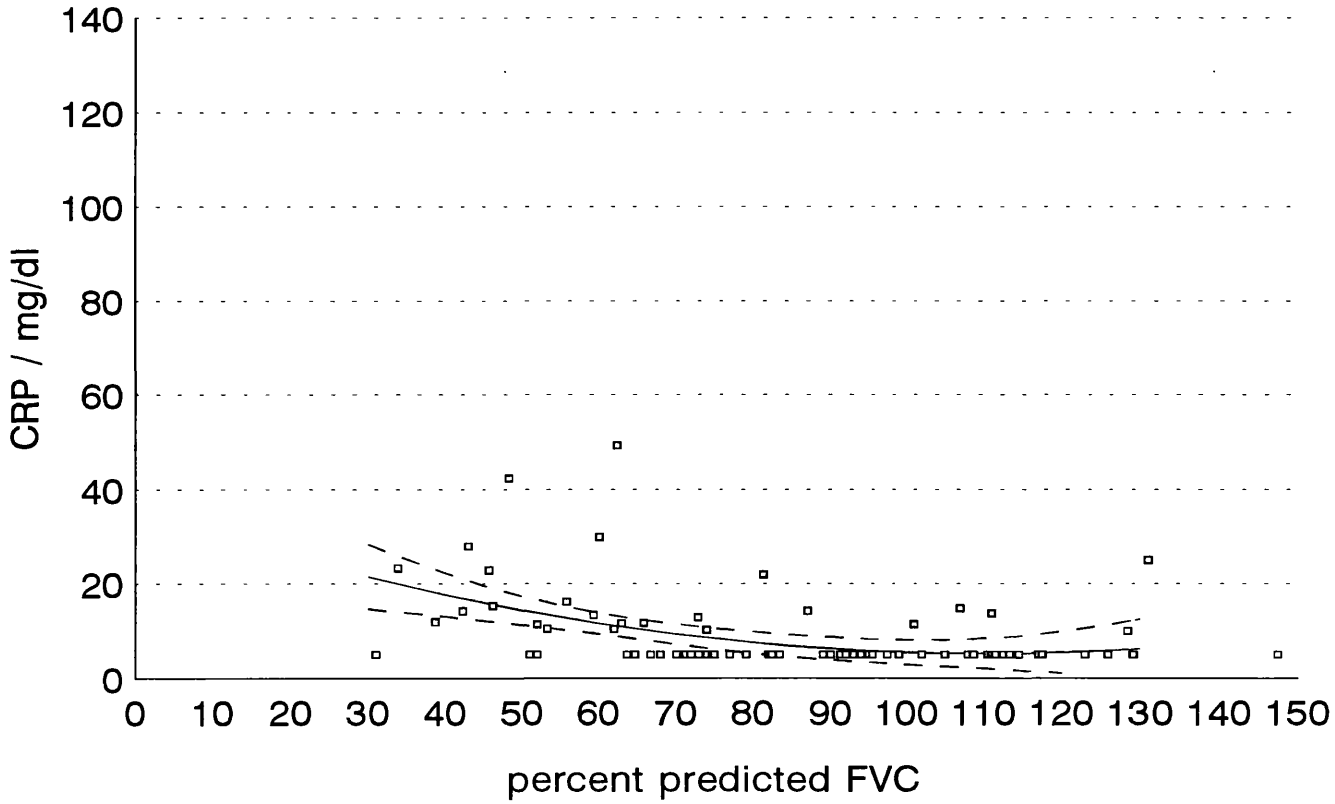


Table 5 - Change in pulmonary function and inflammatory markers with treatment

	pre-treatment	post-treatment	significance level
percent predicted FEV1	49.9 (3.2)	58.1 (26.3)	0.037
percent predicted FVC	70.7 (23.6)	83.0 (25.7)	0.002
WCC 10^9/l	10.60 (3.31)	7.64 (2.76)	< 0.00001
PV mpas	1.80 (0.14)	1.73 (0.15)	0.0021
CRP mg/dl	12.1 (5 - 138)	5.0 (5 - 49)	0.0011

pre-treatment and post-treatment values are mean (SD), except for crp, which are median (range)

significance levels were determined with paired t-tests, except for CRP for which the Mann-Whitney test was used

Table 6 - Correlation (r values) of inflammatory markers with percent predicted FEV1 and FVC

	pre-treatment percent predicted FEV1	post-treatment percent predicted FEV1	pre-treatment percent predicted FVC	post-treatment percent predicted FVC
WCC	0.42	0.31	0.46	0.30
PV	0.52	0.48	0.32	0.34
CRP	0.29	0.28	0.46	0.43

The regression equations were :-

a) WCC vs FEV1

pre-treatment $wcc = 15.4 - 0.145\ FEV1 + 0.000770\ FEV1^2$

post-treatment $wcc = 12.6 - 0.126\ FEV1 + 0.000603\ FEV1^2$

b) WCC vs FVC

pre-treatment $wcc = 10.5 + 0.060\ FVC - 0.000754\ FVC^2$

post-treatment $wcc = 7.82 + 0.041\ FVC - 0.000455\ FVC^2$

c) PV vs FEV1

pre-treatment $pv = 2.08 - 0.00851\ FEV1 + 0.000047\ FEV1^2$

post-treatment $pv = 1.96 - 0.00643\ FEV1 + 0.000036\ FEV1^2$

d) PV vs FVC

pre-treatment $pv = 2.19 - 0.00805\ FVC + 0.000033\ FVC^2$

post-treatment $pv = 2.02 - 0.00474\ FVC + 0.000015\ FVC^2$

e) CRP vs FEV1

pre-treatment $crp = 48.6 - 0.556\ FEV1 + 0.00107\ FEV1^2$

post-treatment $crp = 27.4 - 0.563\ FEV1 + 0.00337\ FEV1^2$

f) CRP vs FVC

pre-treatment $crp = 41.1 - 0.012\ FVC - 0.00297\ FVC^2$

post-treatment $crp = 35.9 - 0.555\ FVC + 0.00252\ FVC^2$

The predicted spirometric values below which values of inflammatory markers are predicted to be elevated by the regression equations are shown in table 7.

Figures 14-19 show the pre- and post- treatment regression curves for each marker and spirometric parameter, plotted together with the relevant upper and lower 95% confidence intervals. Over most of the pulmonary function range the post-treatment curve was shifted downwards and to the right for white cell count and c-reactive protein, as shown by the divergent confidence intervals, but not for plasma viscosity. Table 8 shows the proportion of data points lying within the region of digression of the 95% confidence intervals for WCC and CRP.

Table 7 - Percent predicted spirometric values below which values of inflammatory markers are predicted to be elevated by the regression equations

	pre-treatment percent predicted FEV1	post-treatment percent predicted FEV1	pre-treatment percent predicted FVC	post-treatment percent predicted FVC
WCC	48%	15%	70%	0%
PV	65%	54%	100%	83%
CRP	81%	40%	100%	67%

(Upper limits of normal were WCC 11 × 10^9/l, PV 1.72 mpas, CRP 10 mg/dl)

1 - post-treatment WCC did not exceed 11 × 10^9/l.

Table 8 - Proportion of data points lying within region of digression of 95% confidence intervals

	FEV1	FVC
WCC	81%	93%
CRP	68%	81%

Table 9 - Proportion of patients with normal inflammatory marker values despite pulmonary function lower than that predicted to give rise to raised values

	pre-treatment FEV1	post-treatment FEV1	pre-treatment FVC	post-treatment FVC
WCC	49%	-	23%	-
PV	10%	12%	13%	16%
CRP	38%	9%	14%	47%

Figure 14 - Regression lines and 95% confidence intervals - white cell count vs percent predicted FEV1, pre- and post- treatment

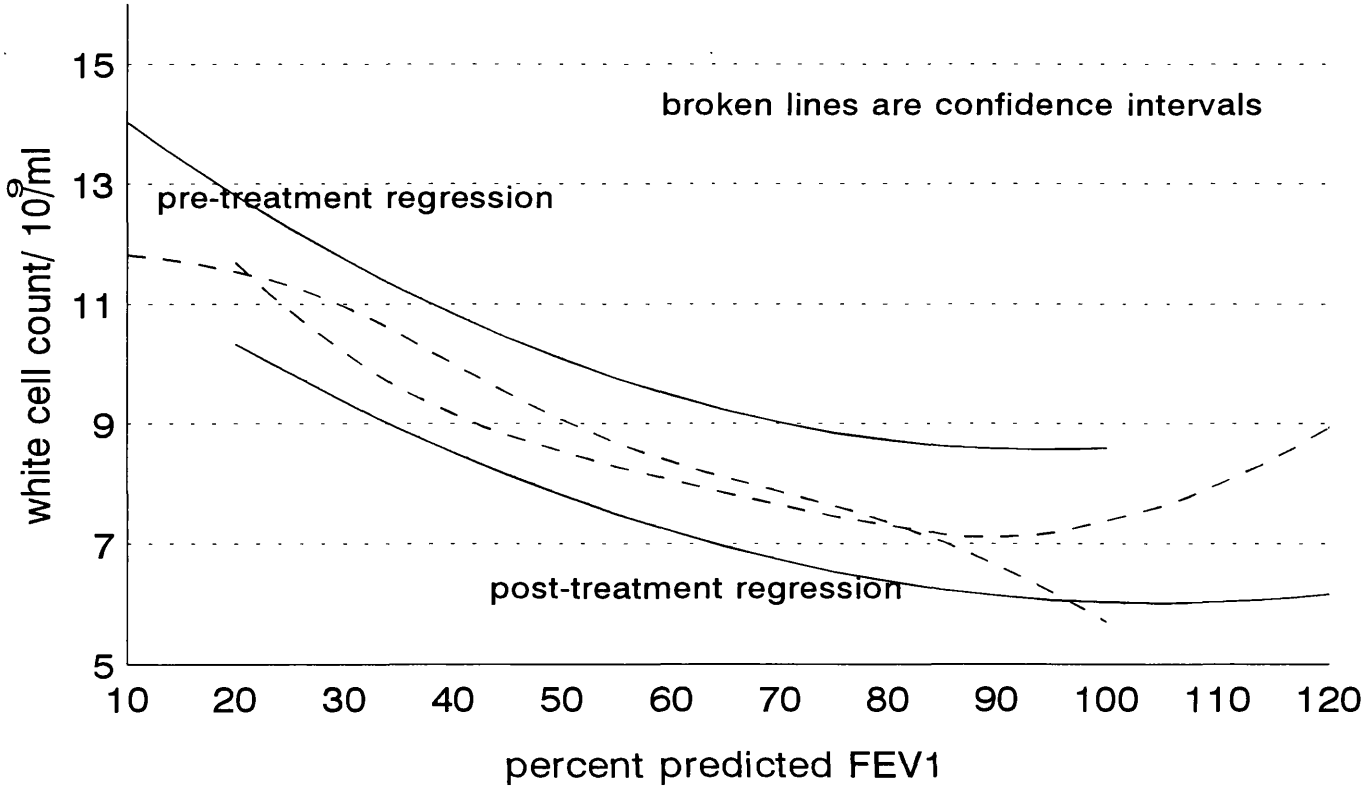


Figure 15 - Regression lines and 95% confidence intervals - white cell count vs percent predicted FVC, pre- and post- treatment

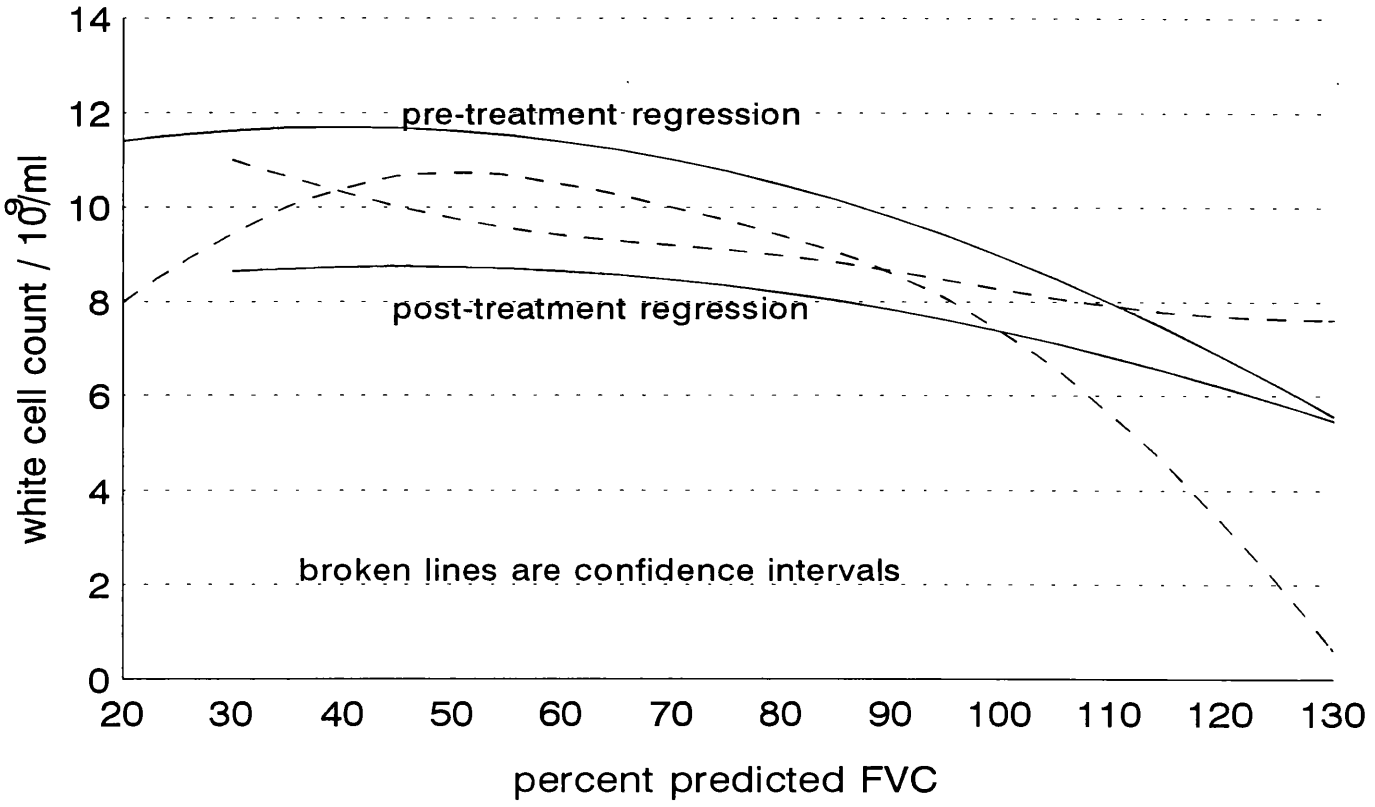


Figure 16 - Regression lines and 95% confidence intervals, plasma viscosity vs percent predicted FEV1, pre- and post- treatment

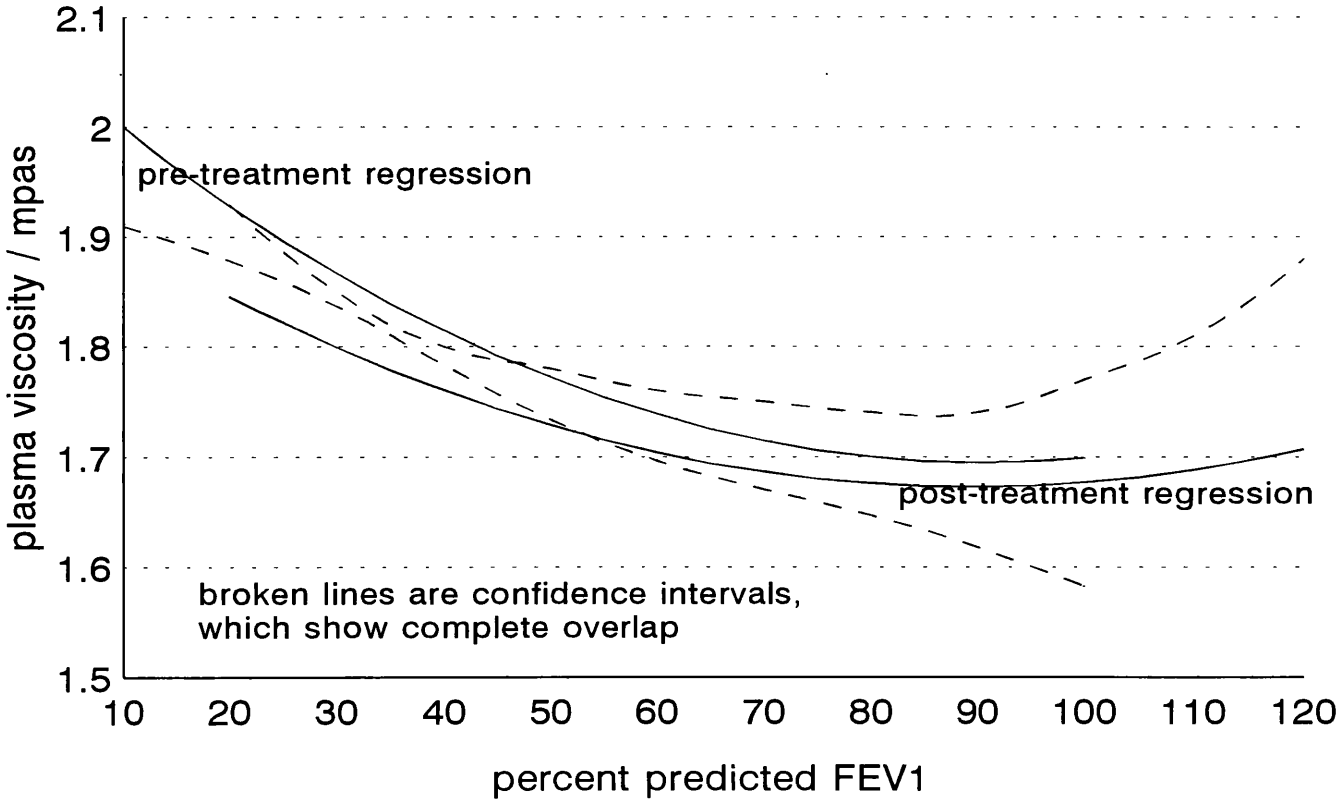


Figure 17 - Regression lines and 95% confidence intervals, plasma viscosity vs percent predicted FVC, pre- and post- treatment

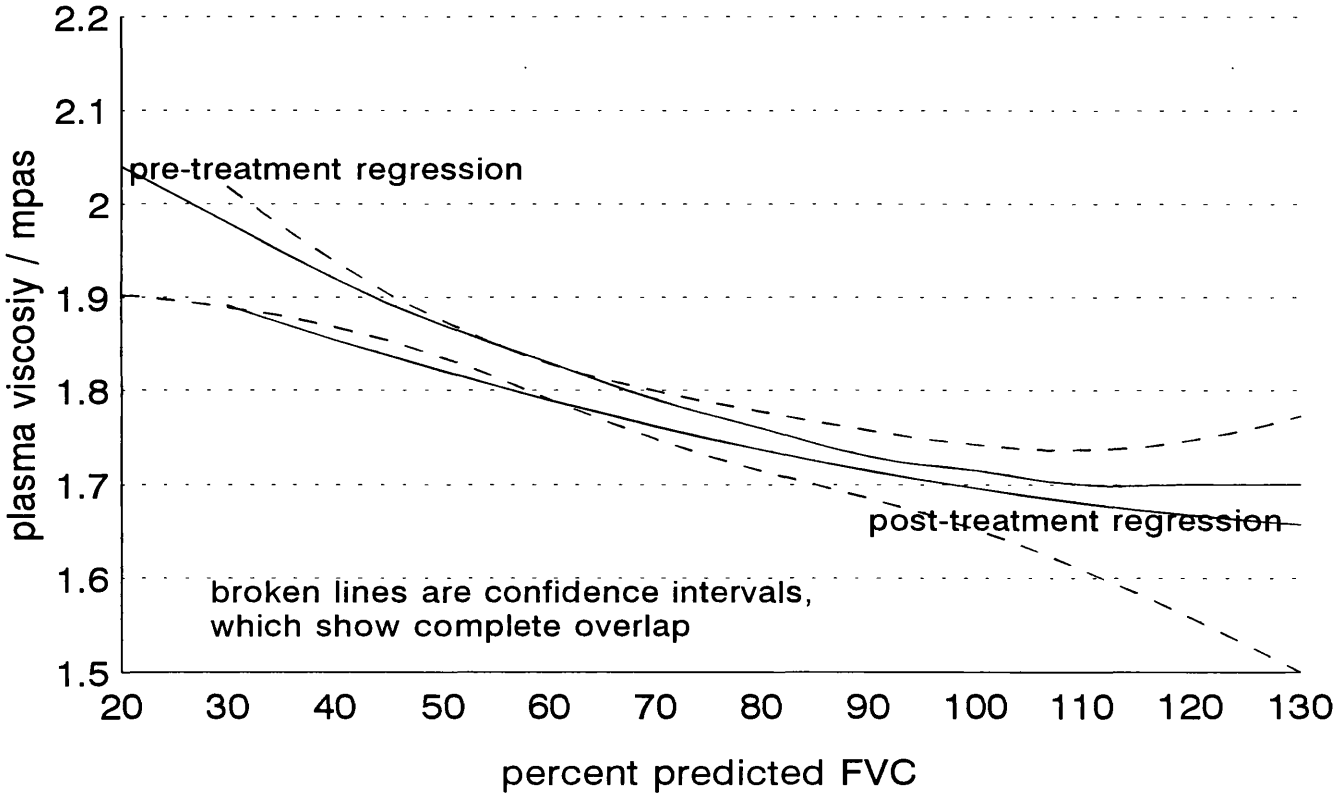


Figure 18 - Regression lines and 95% confidence intervals - CRP vs percent predicted FEV1, pre- and post- treatment

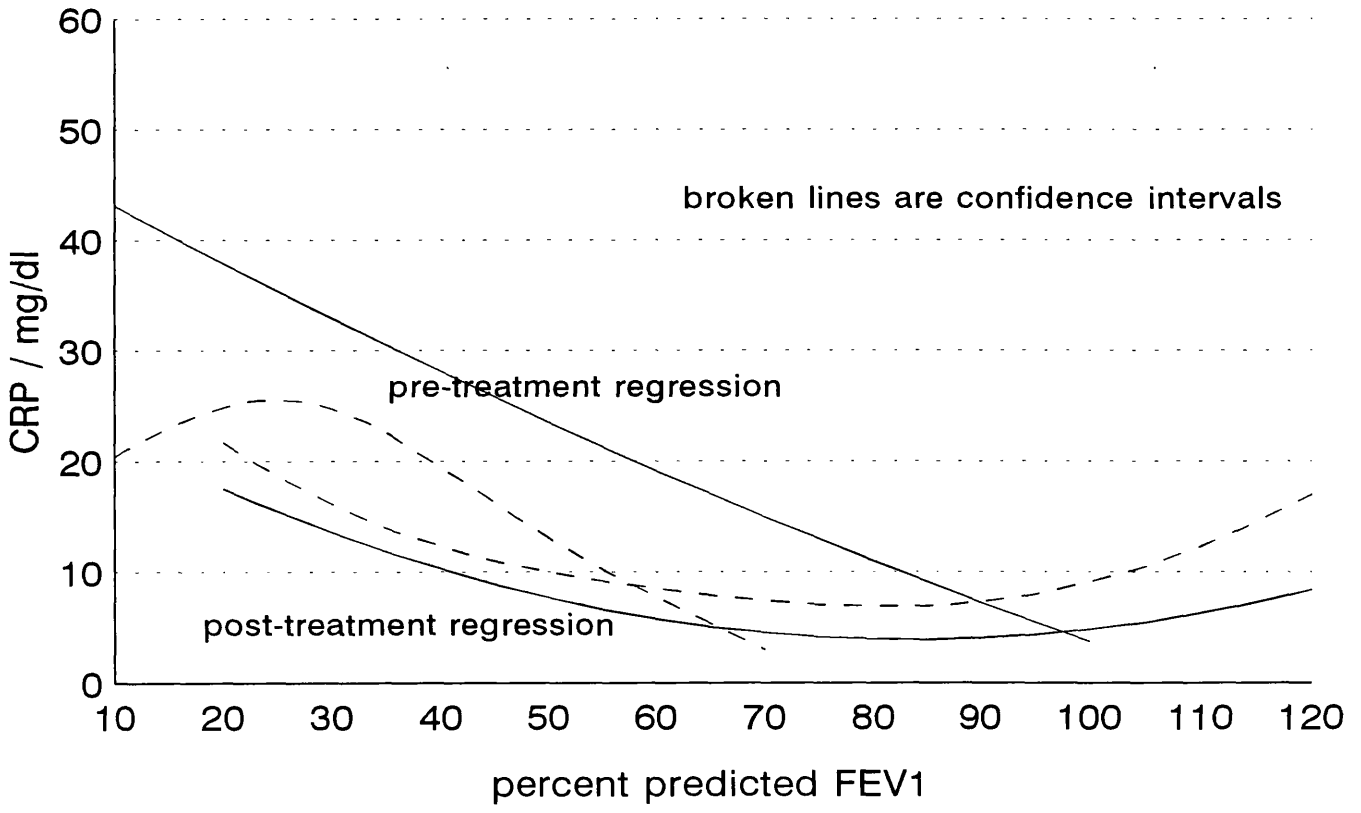
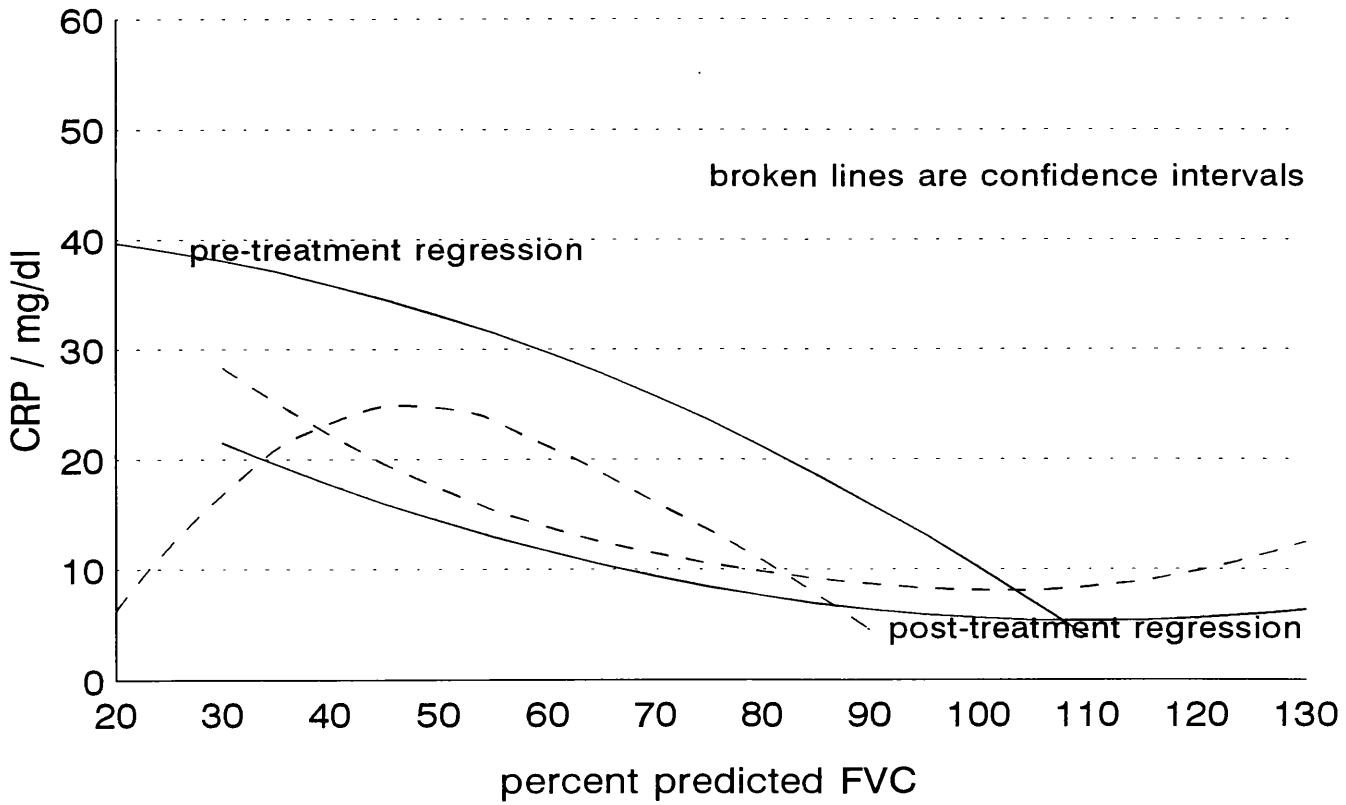


Figure 19 - regression lines and 95% confidence intervals for CRP vs percent predicted FVC, pre- and post- treatment



Discussion

Peripheral markers of inflammation in cystic fibrosis have been investigated in a variety of contexts. Watkin et al found that crp had a sensitivity of only 49% in the determination of symptomatic exacerbation in children not yet colonised with *P aeruginosa* [Watkin 1994]. In six patients crp was elevated at the time of acquisition of *P aeruginosa* [Elborn 1993]. At the other extreme, serum neutrophil elastase, crp, and TNF alpha were more elevated prior to death than in the preceding 6 months [Elborn 1993]. However there is no data relating to the behaviour of inflammatory markers in the majority of adults with CF, viz those chronically colonised with *P aeruginosa* and mild to moderate pulmonary disease.

Because one of the aims was to compare the three inflammatory markers many treatments could not be included in the analysis due to the absence of just one marker. This resulted in the relatively low proportion (64%) of the total treatments that could be included in the analysis. Because the protocol called for measurement of inflammatory markers in all exacerbations, missing data should have arisen randomly. Hence there is no reason to suppose that excluded treatments were in any way untypical.

There was a gradual increase in the levels of all three markers with disease severity, as judged by decreasing predicted spirometry, for both the pre-treatment and post-treatment cases. This provides further support for the

generally held view that the bulk of pulmonary damage arises through host immune responses rather than directly from bacterial toxins, although an alternative but less likely interpretation would be that the increased inflammatory markers represent an epiphenomenon. In the cases of white cell count and plasma viscosity the appearance of the scatter plots suggested that levels were gradually increasing throughout the range of pulmonary function, rather than there being a critical level of pulmonary function below which they began to increase. The increase was evident within the normal range of these markers, as well as at higher levels. This finding is in agreement with the work of Konstans et al, who demonstrated high levels of inflammatory material in BAL from adult patients with well-preserved lung function in periods of clinical quiescence. The crp scatterplots were more difficult to interpret due to the very wide spread of the data.

Elborn at al have shown that several peripheral markers of inflammation are suppressed with intravenous antibiotic treatment of pulmonary exacerbations [Elborn 1991]. All three inflammatory markers showed significant suppression in this study, with a concomitant improvement in pulmonary function. An unresolved question is whether the reduction in inflammatory markers can be accounted for by the general improvement in well-being, as represented by pulmonary function, or whether treatment has an additional, specific effect on the markers of inflammation. In the former case the regression curves would overlap, with the post-treatment

curve shifted to the right. This is the case for plasma viscosity. In the case of a specific anti-inflammatory effect, the post-treatment curve should be shifted downwards, as well as to the right. This is what is seen in the cases of white cell count and CRP. Although in both cases the 95% confidence intervals overlap at the outer edges of the curve, the bulk of the data lies within the region in which the confidence intervals do not overlap (see table 8). Mean white cell count decreased by $3.0 \times 10^9/l$ overall, and mean CRP by 12.3 mg/dl. Roughly 20% of the decrease was explained in each case by improved pulmonary function, the remaining 80% by a specific anti-inflammatory effect.

It is possible to place two polarised interpretations on this result. Firstly, the pre-treatment curve could represent the "true baseline". In this case the specific anti-inflammatory effect arises solely due to the treatment modalities employed. Secondly, the post-treatment curve could represent the "true baseline". In this case the effect arises due to a relative excess of anti-inflammatory activity at the outset of treatment of a pulmonary exacerbation. If this is the correct interpretation it suggests that exacerbations are "real" entities, and not just random deviations from the normal rate of disease progression. In this case it is more likely that specific precipitants of exacerbation may be identified. Most probably, the "true baseline" lies in between the two regression curves, but this is pure speculation. A separate study with additional data collected outside the context of an exacerbation would be required to

resolve the issue (with many more patients, given the smaller effect on inflammatory markers which would be expected, and the small difference in confidence intervals observed in this study).

With all three markers the data scattered widely about the regression lines. Correlation, although statistically significant in all twelve cases, was weak. With each marker several patients demonstrated no increase in levels despite very poor pulmonary function. An index of the sensitivity of each marker is the spirometric value predicted to give rise to raised values of that marker (the higher the predicted value, the more sensitive the marker). These values are shown in table 7. The predicted values for crp are artifactually inflated by the very high values observed at the lower end of the spirometric range. Log transforming the data did not overcome the problem. A further index of sensitivity is the proportion of data with spirometric values below these predicted values in which the markers are not raised. These proportions are shown in table 9.

In summary, plasma viscosity can be considered as a marker of chronic inflammation, because of the absence of a specific treatment effect. Conversely, crp and white cell count act as markers of acute on chronic inflammation. None of the markers was especially sensitive, making clinical interpretation difficult.

VI.3 Neutrophil elastase as an alternative to "standard" inflammatory markers

Introduction

Neutrophil elastase (NE) is released following pulmonary neutrophil degranulation and significant quantities are absorbed into the pulmonary circulation and complexed with α-1-antitrypsin. It has been shown that BAL neutrophil counts are elevated even in periods of quiescence in those patients with mild disease [Konstans 1994]. A priori peripheral neutrophil elastase - α-1-antitrypsin complex (NEATC) should be a more direct marker of pulmonary inflammation than WCC, PV and CRP. It was hypothesised that NEATC might be a more sensitive and reliable peripheral marker of inflammation.

Methods

During the two year study blood was collected from patients at the start and end of intravenous antibiotic treatments. The samples were transported on ice and centrifuged immediately on receipt by the laboratory. Serum was stored at -70^{o}C. NEATC was assayed using a commercially available ELISA (Merck). The original intention was to analyse all the collected samples but this was not logistically possible. Therefore paired samples were selected from thirty patients with varying disease severity, as judged by percent predicted FEV1. NEATC was measured using the

stored samples; the other markers were measured routinely at the time of the exacerbation.

Results

In this patient group none of the variables was normally distributed. This was partly accounted for by a bias towards lower spirometric values, which precluded regression analysis as performed above. Spirometry improved significantly with treatment (see table 10). All the inflammatory markers except plasma viscosity showed a statistically significant improvement. The proportion of the patient sample with raised values of each inflammatory marker is shown in table 11.

Table 10 - Change with treatment in percent predicted spirometry and inflammatory markers (median)

	admission	discharge	Mann-Whitney
percent predicted FEV1	32	44.5	0.04
percent predicted FEV1	62	82	0.009
NEATC (mcg/l)	90.5	60.5	0.005
CRP (mg/l)	23.25	10	0.001
WCC (10^9/l)	11.6	9.11	0.02
PV (mpas)	1.84	1.80	0.15

Table 11 - Percentage of patient sample with raised values of inflammatory markers on admission

	upper limit of normal	percentage on admission	percentage on admission
NE	32 mcg/l	100	100
CRP	10 mg/dl	67	27
WCC	11 × 10^9/l	57	27
PV	1.72 mpas	83	60

Discussion

Measurement of NEATC is complicated by the need to collect samples on ice and centrifuge as soon as possible. Delaying separation in four normal controls by leaving the samples at room temperature for one hour resulted in a 30% increase in NEATC. The effect of delayed separation on samples from CF patients is likely to be similar.

As shown above, plasma viscosity is a marker of chronic rather than acute inflammation. The small changes which would be expected together with the smaller sample size probably account for the lack of statistical significance in this comparison. NEATC, WCC and CRP all showed statistically significant reductions. Despite the sample being somewhat biased towards more severe pulmonary disease CRP was normal in 33% and WCC in 43 % of pre-treatment cases. In each case, 27% of post-treatment values were elevated. In contrast NEATC was elevated in all pre-treatment cases, and was therefore more sensitive than the other markers. In addition NE remained elevated following treatment in all cases. It might therefore be able to act as a satisfactory marker both of acute and chronic inflammation. The smaller number of patients in this study, and a lack of middle-range values of percent predicted FEV1 made pre- and post- treatment regression analysis inappropriate in this study. Long term studies of NEATC with serial measurements in individual patients are therefore justified.

Chapter VII A Model for Defining Pulmonary Exacerbation in Cystic Fibrosis

VII.1 Introduction

Currently there is no consensus on what constitutes a pulmonary exacerbation of CF. Indeed the terminology varies, "infection" and "infectious episodes" being used interchangeably for the same concept. Since all CF patients become chronically colonised with bacteria at an early age "infection" seems an inappropriate term to differentiate between a sudden deterioration and the usual state of health. "Pulmonary exacerbation" is the term usually preferred and will be used in this thesis.

Pulmonary exacerbations are thought to play an important role in the long-term decline in lung function in CF. The very gradual nature of the long-term deterioration in lung function that is seen presents an obstacle to the evaluation of novel therapies, and the frequency with which pulmonary exacerbations occur is a useful, if unvalidated, surrogate. Evaluation of such therapies is likely to require multicentre cooperation. It is important that the term "pulmonary exacerbation" is standardised so that the efficacy of new therapies can be compared.

No published work has yet addressed this fundamental issue. A preliminary paper presented at the Eighth Annual North American Cystic Fibrosis Conference [Ramsay 1994] outlined proposals for a multicentre study to determine the

key features, in terms of symptoms and spirometry, of patient episodes which are considered to be exacerbations by the responsible clinicians. This will provide a consensus statement of what clinicians believe to be exacerbations, not a definition of exacerbation derived from serial measurements.

The most frequently used "default" definition of an exacerbation is the requirement for the use of intravenous antibiotics. This is unsatisfactory for several reasons. Firstly, it is possible to treat with oral rather than intravenous antibiotics. This applies especially to younger patients who have not yet become colonised with *P aeruginosa*, in whom bacterial colonisation is predominantly with *S aureus* and *H influenzae*, but also to 4-quinolone treatment of pseudomonal colonisation in older patients. Secondly, there is inter-patient variation in the threshold of symptoms beyond which intravenous antibiotic treatment will be sought. (This variation is problematic whatever the definition employed, as it will introduce inhomogeneity into the population included under the heading of exacerbation.) Thirdly, there is inter-clinician variation in the clinical threshold for instituting such treatment.

The intensification in the use of intravenous antibiotic administration to combat pseudomonal infection in recent years has further obscured the issue. This followed from the results of Szaff et al, who instituted intravenous antibiotic treatment every three months, regardless of any clinical deterioration, for patients colonised with *Pseudomonas*

aeruginosa in the late 1970's [Szaff 1983]. Retrospective analysis showed dramatically improved survival following the institution of this policy, but this may have been due to other factors such as improved nutrition, improved physiotherapy or the emergence of a wider spectrum of effective anti-pseudomonal agents, at a time when survival was improving in other centres. A British Thoracic Society controlled study to examine whether scheduled intravenous therapy is better than as required therapy is currently in progress. Despite the absence of controlled data, some CF centres now follow the Danish protocol. Because some patients in these centres will receive intravenous antibiotic therapy in the absence of any clinical deterioration it is no longer tenable to define exacerbation solely in terms of the requirement for such therapy.

The adoption of a modified Danish approach at the Leeds unit meant that a substantial proportion of intravenous antibiotic treatment episodes took place in the absence of clinical deterioration. This provided an opportunity to explore the components of exacerbation through the inclusion of the widest possible spectrum of pretreatment changes. Because there is no "gold standard" by which to judge exacerbation the approach was necessarily empirical.

A priori there are four measures which could be used to define exacerbation.

1. Pulmonary function

Serial pulmonary function testing forms the basis of long-term monitoring of CF patients. Most patients will be seen at least at two monthly intervals. Frequently repeated full lung function testing is impracticable and spirometry, which is highly reproducible, is therefore used. Interpretation is not straightforward, as in the short term values vary between a nadir at which antibiotic treatment is commenced and a peak occurring usually at the end of antibiotic treatment or sometimes altogether outside the context of an "exacerbation". In terms of defining exacerbation the most practical approach is to take the difference between the value recorded at the start of treatment and the most recently recorded value when the patient was considered to be clinically well. However this still carries the disadvantage of subjectivity in deciding what constitutes a state of clinical "wellness".

2. Clinical symptoms

Using patients' perception of a change in clinical symptoms as a basis for definition is attractive since this is the immediate problem for the patient at the time of exacerbation. It would also be useful if a definition based on a perceived change in symptoms could substitute for one based on change in pulmonary function, as some pulmonary function data will inevitably be missed. However symptoms are

essentially categorical variables and cannot be treated as continuous variables unless visual analogue scoring is employed, which is unlikely to be reproducible in the long-term. A further disadvantage is that assumptions have to be made at the outset about the relevance of individual symptoms in the context of exacerbation.

3. Markers of inflammation

Because of the absence of published data on the behaviour of peripheral markers of inflammation in adult patients with CF, their use in the definition of exacerbation has not been explored.

4. Quantitative bacteriology

Given the almost universal response to antibiotic therapy it is generally assumed, but not proven, that exacerbations virtually always follow from an increase in the numbers of intrapulmonary bacteria. Defining exacerbation on the basis of serial change in pulmonary colonisation is an attractive concept but there is no method which can directly quantify the total intrapulmonary bacterial load. Sputum bacterial density estimation using quantitative culture techniques is the usually used surrogate, although a minority of patients have difficulty in producing sputum specimens outside the context of an exacerbation. In addition, total bacterial load must depend on volume of secretion as well as

bacterial density. There is no published data concerning the validity of the technique.

VII.2 General methodology of evaluation of the determinants of exacerbation

As noted above, the approach is necessarily empirical. The four potential measures of exacerbation were evaluated sequentially. Changes in pulmonary function were examined first. Symptoms, inflammatory markers and quantitative bacteriology were examined in turn to assess their potential. All courses of intravenous antibiotic treatment administered between 1st May 1991 and 30th April 1993 were included in the analysis, as in chapter VI. As noted above, this included routinely administered courses at approximately 3 month intervals in patients who had agreed to this schedule. Again, home- and hospital- based treatments were included, as they have been shown be equivalent in terms of outcome [Pond 1994].

VII.3 Evaluation of serial pulmonary function as the determinant of exacerbation

Methods

Pulmonary function in an adult cystic fibrosis population varies from over 100% of predicted values down to 20% of predicted values. Patients at the lower end of the

range become quite sick with small absolute decreases in pulmonary function. Consequently, a meaningful definition of change in pulmonary function which is applicable across the whole range of clinical status cannot be based on a change in absolute values. Therefore, to determine whether the change from baseline of FEV1, FVC, or both, is appropriate to define exacerbation, the change in FEV1 and FVC from the previously recorded value when the patient was considered to be clinically well was calculated for each treatment episode during the two year study period, and then expressed as percentage change from baseline. These terms were denoted "dFEV1" and "dFVC" respectively. For mathematical convenience, a deterioration in pulmonary function was assigned a positive value. The agreement between dFEV1 and dFVC was then examined.

A decline in FEV1 or FVC of 10% or more compared to baseline (dFEV1 or dFVC > 10%) was arbitrarily taken as the "gold standard" in order to examine the sensitivity of dFEV1 and dFVC individually. The value of 10% was chosen to represent a clinically significant deterioration, analogous to exacerbations of asthma defined in terms of changes in peak flow of at least 10% from baseline.

To compare the magnitudes of the changes in FEV1 and FVC from baseline, correlation was not an appropriate statistical tool, because FEV1 and FVC are related variables. The situation is analogous to that described by Bland and Altman when comparing two devices to measure a single variable, and a Bland-Altman plot was therefore constructed [Bland 1986].

Because multiple treatment courses were included for each patient, for the purposes of this plot the mean of the various values of dFEV1 and dFVC were first calculated for each individual patient, and the terms denoted "$dFEV1_i$" and "$dFVC_i$", respectively. The plot was constructed by plotting the difference between $dFEV1_i$ and $dFVC_i$, ($dFEV1_i$ - $dFVC_i$), against the mean of both values, ($dFEV1_i$ + $dFVC_i$)/2 (figure 20).

Results

Sequential pulmonary function data were available for 412 out of the total of 459 (90%) courses of treatment in 85 patients. Patients refused to perform spirometry or were not requested to perform it in 23 courses in 12 patients. Patients were too ill to perform spirometry in 13 courses in 8 patients (including 3 spontaneous pneumothoraces). In 11 courses in 11 patients, the treatment course was the first presentation to the unit for that patient, so that there was no pre-existing data for comparison. Figure 21 shows the frequency distribution per patient of the included treatment courses. The distribution was normal.

The boxplots in figure 22 summarise the spread of dFEV1 and dFVC (ie pooled values from all patients, each with varying numbers of treatment courses). The distribution was normal. Mean (SD) dFEV1 was 14.0 (16.0) and dFVC was 14.0 (17.4). Table 12 summarises the findings of dFEV1 and dFVC used individually, compared to dFEV1 and dFVC combined, in

defining exacerbation. In 36 treatment courses in 24 patients dFEV1 was ≥ 10% when dFVC was < 10%. The sensitivity of dFEV1 alone compared to combined dFEV1 and dFVC was therefore 249/285 × 100% = 87.4%. Conversely, dFVC was ≥ 10% when dFEV1 was < 10% in 28 treatment courses in 20 patients. The sensitivity of dFVC alone compared to dFEV1 and dFVC combined was therefore 257/285 × 100% = 91.2%.

Visual inspection of the Bland-Altman plot confirmed that $(dFEV1_i - dFVC_i)$ was not dependent on the absolute value of $(dFEV1_i + dFVC_i)/2$. Overall $(dFEV1_i - dFVC_i)$ was 0.6%, and FEV1 and FVC therefore change by equal proportions around the time prior to antibiotic treatment. The standard deviation of $(dFEV1_i - dFVC_i)$, 5.9, is artificially low, because some of the effect of repeated measurement error has been removed in finding the individual patient means. The true standard deviation approximates to $\sqrt{(2SD^2)}$, which is 8.4. In 95% of treatment courses the difference between dFEV1 and dFVC will lie between (mean $dFEV1_i - dFVC_i$) +/- 2SD, the "limits of agreement". The limits of agreement between dFEV1 and dFVC in monitoring the change in pulmonary function at the time of antibiotic administration are therefore -16.2% to 17.4%. In individual cases, differences of up to 33.6% between dFEV1 and dFVC are therefore expected.

Figure 20 - Bland-Altman plot of difference in percent change from baseline of FEV1 ($dFEV1_i$) and percentage change from baseline of FVC ($dFVC_i$) vs mean percent change from baseline of FEV1 and FVC ($\{dFEV1_i + dFVC_i/2\}$)

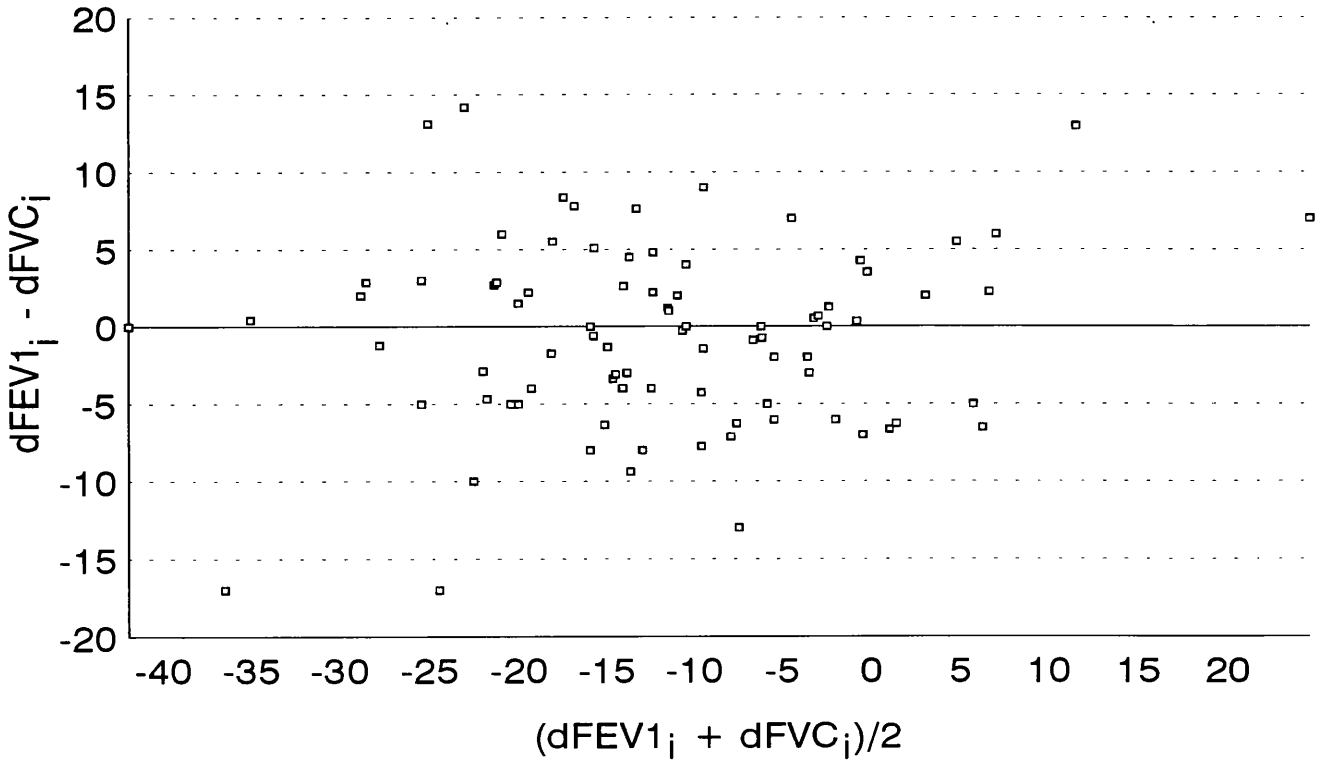


Figure 21- Frequency distribution of the treatment courses included in the analysis

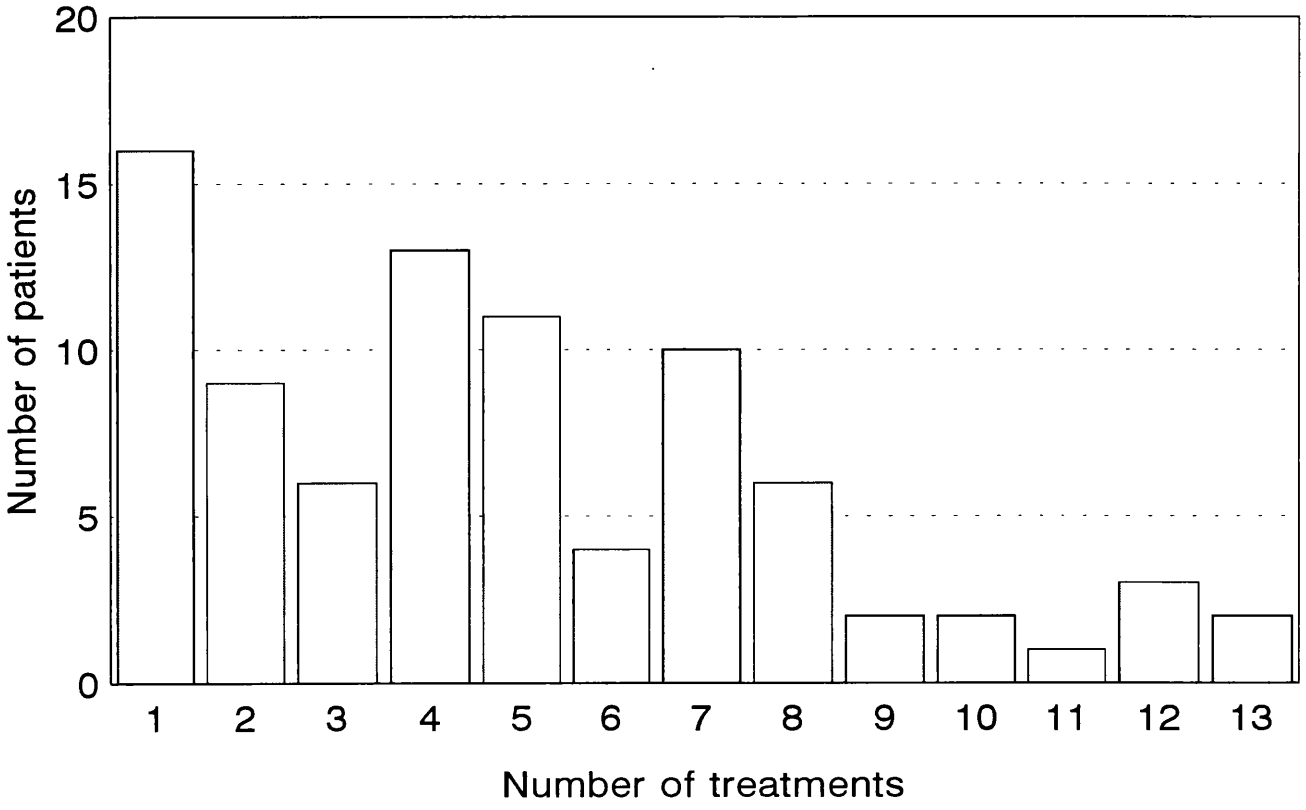


Figure 22 - Boxplots summarising the spread of dFEV1 and dFVC (pooled values from all patients, each with varying numbers of treatment courses)

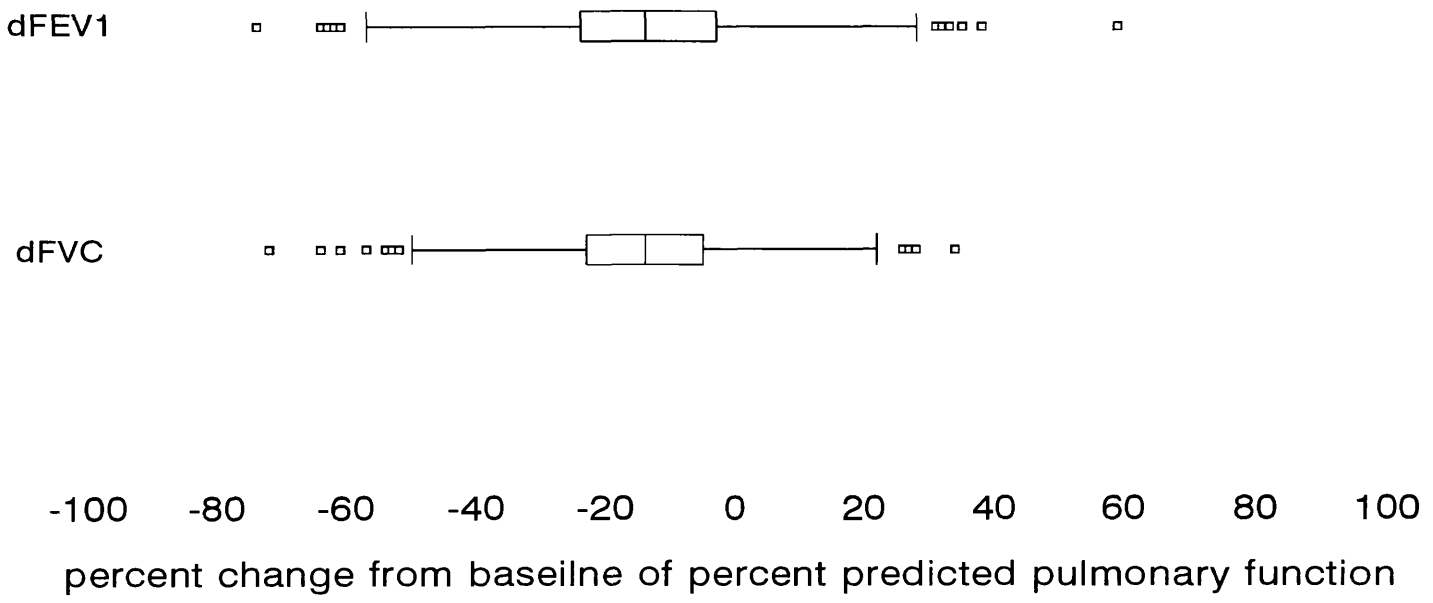


Table 12 - 2 × 2 table of dFEV1 vs dFVC according to presence/absence of exacerbation (dFEV1 or dFVC ≥ 10%)

	dFEV1 ≥ 10%	dFEV1 < 10%	
dFVC ≥ 10%	219 (53%)	36 (9%)	255 (62%)
dFVC < 10%	28 (7%)	129 (31%)	157 (38%)
	247 (60%)	165 (40%)	412

Discussion

In the group as a whole FEV1 and FVC both change by similar proportions before intravenous antibiotic treatment, but reliance on either dFEV1 or dFVC alone misclassifies approximately 10% of episodes in which one pulmonary function parameter declines by at least 10%. This might not be too important if most of the misclassifications were "borderline exacerbations" ie both dFEV1 and dFVC were very close to 10%. However the relatively wide limits of agreement show that this does not hold, with wide variation between dFEV1 and dFVC in individual cases. This variation is not confined to one end of the spectrum of pulmonary disease, as the magnitude of ($dFEV1_i$ - $dFVC_i$) was not dependent on the absolute value of ($dFEV1_i$ + $dFVC_i$)/2. Therefore, in a definition of exacerbation based on pulmonary function, changes in both FEV1 and FVC must be included.

VII.4 Evaluation of pulmonary function + symptoms in defining exacerbation

Methods

Relevant symptoms were recorded in all patients commencing intravenous antibiotic treatment using a structured questionnaire. In addition to lower respiratory tract symptoms, "constitutional" symptoms such as lethargy

and weight loss are recognised to accompany exacerbations of all forms of bronchiectasis, and these were therefore included in the questionnaire. Since most adult patients with CF have residual lower respiratory tract symptoms even when "well", the questionnaire was designed to ascertain recent changes in symptoms by quantifying patients' assessment of individual symptoms at the time of admission, and also their "usual" level of symptoms. The questionnaire proforma is shown in the appendix.

Weight loss was considered to be significant if reported to be of 2kg or more. In order to increase the specificity of the questionnaire, a significant deterioration was considered to have occurred only if at least two of the seven symptoms were worse than normal.

Symptom histories were obtained by either the author or by the admitting doctor (of whom there were twelve through the course of the study).

Results

Of the 412 treatment courses for which sequential pulmonary function data was available symptom questionnaires were lost in 3 cases, and inadequately completed in two. Changes in spirometry could be compared with changes in symptoms in 375 treatment courses. Table 13 summarises the findings.

Table 13 - 2 × 2 table of symptomatic deterioration vs change in spirometry

	symptomatic deterioration	no symptomatic deterioration	
change in spirometry[1]	232 (62%)	26 (7%)	258 (69%)
no change in spirometry	60 (16%)	57 (15%)	117 (31%)
	292 (78%)	83 (22%)	375

1 - defined as dFEV1 or dFVC ≥ 10%.

Discussion

There was no change in symptoms or spirometry in 78 treatments (18% of the total). Symptomatic deterioration was significantly more frequent than change in spirometry (76% vs 66%) ($p < 0.0001$, McNemar's test), suggesting that a symptom-based approach to the definition of exacerbation may be more sensitive than one based on pulmonary function. Symptoms deteriorated in the absence of a change in pulmonary function in 69 (16%) of the treatment courses, but did not change in 24 (6%) treatments in which there was a pulmonary deterioration. This corresponds to 20% and 7% respectively of the treatments in which one or both parameters deteriorated (ie sensitivities of 80% and 93% cf deterioration in either pulmonary function or symptoms).

In summary, symptomatic deterioration usually accompanies a deterioration in pulmonary function, and vice versa, but both symptomatic deterioration and deterioration in pulmonary function can occur independently. Reliance on pulmonary function alone would misclassify 20% of treatments in which there is a deterioration in one or other variable, and reliance on symptoms alone would misclassify 7%. The definition of exacerbation should therefore be expanded to include both pulmonary function and symptoms.

VII.5 Pulmonary function and symptoms as determinants of exacerbation in severely affected patients

Introduction

In severely affected patients who have chronically very low spirometric values, only small absolute increases are observed after intravenous antibiotic treatment. Such patients must also exhibit small absolute decreases in values at the time of an exacerbation. Because of their reduced baseline values such patients might be expected to exhibit a wider variation between dFEV1 and dFVC than that seen in the population as a whole. Similarly, symptoms may be a more sensitive indicator of exacerbation in this subgroup than in the whole group.

Methods

There is no generally accepted definition of severe pulmonary disease in CF. All treatments with a post-treatment predicted FVC of less than 45% were selected for the subgroup analysis, with methods as above.

Results

Convalescent predicted FVC was less than 45% in 40 (9%) of the total of 459 treatments, in 14 (16%) of the 85 patients. dFEV1 and dFVC were not available in 5 treatments. The Bland-Altman plot in shown in figure 23. Mean ($dFEV1_i$ - $dFVC_i$) was -1.1%, uncorrected SD 10.6, corrected SD 14.9. Limits of agreement were therefore -30.9 to 28.7 in this subgroup. Tables 14 and 15 summarise the data categorised according to presence or absence of clinically significant deteriorations.

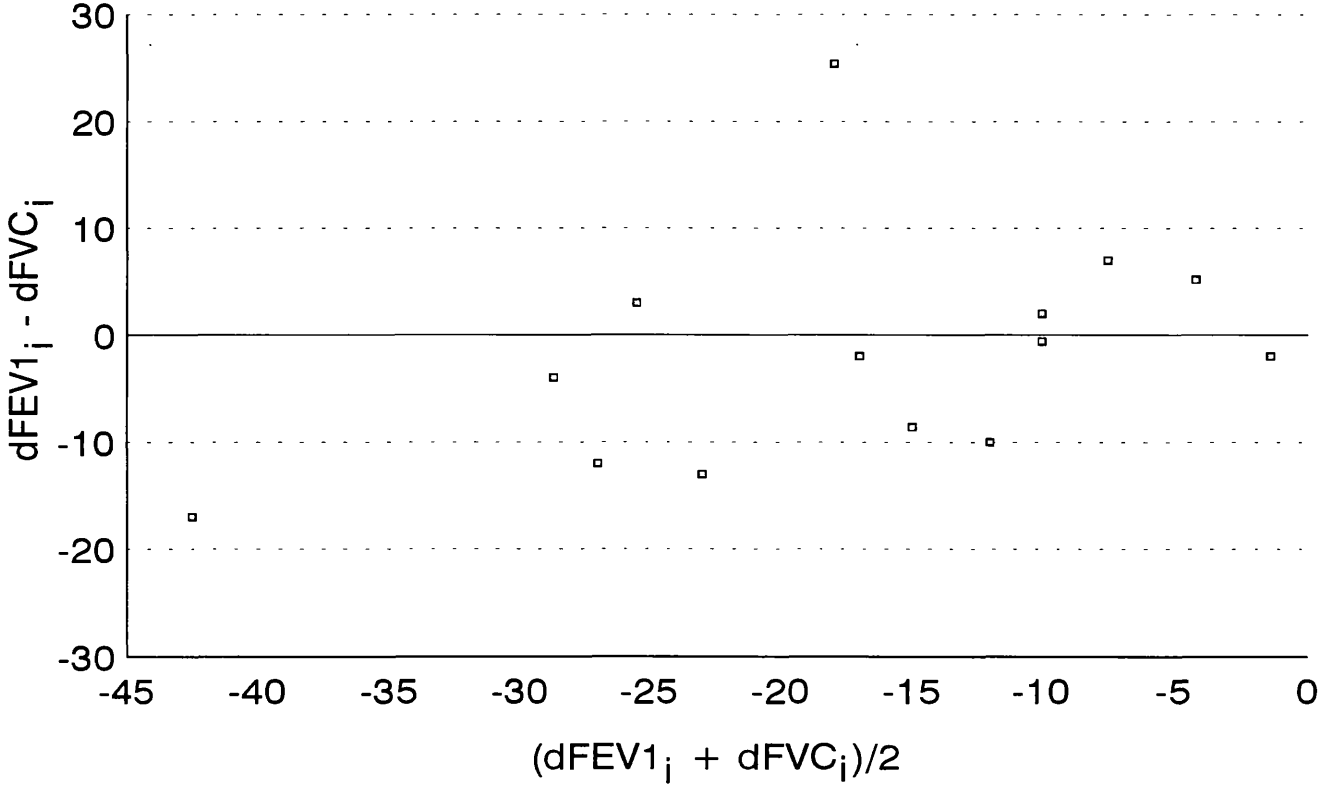
Table 14 - 2 × 2 table of dFEV1 vs dFVC according to presence/absence of exacerbation - severe group

	dFEV1 ≥ 10%	dFEV1 < 10%	
dFVC ≥ 10%	20 (57%)	2 (6%)	22 (63%)
dFVC < 10%	4 (11%)	9 (26%)	13 (37%)
	24 (69%)	11 (31%)	35

Table 15 - 2 × 2 table of dFEV1 vs dFVC according to presence/absence of exacerbation - nonsevere group

	dFEV1 ≥ 10%	dFEV1 < 10%	
dFVC ≥ 10%	199 (53%)	34 (9%)	233 (62%)
dFVC < 10%	24 (6%)	120 (32%)	144 (38%)
	223 (59%)	154 (4%)	377

Figure 23 - Bland-Altman plot of diifference in percent change from baseline of FEV1 ($dFEV1_i$) and percent change from baseline of FVC ($dFVC_i$) against mean percent change from baseline of FEV1 and FVC ($\{dFEV1_i + dFVC_i/2\}$)in severely affected patients



In this severely affected group dFEV1 and dFVC alone both misclassify 3/26 (11%) treatments in which either decreases by 10%. There were no differences between the groups (McNemar's test) in any of the proportions.

Symptom data was available for 38 of the 40 treatments. Both pulmonary function and symptom data were available in 33 treatments. Tables 16 and 17 summarise the symptom and pulmonary function data in the severe and non-severe groups. There were no differences between the groups (McNemar's test) in any of the proportions.

Table 16 - 2 × 2 table of symptomatic deterioration vs change in spirometry

	symptomatic deterioration	no symptomatic deterioration	
change in spirometry	24 (73%)	1 (3%)	25 (76%)
no change in spirometry	2 (6%)	6 (18%)	8 (24%)
	26 (79%)	7 (21%)	33

Table 17 - 2 × 2 table of symptomatic deterioration vs change in spirometry

	symptomatic deterioration	no symptomatic deterioration	
change in spirometry	208 (61%)	25 (7%)	233 (68%)
no change in spirometry	58 (17%)	51 (15%)	109 (32%)
	266 (78%)	76 (22%)	342

Discussion

It is not known whether pulmonary function peaks when a course of intravenous antibiotic treatment is completed or whether there is continued improvement subsequently. In practice, when patients are seen some weeks later as outpatients spirometry is nearly always lower than at the end of the preceding course of treatment. Consequently end of treatment values are the closest to the best attainable for individual patients. Hence these values, rather than the "clinically stable" values were selected to determine the severely affected patients.

In this severely affected subgroup misclassification rates with dFEV1 and dFVC alone were similar to the whole study population, but as expected, limits of agreement between dFEV1 and dFVC were wider. These wider limits of agreement emphasise the need for the inclusion of both dFEV1 and dFVC in the definition of exacerbation in this subgroup with severe disease.

VII.6 Inflammatory markers (white cell count, plasma viscosity and c-reactive protein) compared with the spirometric/symptom definition of exacerbation

Method

The proportion of exacerbations defined according to symptoms and pulmonary function with raised pre-treatment inflammatory markers was estimated for each marker.

Results

Three hundred and seventy treatments fulfilled the criteria for exacerbation. Data were missing for white cell count in 16 of these, for plasma viscosity in 25 and for c-reactive protein in 28. Table 18 summarises the results.

Table 18 - proportion of symptom/pulmonary function defined exacerbations with elevated pre-treatment inflammatory markers

	proportion
PV	72%
WCC	47%
CRP	61%

Discussion

The analysis assumes that a raised inflammatory marker value is necessary to be considered significant. The proportions in table 18 then indicate the maximum possible sensitivity of each marker taking the pulmonary function/symptom model as the standard. [An exact sensitivity, and specificity, could only be derived by arbitrarily defining a clinically significant change in each marker and would require retrospective knowledge of the value at the time of the last recorded spirometry.] The maximum sensitivities are low for all three markers, and it is clear that a definition of exacerbation based on any one of them would exclude many treatments with a significant drop in pulmonary function and increase in symptoms, particularly at the milder end of the disease spectrum. "Conventional" inflammatory markers are therefore too insensitive to be included in a definition of exacerbation. However there could conceivably be a role for NEATC, but this would require further evaluation.

VII.7 Quantitative bacterial culture of sputum

Introduction

To assess whether quantitative bacterial culture of sputum could play a role in the definition of exacerbation a preliminary study was undertaken to assess the method's reliability in terms of the coefficient of variation.

Whether a single sample of sputum, emanating from one lung region, is representative of that from the complete lung field is unknown, and a cause for concern. To address this issue quantitative bacterial culture was compared in two sputum samples collected from the same patient at different times on the same day.

Finally, to assess whether quantitative bacterial culture could be of utility as a marker of exacerbation, sputum samples collected at the start and the end of intravenous courses of antibiotics were compared.

Methods

Sputum specimens were diluted twofold in saline and then liquefied. Liquefied sputum was further diluted 1/100 in saline. Six serial ten fold dilutions were then performed before incubation to log phase on bacitracin chocolate agar. Results were expressed as counts per millilitre of the original specimen.

Sputum samples were collected from patients colonised

with *P aeruginosa* following physiotherapy administered by a physiotherapist. To determine the coefficient of variation the sample was divided into two on receipt in the laboratory. Both halves of each sample were then cultured simultaneously.

To compare sputum samples which may have originated in different areas of the lung one sample was collected following physiotherapy in the morning and a second sample was collected following physiotherapy in the afternoon.

Finally, quantitative bacterial culture was performed on paired sputum samples collected at the start and end of intravenous antibiotic courses.

Results

Forty-one sputum samples were obtained from 24 patients for the determination of the coefficient of variation. Median (range) percent predicted FEV1 in the patients providing samples was 65 (23-122), percent predicted FVC 34 (15-108), and clinical score 10 (2-17). Mean (SD) bacterial count was 6.28 (0.89) $\log_{10}$ units for the whole group, with a coefficient of variation of 20.2%.

Seventeen paired sputum samples were obtained in the morning and the afternoon of the same day from 17 patients. Mean (SD) bacterial count in this group was 6.27 (1.23) $\log_{10}$ units and SD of the differences between the paired samples 1.02, a coefficient of variation of 16%. The 95% confidence interval was +/- 2.04 $\log_{10}$ units.

Paired sputum samples were obtained for quantitative

culture at the start and end of 42 intravenous treatments in 30 patients. Mean (SD) pretreatment count was 7.71 (1.30) $\log_{10}$ units, and post-treatment count 7.55 (1.32) $\log_{10}$ units. Pulmonary function was not distributed normally in this group. Median (range) percent predicted FEV1 was 45 (25 - 103) pre-treatment and 53 (30 - 115) post-treatment, and percent predicted FVC 66 (33 - 112) pre-treatment and 72 (38 - 122) post-treatment. The improvement in FEV1 and FVC with treatment was significant ($p < 0.0001$, Mann-Whitney), as would be expected.

Discussion

The high coefficient of variation of quantitative sputum culture renders it insensitive for the detection/definition of pulmonary exacerbations. That there was no further increase in the coefficient of variation when sputum samples were collected at different times of day suggests that bacterial sputum density is probably reasonably homogeneous, whichever area of lung the sputum sample emanates from, although it is difficult to be sure given the inaccuracy of the method.

Quantitative bacterial culture values did not change with intravenous antibiotic treatment, despite a highly significant improvement in pulmonary function. Thus there was insufficient change in bacterial counts with intravenous antibiotic treatment to overcome the poor coefficient of variation of the method. Since quantitative culture was

unable to detect a change in the extreme case of intravenous antibiotic treatment, it clearly can be of no use in the detection of exacerbations. Furthermore quantitative culture cannot be used either to grade the severity of an exacerbation or to investigate the contribution of increased bacterial numbers in the aetiology of exacerbations.

VII.8 Summary of methods of defining exacerbation

Quantitative bacteriological culture of sputum was not reproducible enough to serve as a marker of exacerbation. White cell count, c-reactive protein and plasma viscosity were insensitive, and inconsistently elevated with increasing disease severity, and were rejected as markers of exacerbation on these grounds. Neutrophil elastase α-1-antitrypsin complex is more sensitive than these markers (see VI.3) and may permit a definition based on inflammatory markers in future.

In a definition based on pulmonary function, changes in both FEV1 and FVC must be included, since they deviate from the baseline inconsistently in relation to each other. An increase in two or more lower respiratory tract symptoms showed good general agreement with a decrease in pulmonary function of at least 10% from baseline. Although a symptom-based definition would be slightly more "sensitive" than one based on pulmonary function, it cannot entirely substitute

for pulmonary function, as some patients demonstrated significant falls in spirometry in the absence of symptoms. A definition based on both pulmonary function and symptoms has the advantage of being able to define those treatments in which data relating to one of the criteria is missing, provided the other criterion is positive.

VII.9 Pulmonary exacerbations and long-term decline in pulmonary function

Introduction

Pulmonary exacerbations are generally assumed to have a detrimental effect on long-term health, through accelerated loss of lung tissue as part of the inflammatory process, although there is no data to support this assumption. To investigate this hypothesis the frequency of pulmonary exacerbations was compared with decline in pulmonary function over the two year period of the study.

Methods

Pulmonary function data, collected both during antibiotic treatment and at routine out-patient visits, were available for 60 patients for the entire two year period. For each patient all values of percent predicted FEV1 and FVC recorded during the two year study period were plotted

against time. Regression was then performed on each individual patient plot to estimate the values on 1st May 1991 and 30th April 1993. The change in pulmonary function over the two year period was then taken as the difference between the two values. As well as absolute change in pulmonary function, the percentage decline in pulmonary function was calculated. Pearson correlation coefficients were then calculated for all measures of change in pulmonary function vs number of exacerbations. For completion, correlation coefficients were also calculated for change in pulmonary function vs number of exacerbations with exacerbation defined as change in pulmonary function only, and vs total number of treatments.

Results

Results are shown in tables 19 and 20. All the variables were distributed normally. The spread of the pulmonary function data is illustrated by the boxplots in figure 24.

Table 19 - Change in pulmonary function over two years, and number of exacerbations and treatments per patient in two years

absolute change in percent predicted FEV1	-5.0 (11.9)
percent change in percent predicted FEV1 over baseline	-9.3 (22.8)
absolute change in percent predicted FVC	-2 (18.4)
percent change in percent predicted FVC over baseline	-2 (23.7)
number of exacerbations - pulmonary function and symptoms	4.3 (3.3)
number of exacerbations - pulmonary function only	3.5 (3.1)
total number of treatments	5.3 (3.6)

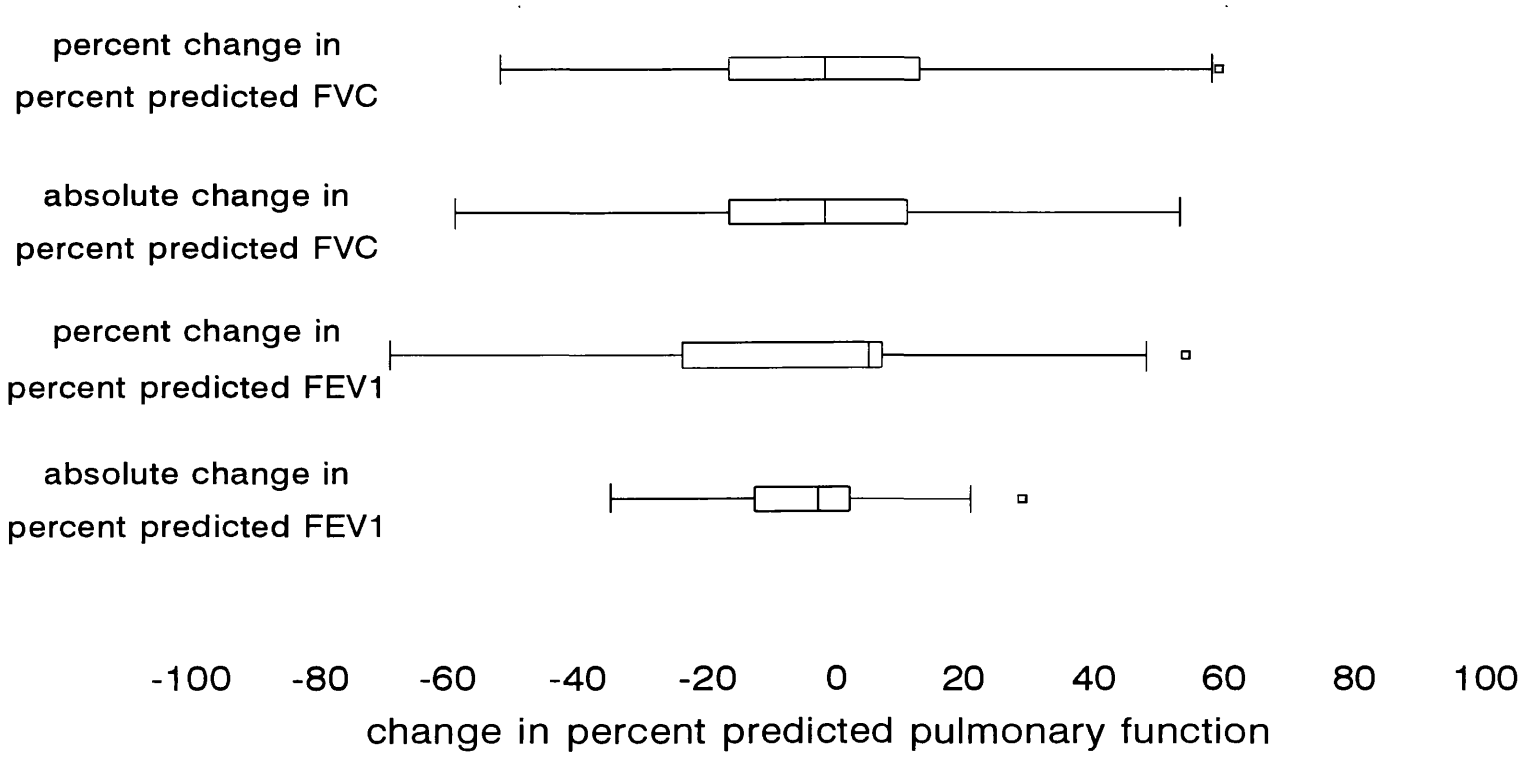
All values are mean (SD). Negative values indicate decline in pulmonary function. Number of patients = 60

Table 20 - Correlation of number of exacerbations/treatments with change in pulmonary function

	Number of exacerbations (pulmonary function and symptoms)	Number of exacerbations (pulmonary function only)	Total number of treatments
absolute change in percent predicted FEV1	0.10	0.08	0.09
percent change in percent predicted FEV1 from baseline	0.25	0.23	0.23
absolute change in percent predicted FVC	0.22	0.21	0.20
percent change in percent predicted FVC from baseline	0.23	0.22	0.23

All correlation coefficients non-significant. Number of patients = 60

Figure 24 - Boxplots to show change in pulmonary function over two years in the cohort of 60 patients with cystic fibrosis



Discussion

The spread of the data relating to change in pulmonary function was extremely broad (see figure 24). Approximately one third of the cohort showed improvements in pulmonary function over the two year period. Pulmonary function varies widely in the short term, and simply subtracting the last recorded from the first recorded value in a longitudinal study of pulmonary function is likely to result in inaccuracy, depending on the clinical context of the recording. Performing individual regression analyses is a fairly robust way of circumventing this problem and the very wide spread of the data is therefore unlikely to be artifactual.

There was no correlation between the number of exacerbations and change in pulmonary function over the two year period. Likewise there was no correlation between total number of treatments and change in pulmonary function. A possible explanation could have been that the definition of exacerbation was too sensitive. Therefore the data were reanalysed to include just those exacerbations with a significant change in pulmonary function, but the correlation was unaffected. This complete absence of correlation is surprising, and implies that in general pulmonary exacerbations, provided they are treated, do not result in long-term loss of pulmonary function. To substantiate this conclusion post-treatment pulmonary function was compared with pre-exacerbation baseline pulmonary function.

VII.10 Recovery of peri-exacerbation loss of pulmonary function - post-treatment compared with pre-exacerbation baseline

Methods

For all exacerbations with complete pre-exacerbation baseline, pre-treatment and post-treatment pulmonary function data, post-treatment pulmonary function was compared with pre-exacerbation baseline values. Since there were variable numbers of treatments for each patient the pattern of the data was non-orthogonal, and ideally should be analysed with general linear modelling (equivalent to ANOVA), with percent predicted pulmonary function as the response variable, treatment as a factor and within-patient repeated treatments as a factor nested within the main treatment factor. Unfortunately the calculation proved too large for a personal computer to compute. Each exacerbation was therefore treated as a separate entity, without taking interindividual patient differences into account. The data were not normally distributed and were compared with the Mann-Whitney U-test. The analysis was performed separately, once for exacerbations defined by pulmonary function and symptom criteria, and once for exacerbations defined by pulmonary function alone.

Results

Data was complete for 344 exacerbations defined by both parameters in 80 patients, and for 274 exacerbations defined by pulmonary function alone in 71 patients. Tables 21 and 22 summarise the data.

Table 21 - Post-treatment pulmonary function compared with pre-exacerbation baseline - exacerbation defined by symptom and pulmonary function criteria

	pre-treatment	post-treatment	significance level
percent predicted FEV1	42 (13 - 124)	43 (16 - 119)	p = 0.56
percent predicted FVC	74 (29 - 142)	75 (24 - 148)	p = 0.59

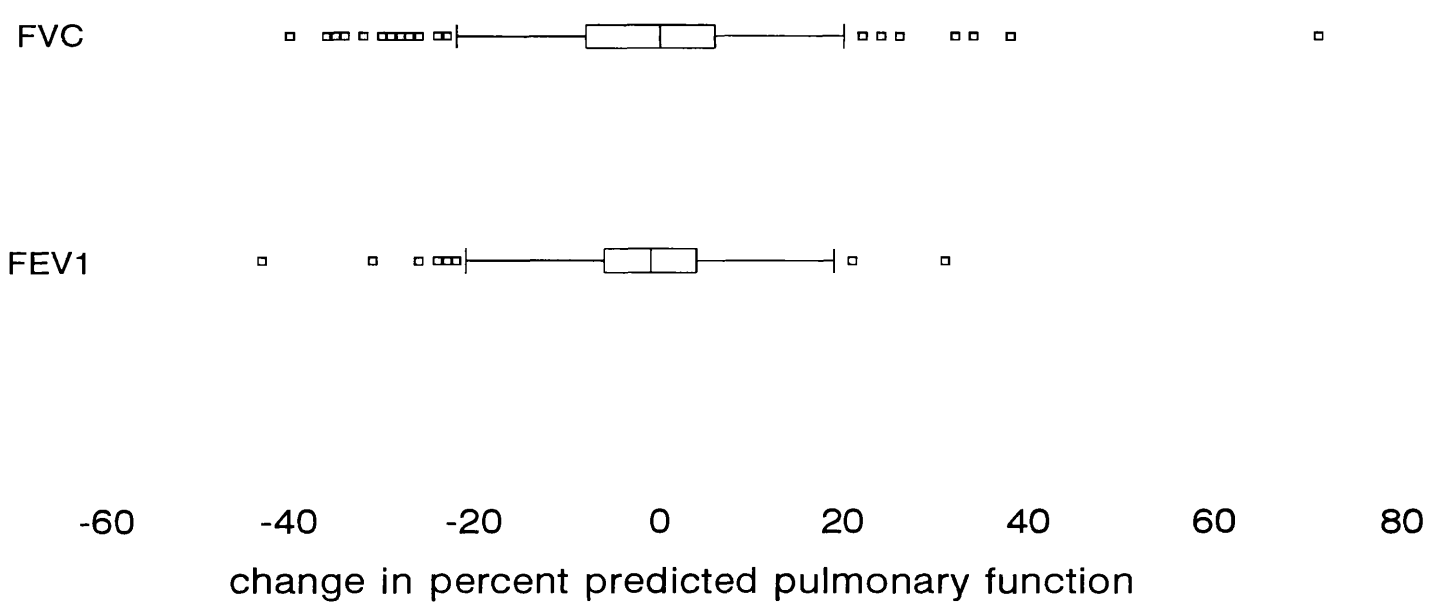
values are median (range)

Table 22 - Post-treatment pulmonary function compared with pre-exacerbation baseline - exacerbation defined by pulmonary function criteria alone

	pre-treatment	post-treatment	significance level
percent predicted FEV1	41 (13 - 124)	41 (16 - 119)	p = 0.95
percent predicted FVC	74 (29 - 142)	74 (24 - 148)	p = 0.64

values are median (range)

Figure 25 - Boxplots summarising the spread of {pre-treatment - post-treatment} pulmonary function (pooled values from all patients, each with varying numbers of treatment courses)



Discussion

The analysis confirms that in general treated pulmonary exacerbations, whether defined solely by pulmonary function criteria, or additionally with symptom criteria, do not result in significant loss of pulmonary function, although the spread of the individual {post-treatment - pre-treatment} differences was wide (see boxplots in figure 25). Although the statistical treatment was not ideal, the pre- and post-treatment medians were virtually identical and it is very unlikely that repeated exacerbations in some individuals would have biased the result. It is important to emphasise that the population studied constitutes patients who presented for treatment of exacerbations. No conclusions can be drawn about untreated exacerbations from this data, although one could speculate that there would be residual loss of function after an arbitrary length of time without treatment.

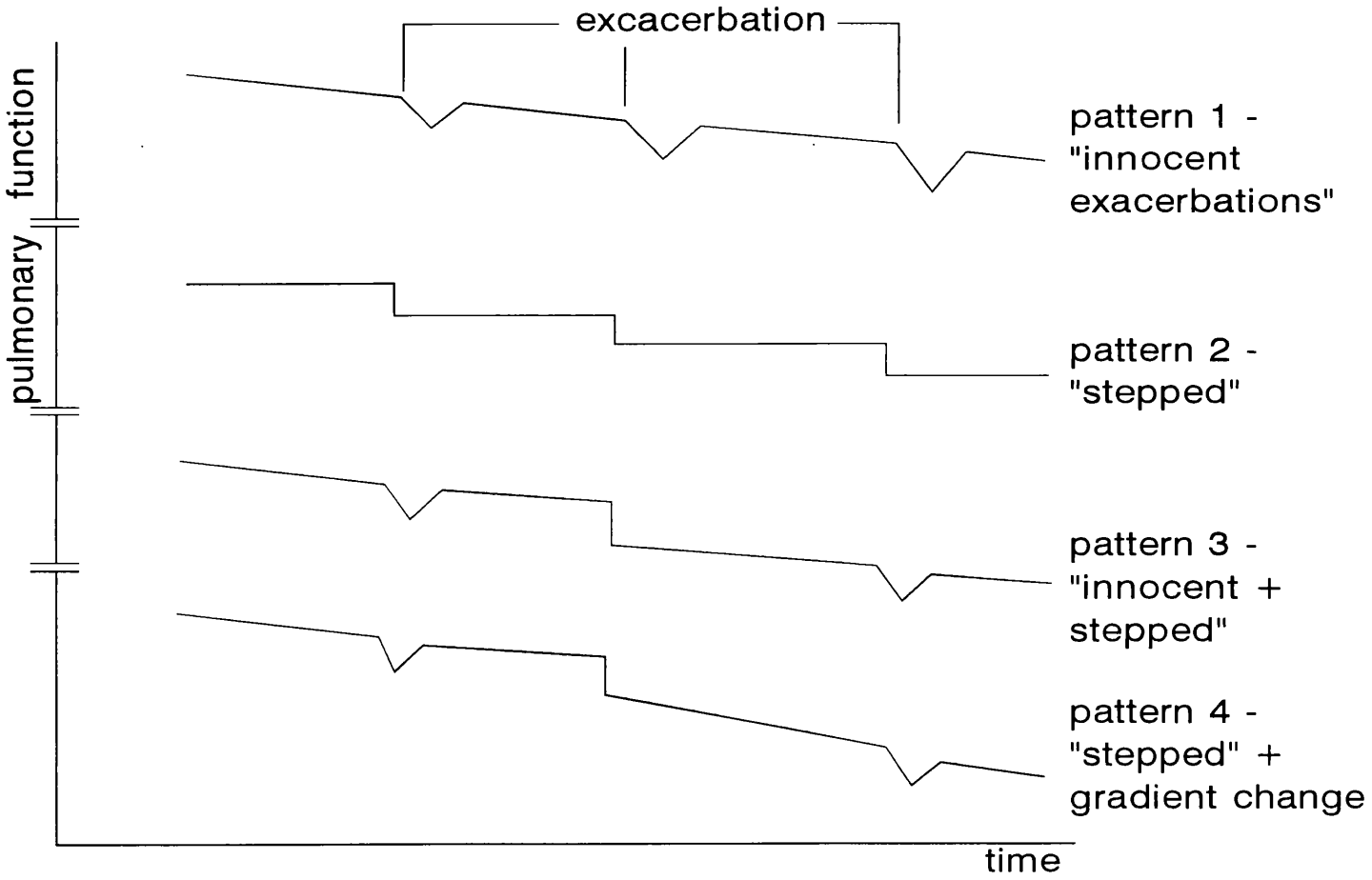
The most likely model for the long-term decline of pulmonary function in an individual is that its rate is relatively stable. Pulmonary exacerbations would then represent temporary fluctuations from the baseline rate of deterioration, with a return back to the baseline following treatment. The pattern would approximate to pattern 1 in figure 26 rather than pattern 2. An unresolved question is, "why do patients with CF develop exacerbations ?" The subsidiary question is "are there specific precipitants of exacerbation eg co-infection with respiratory viruses and/or

atypical bacteria ?" The latter question is further addressed in succeeding chapters. For the moment it is worth noting that pattern 1 is entirely compatible with exacerbations merely representing random fluctuations from the baseline rate of deterioration. Furthermore it should be noted that the above analysis describes the general situation - deterioration as in patterns 3 and 4 in figure 26 may have occurred in some individuals ie it is possible that a minority of exacerbations do have a significant effect on long-term pulmonary function. Conceivably it is these exacerbations that are associated with co-infection.

It was shown in the previous chapter that intravenous antibiotic treatment results in greater improvements in white cell count and c-reactive protein than can be accounted for by the observed change in pulmonary function. The apparently innocent nature of pulmonary exacerbations would tend to support a specific effect of treatment rather than an excessive pre-treatment build up of these inflammatory markers to account for their "excess reduction", although this remains speculative.

Pulmonary exacerbations are important end-points in longitudinal CF studies as they represent a significant separate morbidity for patients and a substantial economic drain on both health care resources and the patients' own resources. However the finding that pulmonary function is completely recoverable following treatment of exacerbation means that exacerbations must be treated as end-points in their own right, and that they cannot serve as surrogates of

Figure 26 - potential patterns of long-term decline in pulmonary function



long-term morbidity and mortality.

This finding also brings into question the rationale for administering intravenous antibiotics to patients outside the context of pulmonary exacerbations. Chronic administration of nebulised antibiotics has been shown to reduce the rate of loss of pulmonary function [Mukhopadhay 1996]. However, if pulmonary function is fully recoverable after symptomatic exacerbations, and the frequency of exacerbations has no relation to the rate of decline of pulmonary function, it is hard to see what extra benefit would be derived from additional "asymptomatic treatments", of just one to two weeks duration. There may however be an exception to this argument (see XII.3).

VII.11 General conclusion

Any definition of pulmonary exacerbation has to be to some extent arbitrary. A universally accepted definition of pulmonary exacerbation is highly desirable, but must be decided by consensus. It is suggested that a definition of a decrease in FEV1 or FVC of at least 10% from baseline, or an increase in at least two lower respiratory tract symptoms be adopted. This is the definition of exacerbation that will be employed for the remainder of this thesis.

Pulmonary function is completely recovered after treatment of pulmonary exacerbations. Treated pulmonary exacerbations have no overall effect on long-term pulmonary function.

Chapter VIII Incidence of viral infection, indirect evidence and conventional diagnostic procedures

VIII.1 Introduction

Clinical experience suggests that pulmonary exacerbations frequently are associated with upper respiratory tract symptoms suggestive of respiratory viral infection. The hypothesis that respiratory viral or atypical bacterial infections are responsible for some, or even most, exacerbations therefore deserves testing. At the outset of this study the hypothesis was that such respiratory co-infection causes significant permanent loss of pulmonary function, and by implication reduced longevity, through precipitation of pulmonary exacerbations. However it was shown in the last chapter that carefully defined pulmonary exacerbations treated during the course of the study did not have any effect on two-year changes in pulmonary function, although they still represent recurring episodes of excess morbidity requiring treatment in their own right, with economic consequences for the patient and the health-care provider. From this data alone it is possible to state that respiratory co-infection cannot result in significant long term morbidity in an adult CF population through the induction of exacerbations: pulmonary function is very largely recoverable in the short term following treatment of exacerbations. However there may still be a role for such co-infection in the induction of exacerbations with

recoverable pulmonary function, or possibly in inducing irrecoverable loss of function in a small number of cases.

The aims of this part of the study were as follows:-

1. To estimate the frequency with which patients present for treatment of exacerbations with a history suggestive of non-bacterial infection, and to identify any excess of such symptoms during the winter months.

2. To identify any excess in exacerbations during the winter months when respiratory viral infections should be more common.

3. To document the incidence of positively identified non-bacterial infections in pulmonary exacerbations.

4. In case of a significant number of patients presenting late for treatment of exacerbations, to estimate the frequency of confirmed non-bacterial infection in patients reporting suggestive symptoms outside the context of an exacerbation.

5. To compare the frequency of respiratory tract symptoms suggestive of non-bacterial infection, and the frequency of confirmed non-bacterial infection, between patients, family controls and controls with asthma and non-CF bronchiectasis.

VIII.2 Frequency of symptoms suggestive of non-bacterial infection in patients presenting for treatment of pulmonary exacerbations.

Methods

All patients presenting for intravenous antibiotic treatment during the two year period May 1st 1991 to April 30th 1993 were asked specifically whether they had recently developed any of the following symptoms -

sore throat
nasal congestion
lassitude
fever
myalgia
earache
rash
arthralgia\arthritis

During the first year of the study all patients were interviewed personally by the author. During the second year responses were recorded on a questionnaire either by the author or by the admitting doctor. Final classification was undertaken in all cases by the author.

The following symptoms were considered to be suggestive of non-bacterial infection :-

1 sore throat alone

2 nasal congestion + one of lassitude, fever or myalgia

3 earache alone

4 sore eyes alone

5 rash alone

6 two of lassitude, fever, myalgia, or arthralgia/arthritis

Patients positive on the basis of these criteria were classed as having "symptoms of non-bacterial infection", abbreviated to SNBI. The frequency of SNBI was determined for those with and without exacerbations.

Results

Data with completed categorisation according to presence/absence of exacerbation and presence/absence of SNBI were available for 401 treatments in 88 patients. One patient with just one treatment that could not be classified as to the presence\absence of exacerbation could not be included. The data are summarised in table 23. SNBI was very strongly associated with exacerbation. Only one SNBI was reported in the absence of exacerbation.

Table 23 - 2 × 2 table of exacerbation vs SNBI

	Exacerbation	No exacerbation	
SNBI	154	1	155
no SNBI	189	57	246
	343	58	401

Chi square $p < 0.0000001$

Table 24 - 2 × 2 table of SNBI vs exacerbation (pulmonary function criteria only)

	Exacerbation	No exacerbation	
SNBI	113	29	142
no SNBI	145	88	233
	258	117	375

Chi square $p = 0.0007$

Discussion

SNBI occurred in 45% of exacerbations and only once (2%) in the absence of exacerbation. This extremely strong association might be accounted for by the inclusion of fever and malaise in the symptom criteria for both exacerbation and SNBI. To ensure that the association was not due to confounding caused by inclusion of common symptoms in the respective definitions, SNBI was further compared with exacerbations defined solely by pulmonary function criteria. The results are summarised in table 24, and the association remains highly significant.

Detection of non-bacterial infection in any patient with bronchiectasis is difficult. "Constitutional" symptoms such as fever and malaise frequently accompany exacerbations, and are probably caused by enhanced levels of circulating inflammatory mediators, possibly tumour necrosis factor alpha. In addition, the almost universal presence of chronic upper respiratory tract disease in these patients means that new upper respiratory symptoms may arise at the same time as lower tract symptoms during an exacerbation, without there necessarily being a specific non-bacterial trigger. An individual SNBI may therefore just be an epiphenomenon, rather than a true indicator of non-bacterial infection.

VIII.3 Seasonal distribution of exacerbations and SNBI

Introduction

Johansen and Hoiby, in their large retrospective survey [1992], identified the onset of chronic *Pseudomonas aeruginosa* infection as occurring more frequently during the winter months, and postulated a respiratory viral aetiology. Using a similar approach, it is possible to draw inferences about the likely frequency of respiratory viral infection in exacerbations of CF, and also as to the significance of SNBI.

Methods

Only those exacerbations and SNBI in the cohort of patients who were followed for the whole duration of the two year study were analysed. Exacerbations and SNBI were divided into those occurring during the winter (October to March) and summer (April to September) months (after Johansen and Hoiby). The proportions of the total number of exacerbations and SNBI occurring during each period were compared for statistical significance by calculating the standardised normal deviate.

Results

Sixty patients were followed for the whole two year study period. Completed data were available for the classification of SNBI and exacerbation in 303 of 320 treatments. Two hundred and fifty one treatments were classified as exacerbations. The numbers of exacerbations and SNBI occurring each month are shown in figures 27 and 28, and the numbers occurring in the winter and summer months are shown in table 25.

Figure 27 - Bar chart to show distribution of exacerbations by month

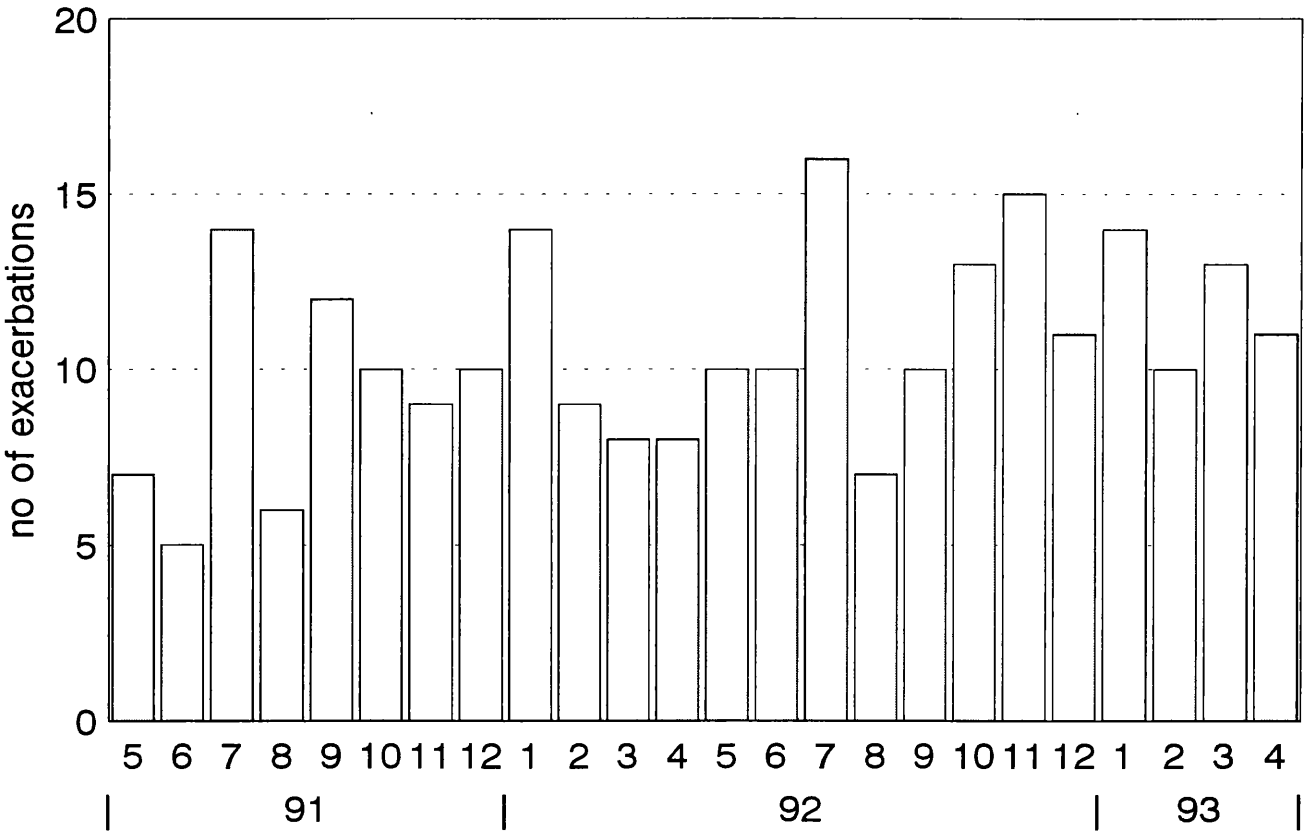


Figure 28 - Bar chart to show distribution of SNBI by month

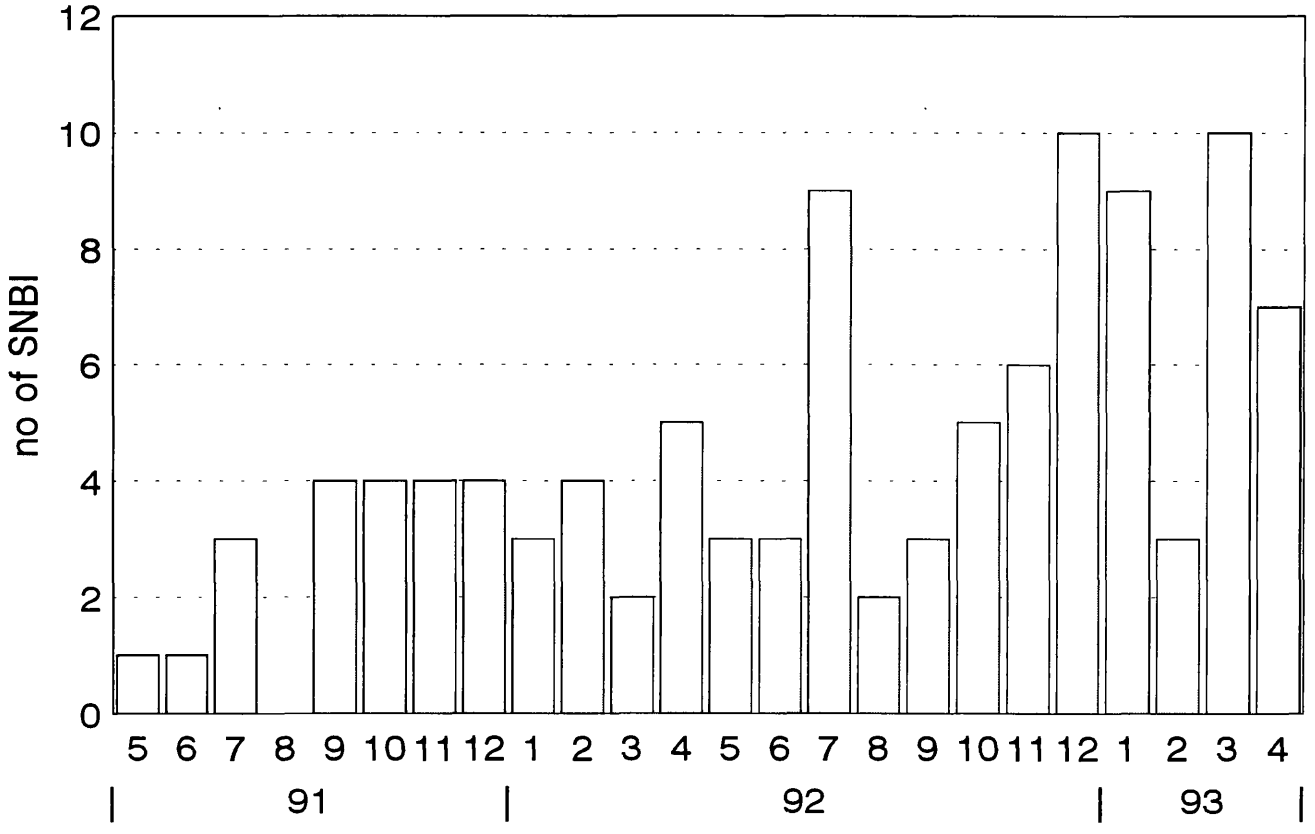


Table 25 - seasonal distribution of exacerbations and SNBI

	winter	summer	significance
exacerbations	135 (54%)	116 (46%)	p = 0.25
SNBI	61 (60%)	40 (40%)	p = 0.003

Discussion

Confirmatory evidence of the seasonality of respiratory viral infections is very limited. Foy et al [1979] looked specifically at the incidence of *M pneumoniae*, which was not seasonal. Anderson [1989] studied RSV infection only in a cohort with age less than 5 years, and found a peak incidence in winter. Carlsen et al's retrospective study [1988] showed winter and spring peaks of RSV and both influenza A and B, but no peaks in parainfluenza and adenovirus infection. Their study group was predominantly paediatric, and when the adult group was analysed separately the most frequent isolates were influenza B and adenovirus. Kellner et al's two year prospective study was confined to a paediatric age group, and identified winter and spring peaks in RSV infection, and autumn and spring peaks in rhinovirus infection, but there was a background of rhinoviral infection throughout the year. Thus there is no prospective data regarding seasonality of respiratory viral infections in adult patients, and no data whatever regarding coronaviral infection. A best guess based on this data would be a slight excess seasonality of non-bacterial infection in winter, superimposed on a background year long incidence.

There is no discernible pattern in the bar charts of the monthly frequency of exacerbations. There appears to be an excess of SNBI during the winter months, particularly in the second year of the study. With classification according to season there was a winter excess of nineteen exacerbations

(8% of the total number), but this was not statistically significant. There was a winter excess of twenty one SNBI (20% of the total number), which was statistically significant. The close concordance of these excess values provides strong circumstantial evidence of a non-bacterial aetiology in at least these "excess" exacerbations. (Although the excess number of exacerbations was not statistically significant, this could nevertheless represent a real, but small, effect.) Because symptoms suggestive of non-bacterial infection may occur simply due to excess inflammation during an exacerbation, it is not possible to draw firm conclusions about the precise impact of non-bacterial infection in precipitating exacerbations. Nevertheless if most non-bacterial infections were to occur in the presence of suggestive symptoms it would be reasonable to suppose that the true proportion of exacerbations with a non-bacterial co-infection lies between 8% (the winter excess) and 40% (the total proportion of exacerbations associated with SNBI).

VIII.4 Confirmed non-bacterial infection in patients presenting with pulmonary exacerbations

Introduction

Antigen detection methods for the diagnosis of respiratory viral infections are probably no more sensitive than viral culture, although they are more rapid. There have

been few comparative studies, and almost all have been confined to RSV. RSV antigen can be detected reasonably reliably, but generally the sensitivity has been no better than culture [Halstead 1990; Waner 1990]. The advantage of identifying RSV infection early is that it allows intervention with ribavirin in the treatment of severe bronchiolitis. Direct detection methods are available for other respiratory viruses, but they have not been validated against viral culture methods, which currently remain the "gold standard". Therefore antigen detection methods were not used in this study.

There is little guidance available from the literature as to the sampling site with the highest yield, since it is dominated by paediatric studies. Most of these have favoured naso-pharyngeal aspirates in studies of rhinovirus and RSV infection. However the procedure is unpleasant, particularly in adults, and it was not felt to be a practical sample to obtain repeatedly in young adults with cystic fibrosis. The underlying hypothesis being tested was that viral upper respiratory tract infection could exacerbate the chronic inflammatory state in the lower respiratory tract. No assumptions were made at the outset as to the detailed mechanism involved, but this was assumed to involve direct viral infection of the lower airways. Throat swabs therefore seemed to be an appropriate sample to obtain, and they are generally held to be an acceptable substitute [Parker 1990] for most known respiratory viruses. They are relatively easy to obtain, provided care is taken to make contact with the

posterior pharyngeal wall. Viruses can sometimes be cultured from sputum, but it was felt that the colonisation with *P aeruginosa* would inhibit viral replication to such an extent as to make the attempt at isolation from this source futile. Consequently throat swabs alone were collected for viral isolation.

Methods

In all patients throat swabs were obtained by experienced infectious diseases nurses or the author on the day that intravenous antibiotic treatment was started, except for patients presenting at weekends after Saturday morning, when sample collection was delayed until the following Monday. Patients gave verbal consent before collection of the swab. Swabs were immersed in transport medium containing amphotericin and ciprofloxacin, and transferred to the virology laboratory on the same day, or the following morning at the latest.

Blood was obtained for baseline serology at the start of the study. Sera were also obtained at the start and end of all treatments. In addition, in those patients who did not receive intravenous antibiotic treatment in any six month period, a separate serum sample was obtained at six months to identify asymptomatic seroconversions.

To avoid confusion in the collection of samples, all treatments carried out during the two year study (May 1st 1991 to April 30th 1993) were included in the protocol. This

meant that patients recruited to the CF unit after the study commenced were included. Data from these patients were included in the estimate of the proportion of exacerbations with confirmed non-bacterial co-infection. Data from the cohort of patients who attended the CF unit throughout the two year study period were also analysed separately. Treatments where there was no exacerbation, and asymptomatic seroconversions (ie seroconversion occurring between the previous convalescent sample and the acute sample, or routine six month sample) were treated as "negative controls", to estimate the significance of the association of non-bacterial infection with exacerbations.

As well as for respiratory viruses, serology was performed for cytomegalovirus, not because it was thought likely to be a pathogenic co-infecting organism, but in order to document the rate of seroconversion in view of the requirement for CMV status matching in pulmonary transplantation.

Viral isolation methods

On receipt in the laboratory the transport medium was inoculated into cultures of primary baboon kidney (parainfluenza), HEp 2 (adenovirus) and human embryonic fibroblast (rhinovirus) cells. They were then inspected at appropriate intervals for cytopathic effect. In addition, each sample underwent a haemadsorption test (influenza A and B).

Serology

Complement fixation tests were carried out for influenza A and B, adenoviruses, RSV, *M pneumoniae*, *C Burnetti*, and *chlamydia* species. It should be noted that the complement fixation test for chlamydia is not species specific, and identifies *C psittaci* and *C pneumoniae*. Most positive sera are likely to be due to *C pneumoniae*. Indirect fluorescent absorbence was carried out for *L pneumpophila*. A latex agglutination test was performed to screen for CMV. Where this was positive a fluorescent antibody test and complement fixation test were performed for confirmation. In all cases a rise in titre of at least fourfold compared with the previous titre was considered to be diagnostic. Influenza infection was diagnosed only when an increase in titre occurred in the absence of vaccination against influenza.

VIII.4.i CMV seroconversion

Results

Eleven (18%) of the cohort had demonstrable antibodies vs CMV at the outset of the study. However most of these patients had modest titres of 1/16 or 1/8, and nine became antibody negative at some point during the study. Three patients who were initially antibody positive became antibody negative before becoming antibody positive at low titre once more.

Three patients who were initially antibody negative developed antibodies to CMV, a seroconversion rate of 2.5% per year. In all three cases seroconversion was associated with exacerbations of CF. All three patients remained antibody positive for the remainder of the study.

Discussion

Although not usually thought of as a respiratory virus, the temporal association of CMV seroconversion with exacerbation, and the absence of asymptomatic seroconversion, suggests that acute CMV infection is capable of precipitating pulmonary exacerbations. The data from these three exacerbations have therefore been included with the remainder of the serological data (see below).

CMV infection post-transplantation is a major cause of morbidity and mortality. In addition to the direct effects of pneumonitis, superadded infection with bacteria and fungi is more common following CMV infection, as is chronic rejection [Duncan 1992]. Using regular post-transplant surveillance with transbronchial biopsy, Ettinger et al [1993] showed CMV infection in 92%, and CMV pneumonitis in 75%, of patients who were either donor or recipient CMV antibody positive, but antibody negative patients with antibody negative donors were completely unaffected. No relation between CMV infection and obliterative bronchiolitis was observed in this study. Although the effects of CMV infection are more pronounced in CMV antibody negative recipients of CMV antibody positive

donors reactivation of latent CMV infection in antibody positive recipients is also a significant problem [Wreghitt 1989; Ettinger 1993]. Three studies have found positive donor antibody status to be an adverse influence on survival [Wreghitt 1989; Ettinger 1993; Novick 1993]. Sharples et al [1994] found an odds ratio of 3.24 for death within 90 days of transplant for positive donor or recipient antibody status compared with negative donor and recipient antibody status. Most of these authors have concluded that recipients of lung transplants should be matched for CMV antibody status with the donor, but the only reported series, of 131 lung transplants, in which matching was carried out did not demonstrate any benefit [Cooper 1994].

In this study seroconversion to CMV was often transitory. Antibodies were not detected for a period of time, before becoming detectable at low titre once more. The usual wait between referral for transplantation and receipt of a transplant is about two years, the length of this study. During this time 77% of patients remained consistently antibody negative, and if being considered for transplantation might be expected to have very little CMV infection if matched with antibody negative donors. In nearly all of the remainder of the study population CMV antibody status varied. Thus a single estimate of CMV status at the time of assessment for transplantation is likely to be 80% accurate at the time of the actual transplant. Periodic reassessment of CMV antibody status during the period of waiting for transplantation would improve the accuracy of

matching in transplant centres which follow this policy.

VIII.4.ii All viruses and atypical bacteria

Results

A) All exacerbations

Sample collection was 95% complete. Three hundred and seventy three treatments were classified as exacerbations, sixty-two as non-exacerbations, and 24 were unclassifiable, in a total of 89 patients. There were no viral isolates. Twelve seroconversions occurred, including the three seroconversions to CMV, all in different patients. The details of these are listed in table 26. Frequent minor increases in titres to *L pneumophila* were observed, but never a fourfold increase.

Table 26 - Characteristics of seroconversions

Organism	Month	Exacerbation	SNBI
adenovirus	January	yes	no
adenovirus	March	yes	no
adenovirus	July	yes	no
adenovirus	October-January*	no	no
influenza A	January	yes	no
influenza A	December	yes	yes
influenza B	March	yes	yes
RSV	January	yes	no
RSV	March	yes	yes
CMV	January	yes	yes
CMV	May	yes	yes
CMV	September	yes	no

* this seroconversion occurred between treatments at the beginning of October and the end of January

B) Cohort followed for the full two year period

Two hundred and fifty-five treatments were classified as exacerbations, fifty-two as non-exacerbations, and thirteen were unclassifiable, in 60 patients. There were ten seroconversions in this group, including the asymptomatic seroconversion. The rate of seroconversion was therefore 0.083 per patient per year.

Discussion

There were no seroconversions to any of the atypical bacteria. The minor increases in titre to *L pneumophila* were probably due to cross-reaction with antibodies directed against *P aeruginosa* [Collins 1984; Wang 1987]. The lack of any seroconversion to *chlamydiae* was unexpected, as *C pneumoniae* is thought to be a reasonably common respiratory pathogen, although the sensitivity of the complement fixation test is limited. More surprising was the absence of seroconversions to *M pneumoniae*, since the first year of the study was a year of peak incidence for the organism, which follows a four year cycle. This total absence of seroconversion to atypical bacteria may be due to the dominance of *pseudomonas* species, primarily *P aeruginosa*, in the colonisation of the lower respiratory tract in adult patients with CF. The only other bacterial species cultured with any regularity from sputum is *S aureus*, and co-recovery of the two organisms gradually gives way to recovery of

pseudomonas species alone (data on file).

The rate of positive identification of respiratory viral infection is highly dependent upon age. Wang et al [1987] described 105 seroconversions to respiratory viruses in 49 patients over two years, but their cohort certainly included some children, as the mean age was 13.7 years. The inclusion of older patients in the late twenties tends to skew mean ages, and it is likely that this cohort was in fact predominantly paediatric. A similar caveat applies to the study of Ramsey et al [1989] in which the mean age was 13.4 years in both their patient and sibling control groups. Sixty-eight seroconversions to respiratory viruses were documented in the thirty individuals during two years. Hordvik et al [1989] only found ten seroconversions in ten patients with mean age 14.1 years over twenty months. These three studies all give an annual seroconversion rate of roughly 1/patient/year.

The low rate of seroconversion in our study, 0.083/patient/year, was similar to that observed by Efthimiou et al [1984] in a similar group of CF patients. They found one *mycoplasma* and one *chlamydia* seroconversion, and five respiratory viral seroconversions in 46 patients during one year, a seroconversion rate of 0.15/patient/year. Ong et al [1989] found eleven seroconversions in 36 adult patients in one year. This would be a rate of 0.3/patient/year, but must represent an overestimate as patients were initially recruited to their study only when they reported "flu-like" symptoms. The rate of seroconversion thus varies between

primarily paediatric and adult populations by around one order of magnitude.

The total absence of positive viral culture was disappointing, but in keeping with previous workers' experience. Viral isolation, like seroconversion, is dependent on age and is generally held to be more difficult in adults than in children. This is usually taken to mean that the culture methods are less sensitive in adults, but could just as easily be due to a genuinely lower incidence of respiratory viral infection. In the two large studies in CF patients which were confined to adults, Efthimiou et al isolated two adenoviruses, and Ong et al isolated one coxsackie virus, of doubtful significance. Hordvik's small study was unusual in isolating four influenza B viruses and one rhinovirus. No coronaviruses were isolated in any of the studies of cystic fibrosis patients, despite the belief that these viruses account for about 30% of all respiratory viral infections. Viral isolation was not attempted in the study of Thom et al [1990], in students with lower respiratory infections. Wang et al's failure to isolate a single virus from over one thousand samples in a young CF cohort led to the speculation that there may be inhibitors of viral replication present in the milieu of the CF oropharynx, possibly as a result of oropharyngeal staphylococcal/pseudomonal colonisation.

In this study 11 of the 12 seroconversions were associated with exacerbations, and there was only one asymptomatic seroconversion. Only five of the eleven

exacerbation-associated seroconversions were associated with symptoms suggestive of non-bacterial infection. Thus seroconversion to respiratory viruses is associated with exacerbation, but the resulting symptoms may be non-specific. One adenoviral infection resulted in an exacerbation defined only by deteriorated pulmonary function, with no accompanying symptoms.

VIII.5 Confirmed non-bacterial infection in CF patients presenting early with SNBI

Introduction

The failure to culture respiratory viruses at the time of treatment of exacerbation might have been due to late presentation for treatment following viral infection. In this case patients might have delayed seeking treatment in the hope of spontaneous improvement in their symptoms. Alternatively any symptomatic deterioration could take some days to develop after the initial infection, at which time infecting virus might not be present in sufficient numbers to allow isolation.

Methods

During the second year of the study CF patients living close to the CF unit were asked to report to the author as

soon as possible after the onset of any of the following symptoms :-

sore throat

more cough or change to darker colour of sputum

more breathlessness than usual

weight loss

runny or blocked nose

feeling feverish

feeling generally unwell

generalised muscle aches and pains

earache

sore eyes

rash

Symptoms were then evaluated by the author, and if fulfilling the criteria for SNBI (definition as in VIII.2) or a respiratory exacerbation, a throat swab was obtained as soon as possible, by the author. Paired specimens for serology were also obtained in each case. Culture and serology methods were as described above.

Results

Throat swabs were collected and sent to the laboratory within 48 hours of the onset of symptoms in sixty SNBI in 39 patients. Despite early collection there were no positive cultures. There were two seroconversions, one to adenovirus and one to CMV.

Discussion

Ensuring that throat swabs were collected early made no difference to the absence of positive cultures.

VIII.6 Frequency of SNBI and confirmed non-bacterial infection in CF compared with other chronic respiratory disease - community based study

Introduction

In view of the continued absence of positive viral culture despite early sample collection, it remained possible that viral isolation might be specifically inhibited in patients with CF, or that such patients might have an exceptionally low incidence of upper airway viral infection. To determine whether viral isolation and seroconversion differed between patients with CF and patients with other chronic respiratory disease, a one year study was undertaken to document the incidence of SNBI and confirmed non-bacterial infection. To determine whether there was a difference specific to CF, a group of patients with non-CF bronchiectasis was included. To determine whether inhibition was due to the bronchiectatic state (and possibly a contaminated oropharynx) a group of patients with different pathophysiology was required. In order to age match this group with the CF patients a group of young asthmatics was

selected. Since chronic oral steroid consumption might have influenced susceptibility to infection, steroid-dependent asthmatics were excluded.

Patients

All patients in the CF cohort were invited to take part in the prospective study. Twenty-five agreed to take part, but two subsequently dropped out. Patients with asthma and non-CF bronchiectasis were selected from the records of the Leeds Chest Clinic. Patients with asthma were excluded if over 40 years of age, to roughly age match them with the CF patients. Steroid-dependent patients were excluded. As expected, it was not possible to age-match the non-CF bronchiectatic patients, but patients over the age of 60 were excluded. Sweat tests and genotyping were not performed in these patients prior to commencement of the study, but no patients had extrathoracic symptoms or a distribution of thoracic disease suggestive of CF. In all three groups household contacts served as controls, after ensuring the absence of respiratory disease. It was intended to recruit 20 to 25 households in each category, but recruitment difficulties and early drop-outs were encountered in the non-CF bronchiectasis and asthma groups. The details of the participating households are summarised in table 27. Most CF household contacts were parents, while in the other two groups most contacts were partners. Three non-CF bronchiectasis patients lived alone.

Mean (SD) baseline percent predicted FEV1 was 58.1 (25.8), and percent predicted FVC 88.1 (25.9) in the CF patients and 66.7 (22.8) and 86.9 (22.7) respectively in the non-CF bronchiectasis patients. All the asthmatic patients had predicted spirometric values of 100% or greater.

Methods

Baseline blood for serology was obtained from all study participants. All participants were then asked to contact the author in the event of symptoms suggestive of non-bacterial infection, and in the case of the various patient groups also in the event of an exacerbation of their underlying respiratory disease, as detailed below. Patients and household contacts were also contacted at two weekly intervals by telephone and asked specifically about the relevant symptoms.

Table 27 - Characteristics of household groups

	number	age at entry
CF patients	23	20.5 (2.5)
CF household contacts	39	41.3 (8.6)
Non-CF bronchiectasis patients	14	50.6 (8.1)
Non-CF bronchiectasis household contacts	11	47.0 (16.1)
Asthma patients	17	28.8 (8.9)
Asthma household contacts	17	36.1 (10.2)

Controls were asked to contact the author if they developed any of the following symptoms :-

sore throat
new onset of cough
runny or blocked nose
feeling feverish
feeling generally unwell
generalised muscle aches and pains
earache
sore eyes
rash

Patients with asthma were asked to contact the author in the event of any of the above symptoms, or in the event of breathlessness associated with a drop in morning PEFR of at least 10% from baseline, which was considered to be an exacerbation of asthma. Baseline PEFR was established from a two week run-in in each asthmatic patient, and was taken to be the best recorded morning value.

Patients with CF and bronchiectasis were asked to contact the author in the event of symptoms as described in the previous section.

Symptoms were then evaluated by the author, and if fulfilling the criteria for SNBI (definition as above) or a respiratory exacerbation (symptom criteria for respiratory exacerbation in the non-CF bronchiectatic patients were identical to those for CF patients) a throat swab was

obtained as soon as possible from the index case by the author. In addition blood was taken for serology from all the household contacts at this stage. If the other household contacts subsequently developed positive symptom criteria within 10 days of the index case throat swabs were obtained as soon as possible after the development of symptoms. Finally, convalescent serum was obtained 14 days after the onset of symptoms in the last affected member of the household.

If positive symptom criteria did not develop within 10 days of the onset of symptoms in the index case, a throat swab and convalescent serum were obtained on the 14th day following the onset of symptoms in the index case ie the household contacts were considered not to have developed symptomatic infections as a result of exposure to the index case, but were screened for asymptomatic infection.

If any of the households were free of symptoms and exacerbations for six months, blood for serology was collected at that time, to screen for asymptomatic seroconversions. A final blood sample was obtained from all participants at the end of the twelve month period. Viral isolation and serology methods were as described for the study of exacerbations in CF. All participants gave written informed consent.

Results

Symptom episodes and SNBI for each group are shown in table 28. The excess of symptoms over SNBI in the patient group represent symptomatic exacerbations of the underlying disease, without meeting the criteria for SNBI. For the household contacts with no underlying respiratory disease these values are of course equivalent. The excess of SNBI/person in the patients compared to the household contacts was statistically significant (standardised normal deviate $p < 0.0001$) in all three groups.

Throat swabs were obtained in 113 of the total of 127 symptomatic episodes. The missing data arose through late reporting of symptoms. Again, there were no positive cultures. There were ten seroconversions, details of which are shown in table 29.

Table 28 - Symptom episodes, absence of symptoms, SNBI, and rate of SNBI per person, by group

	symptoms	no symptoms	SNBI	SNBI/ person
CF patients	41	7	31	1.35
CF household contacts	22	67	22	0.56
non-CF bronchiectasis patients	21	1	15	1.07
non-CF bronchiectasis household contacts	8	17	8	0.73
Asthma patients	24	4	20	1.18
Asthma household contacts	11	21	11	0.65
Total	127	117	107	

"no symptoms" = absence of symptoms despite presence of symptoms in other household member.

Table 29 - Characteristics of seroconversions

organism	month	category	exacerbation	SNBI
adenovirus[1]	November	CF patient		
chlamydia[2]	February - June	non-CF bronchiectasis contact	NA	no
influenza A	January	CF contact	NA	yes
influenza A	January	CF contact	NA	yes
influenza A	January	CF contact	NA	yes
influenza A	January	non-CF bronchiectasis patient	yes	yes
influenza A	March	non-CF bronchiectasis patient	yes	yes
influenza A	December	Asthma contact	NA	yes
RSV	January	CF patient	yes	yes
RSV	November	Asthma patient	yes	yes

NA = not applicable

1 - this represents the same patient, and asymptomatic seroconversion, in the table relating to CF exacerbations (table 26)

2 - asymptomatic seroconversion

Discussion

In all three groups, patients were much more likely to report symptoms than contacts. This is unlikely to represent reporting bias, since, in the absence of spontaneous reporting, all households were contacted at two week intervals by the investigator. Reporting behaviour was remarkably consistent across all three groups. Twenty to thirty percent of reported episodes in the patients were for exacerbations of the underlying disease without symptoms of SNBI. Conversely, seventy to eighty percent of reported episodes were for symptoms of SNBI. Ten seroconversions were observed, equally divided between patients and household contacts. There were two asymptomatic seroconversions, one occurring between February and June. All the other seroconversions occurred during the winter months. The overall rate of seroconversion, 0.083/individual/year, was precisely the same as that seen in the exacerbations of CF. The absence of positive viral culture in all patients and household contacts in the presence of seroconversions is likely to mean that the method is insensitive in all adults, not just those with CF or non-CF bronchiectasis.

The mean rate of reported SNBI among all the household contacts was 0.6/contact during the year, and among all the patients was 1.2/patient. If all these SNBI represent real infection, then the rate is roughly doubled in patients with underlying respiratory disease compared to household contacts. More likely, the true rate of infection is equal

between patients and household contacts, the excess symptom episodes in the patients being due to their underlying disease. It is not possible to infer anything from the observed seroconversions, as these are likely to represent 14% of infections, if the true infection rate is 0.6/patient/year. Serology itself may be a relatively insensitive method of detection.

If the true infection rate is indeed 0.6/patient/year, irrespective of the presence or absence of respiratory disease, then in the two year cohort study of exacerbations of CF the true number of infections would be $0.6 \times 60 \times 2 = 72$. Assuming that all co-infections with respiratory viruses or atypical bacteria result in exacerbations, the proportion of exacerbations with associated co-infection would then be $72/252 \times 100\% = 28.6\%$.

VIII.7 Compliance with influenza vaccination

Introduction

Yearly vaccination against influenza is recommended for all patients with chronic respiratory disease in the UK [Calman 1993]. Knowledge of the clinical effects of influenza infection in patients with CF is confined to the case reports of Conway et al [1992], who documented clinical deterioration in three patients with CF following infection with influenza. In both years of this study all patients were encouraged to

seek vaccination against influenza from their general practitioners.

Methods

An initial letter asking patients to obtain influenza vaccination from their general practitioners was sent out in September of each year, and this was followed up with informal reminders when patients were seen at the CF centre. When a rise in titre against influenza A or B was demonstrated, this was taken as evidence of vaccination, or as evidence of infection, in the absence of a history of vaccination.

Ong et al demonstrated the efficacy of vaccination in a prospective study of patients with CF, observing a significant antibody response in 80% of vaccinated patients [Ong 1991]. Since 20% of CF patients would not be expected to develop a significant antibody response, the rate of seroconversion was adjusted by a factor of 1.25 to derive the estimated rate of compliance. Analysis was confined to the cohort of 60 patients who completed the two year study.

Results

Eleven seroconversions attributable to vaccination were observed in the first year of the study and seven in the second year. Estimated compliance with vaccination was therefore 23% in the first year and 15% in the second year.

Discussion

Rates of uptake of immunisation against influenza of 10-40% have been reported in high risk patients, including the elderly and those with cardiac disease [Nicholson 1987; Kurinczuk 1989; Lennox 1990; Nguyen-Van-Tam 1993]. The rate of immunisation in this study was therefore in the lower end of this range. A particular problem is the perception of intercurrent illness as a relative contraindication to vaccination. Such illness, in the form of worsening respiratory symptoms, is very common in adult patients with CF. Patients may then be reluctant to make an extra visit to their local practitioner in the intervening period of good health. The perception that influenza vaccination can precipitate flu-like illness is a further factor limiting uptake of vaccine, although vaccination does not lead to pulmonary exacerbations [Ong 1991]. The lack of protection against respiratory viruses other than influenza A and B is a further drawback in encouraging uptake. In view of these particular problems, compliance with influenza vaccination should not be taken as a general guide to compliance with other modalities of treatment.

In this adult CF population, an immunisation programme against influenza delivered in the community was ineffective. A programme delivered within the CF centre is likely to be more successful. For the purposes of the virological surveillance study, most of the patients with CF were unprotected against infection with influenza.

Chapter IX PCR Methodology

IX.1 Introduction

The aim of the PCR work was to develop a system which would identify respiratory viral nucleic acid in respiratory secretions. This is either a three (DNA viruses) or four (RNA viruses) stage procedure:-

1. Nucleic acid must be extracted from the clinical sample.
2. Reverse transcription of RNA to cDNA (RNA viruses only)
3. Polymerase chain reaction
4. Identification of polymerase chain reaction products

A variety of nucleic acid extraction techniques have been employed. Studies which have successfully identified viruses in respiratory secretions have hitherto used organic acid extraction techniques [Johnston 1993; Nicholson 1993; Smyth 1995].

RNA viruses require reverse transcription to DNA before proceeding to the PCR stage. This is achieved by incubating extracted nucleic acid with reverse transcriptase, free nucleic acid bases and specific oligonucleotides, which may be the same as those used in the PCR reaction.

The PCR reaction itself is a cycle of 3 separate reactions repeated, usually, 30 to 40 times. In the first, denaturation, reaction, double stranded DNA is heated to just

below the boiling point of water. This causes reversible breakdown of the hydrogen bonds which hold the two single strands together, yielding two single strands. In the subsequent annealing reaction a pair of specific oligonucleotides hybridise with base sequences flanking the DNA region of interest. The oligonucleotides are added in great excess to ensure that the original single strands of DNA do not simply re-anneal with each other. The polymerisation reaction then follows, in which synthesis of the DNA fragment complementary to the region of interest is completed by in-filling of bases (deoxynucleoside triphosphates, dNTPs, added to the reaction mixture) between the annealed oligonucleotide "primers", a process catalysed by the heat-stable DNA polymerase isolated form *Thermus aquaticus*, known as "Taq". Polymerisation depends on the presence of magnesium ions, the concentration of which critically affects the yield. All 3 reactions require different temperatures and the cycle is therefore carried out using a thermal cycler. Since a new fragment of DNA is synthesised each cycle from each pre-existing fragment, the amount of DNA should theoretically double during each cycle. In practice, towards the end of the cycle the yield declines dramatically, partly through progressive decline in the concentration of primers and dNTPs, and through progressive loss of Taq activity.

Following completion of the PCR reaction, the final stage in diagnosis is to identify the fragment that has been polymerised. This can be done by sequencing the fragment,

hybridising it with labelled oligonucleotides complementary to sequences internal to the initial "priming" oligonucleotides, or more simply (but less specifically) by measuring the length of the fragment after running a gel electrophoresis.

Since PCR can theoretically detect a single copy of the organism sought, the method offers potential advantages in the context of organisms difficult to isolate by conventional culture or antigen detection. PCR methods could be particularly useful in the context of adult patients with CF as viral isolation is generally considered to be difficult in the adult age group [Johnston 1993; Nicholson 1993], and particularly so in patients with cystic fibrosis [Wang 1984].

There are two major problems with PCR-based diagnostic systems in diagnosing infection. The first is the potential for false positive results, either from cross contamination from other specimens or from positive controls. This is a particular problem in PCR because of the positive feedback nature of the system - a minute amount of contaminant will result in false positive results. It is possible to overcome this problem with rigorous attention to detail in the laboratory.

Secondly, the isolation of viral nucleic acid may not necessarily indicate infection. It is known from volunteer studies that not all subjects inoculated with respiratory viruses develop clinical symptoms. Patients without symptoms in this situation have then usually been excluded from the subsequent protocol on the grounds that they have not become

infected. Nevertheless it would be expected that appropriate respiratory swabs from such "uninfected" subjects would be PCR positive. In some individuals it is possible to isolate adenovirus for several weeks following the cessation of symptoms [Fox 1977]. Following respiratory viral infection it is not known for how long respiratory secretions remain PCR positive.

There are no published PCR methods for the simultaneous identification of more than one respiratory virus, but where there is known base pair sequence homology, it is possible to identify many serotypes from the same species, most notably with rhinovirus [Gama 1989]. A comprehensive "respiratory viral" PCR-based system requires the simultaneous identification of several different viral species. In order to identify the most common respiratory tract viral pathogens, a minimum of six PCR reactions is required : adenovirus DNA, and influenza, RSV, rhinovirus, parainfluenza and coronavirus RNA, must be identified from respiratory secretions. Of these viruses only adenovirus has a DNA-based genome: therefore most respiratory viruses require a reverse transcription step before the PCR reaction. A third general problem with PCR identification is its labour intensity, even when looking for a single organism. In the context of looking for six organisms, reducing specimen manipulation to a minimum was an important consideration in the development of the methodology.

Although the basic principles of the polymerase chain reaction are well understood, the precise chemistry is not.

The exact composition of the oligonucleotides fundamentally affects the dynamics of the reaction sequence. Consequently the conditions for each PCR reaction that utilises different oligonucleotides have to be individually optimised. Again, this is a particular problem in the context of developing a system capable of diagnosing several different organisms, which necessarily requires the use of different oligonucleotides. Optimisation is very much a trial and error process, and failure to successfully amplify a desired fragment could be due to a problem with any of the stages outlined above, or to defective "known positive" specimens.

IX.2 Oligonucleotides

IX.2.i Rhinovirus

Gama et al first described successful PCR amplification of rhinoviruses [Gama 1989]. The 5' noncoding regions of rhinoviruses and enteroviruses are highly conserved and include some blocks of almost total homology. One of these is located at position 547-562 [Gama 1989]. Oligonucleotide OL27 is complementary to this sequence and can in principle be used to prime reverse transcription of any rhinovirus or enterovirus to give cDNA stretching to the 5' end of the genome. This cDNA can be copied using the universal primer OL26. This primer pair gave positive results when tested on human volunteers infected with HRV2, HRV9 and HRV14. Culture grown HRV3 and HRV85 and several coxsackie A viruses also

gave positive results [Gama 1989]. Further evaluation of this primer pair and two others by Johnston et al showed OL26/OL27 to be the most sensitive in detecting clinical rhinoviral infection. Consequently this primer pair was selected for the present study. It has the particular advantage that the more easily handled coxsackie viruses can be used as positive controls rather than rhinoviruses. The oligonucleotides have the following sequences (5' - 3').

OL26	GCA CTT CTG TTT CCC C
OL27	CGG ACA CCC AAA GTA G

The OL26 - OL27 fragment is approximately 380 nucleotides in length.

IX.2.ii Influenza

The influenza genus is divided into three types A,B and C, based on specific epitopes on the internal nucleoprotein and the matrix protein. Human influenza A virus strains are further classified into subtypes H1N1, H3N2 and H2N2, based on the antigenic differences of the haemagglutinin (H) and neuraminidase (N) surface proteins. In selecting influenza primers, Claas et al reviewed the known sequence data of homologous segments of influenza A and B viral genomes and concluded that genus-specific oligonucleotides could not be selected [Claas 1992]. Highly conserved sequences were apparent for influenza A (segment 8), influenza B (segment

8), and influenza C (segment 7), and primers were selected for these regions.

Twenty two influenza A H1/N1, 9 influenza A H2N2 and 30 influenza H3N2 cell-cultured strains all gave fragments visible on gel electrophoresis. All 11 influenza B and all 3 influenza C strains tested also gave visible fragments. Specificity was confirmed with negative results using primers with opposite viral types (ie A primers with influenza B etc) and with other respiratory tract viruses and bacteria.

As genus specific primers were unavailable, it was decided at the outset not to include influenza C primers in the present study, since influenza C is rarely isolated in clinical specimens. The oligonucleotides described for influenza A and B were selected, and have the following sequences (5' - 3').

A1 (cDNA) AAG GGC TTT CAC CGA AGA G
A2 CCC ATT CTC ATT ACT GCT TC

B1 (cDNA) ATG GCC ATC GGA TCC TCA AC
B2 TGT CAG CTA TTA TGG AGC TG

The A1 - A2 fragment is 190 base pairs in length.
The B1 - B2 fragment is 241 base pairs in length.

NB The appellations "A1,A2,B1,B2" have been adopted for the convenience of the current study. These primers were not named in the original study.

IX.2.iii Parainfluenza

No oligonucleotides have previously been described for these viruses. Examination of known sequence data revealed areas of homology between serotypes 1 and 3, and between serotypes 2 and 4, but not for the whole genus. Consequently two sets of primers were designed, both from the NP gene region. The oligonucleotides have the following sequences (5' - 3').

PI 1/3-1 GGC CAG CTG CAG GAT AGT TGC CTG

PI 1/3-2 GGC CAG CTG CAG GCG TTC AGA CAA G

P1 2/4-1 GAT TCG AAT TCC AAC/T NCC CAT GGC

P1 2/4-2 GAT TCG AAT TCT ATG CT/CA TGG TGG

The expected PI 1/3-1 - PI 1/3-2 fragment length was calculated to be 520 bp.

The expected PI 2/4-1 - PI 2/4-2 fragment length was calculated to be 350 bp.

IX.2.iv Coronavirus

No oligonucleotides had been described for these viruses at the time of the study, and there is little information available on the sequence of the coronavirus genome. Primers were selected on a best guess basis from the small amount of sequence data available, from the NP gene region. The oligonucleotides have the following sequences (5' - 3').

Cor1 TCG AAT TCT AGA ATT CCA GAA CCA G

Cor2 GAT TCG AAT TCT AGT AGG TGA AAG

The expected Cor 1 - Cor 2 fragment length was calculated to be 350 bp.

IX.2.v Respiratory Syncytial Virus

The gene coding for the major nucleocapsid protein of RSV was sequenced in 1985 [Collins]. Cane and Pringle subsequently described oligonucleotides N1 and N2, which amplify a fragment of the N gene between nucleotides 858-1135 [Cane 1992]. The oligonucleotides have the following sequences (5' - 3').

N1 GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC

N2 CTT CTG CTG TCA AGT CTA GTA CAC TGT AGT

The N1 - N2 fragment is 277 bp in length.

IX.2.vi Adenovirus

No oligonucleotides have previously been described for these viruses in the published literature, but oligonucleotides have been used successfully at the University of Warwick for PCR of culture grown Adenovirus serotypes 2 and 5. These primers have not previously been used directly on clinical samples. The oligonucleotides have the following sequences (5' - 3').

Ad1 CGC TTT GCT ATA TGA GGA CCT GTG G

Ad2 CTG GTT AAG CAA GTC CTC GAT AC

The Ad1 - Ad2 fragment is 424 bp in length.

Table 30 - Summary of Oligonucleotides

	Oligonucleotide names	Expected fragment size/bp	Melting temperature (T_m/°C)*
Rhinovirus	OL26 OL27	380	50 50
Influenza A	A1 A2	190	62 58
Influenza B	B1 B2	241	62 58
Parainfluenza 1 & 3	PI 1/3-1 PI 1/3-2	520	68 70
Parainfluenza 2 & 4	PI 2/4-1 PI 2/4-2	350	63 60
Coronavirus	Cor1 Cor2	68	60 58
RSV	N1 N2	277	63 65
Adenovirus	Ad1 Ad2	424	76 68

* Melting temperature is derived from the formula :-

$$T_m = 69.3 + 0.41.(G + C)\% - 650/L$$

where (G + C)% is the content of G and C bases as a percentage of total bases, and L is the length of the oligonucleotide in nucleotides

For brevity, primer pairs will be referred to in subsequent text as follows:-

Rhinovirus	OL
Influenza A	A
Influenza B	B
Parainfluenza 1/3	PI 1/3
parainfluenza 2/4	PI 2/4
Coronavirus	Cor
RSV	N
Adenovirus	Ad

IX.3 Development of PCR technique - basic methodology

The details of the RT-PCR procedure were worked out on the basis of initial results with known positive sources of viral RNA and DNA. The techniques used in development are therefore given in outline here. A detailed description of the final procedure is given at the end of this chapter.

Two specific problems with PCR techniques required general precautions. The potential for contamination with environmental DNAase and RNAase was minimised by the use of molecular biology grade reagents, separately aliquoted for each batch of samples. Contamination with source RNA, DNA and cDNA was avoided by the use of separate pipettes for extraction, reverse transcription, polymerase chain reaction, and gel loading. Extraction, preparation of reverse

transcription reactions and polymerase chain reactions, and the polymerase chain reaction itself, were performed in three separate rooms. Gels were run in a room away from the thermal cycler.

IX.3.i Nucleic Acid Extraction

Of the many extraction techniques described only organic acid extraction followed by ethanol precipitation has been successfully applied to respiratory viral diagnosis in published work [Gama 1989; Ireland 1993; Johnston 1993]. This method was therefore adapted for the current study. Samples were first subjected to proteinase K digestion to break down cellular and viral protein structures, leaving free nucleic acid. Carrier tRNA derived from bakers' yeast was included in this step to prevent endogenous RNAase from digesting the RNA of interest. The principle is to add an excess of RNA to act effectively as a competitive inhibitor of RNAase.

For the proteinase K digest 225 mcl of cell culture grown virus, cell culture (as negative control) or clinical samples (in throat swab transport medium) were transferred immediately on thawing to 2.5 ml reaction tubes containing:-

25 mcl (1 mcg/mcl) proteinase K
1 mcl (1 mcg/mcl) carrier tRNA
250 mcl 2× proteinase K buffer

The reaction tubes were heated in a water bath at 37°C for 30 minutes. Samples were then extracted once with an equal volume (500 mcl) of phenol/chloroform.

For nucleic acid precipitation 400 mcl of the aqueous phase (containing nucleic acid) was added to 1.5 ml reaction tubes containing:-

33 mcl 3M sodium acetate (final concentration 0.25M)
2.5 vols (1 ml) 100% ethanol

The reaction tubes were then left at -70°C for thirty minutes. The reaction tubes were then centrifuged for five minutes and the ethanol discarded. The resultant pellet of nucleic acid was washed once with 70% ethanol to remove any residual solutes and air dried. The final pellet was resuspended in water and transferred to the reverse transcription reaction or directly to the PCR reaction (adenovirus, and adenoviral and RSV cDNA positive controls).

IX.3.ii Reverse Transcription

Initial failure to successfully PCR any RNA viruses was found to be due to defective "superscript" reverse transcriptase, after the successful PCR of adenoviral cDNA confirmed the integrity of the PCR reaction (vide infra). Substitution with M-MLV reverse transcriptase resulted in successful PCR of coxsackie A and B, influenza A and B, and

RSV from positive controls. M-MLV reverse transcriptase was then used throughout the remaining PCR work.

Reverse transcription was carried out in 1.5 mcl reaction tubes containing:-

M-MLV reverse transcriptase	1 mcl
RNasin	1 mcl
dNTP	1 mcl
DTT	2 mcl
M-MLV RT reaction buffer	10 mcl
primers	1 mcl
water	4 mcl
(total volume	50 mcl)

Even the purest commercially available reverse transcriptase can be contaminated with RNase. RNasin, a potent RNase inhibitor derived from human placenta, was therefore included in the reaction mixture. The reaction tubes were heated at 37°C for 1 hr to yield cDNA in a volume of 50 mcl.

IX.3.iii Polymerisation Chain Reaction

This is the most critical step to optimise. The final yield of each primer pair is dependent on the concentration of Taq, the concentration of magnesium, the concentration of primers, and the temperatures of the reaction cycle. The most

critical temperature is that of the annealing reaction - optimum annealing of any given primer usually occurs at a temperature 5°C less than its melting temperature.

A derivative of Taq polymerase, "Supertaq", was obtained locally and used throughout the PCR work.

PCR optimisation experiments were performed with a total volume of 50 mcl. 1.5 mcl reaction tubes contained the following:-

10 mcl cDNA (but only 1 mcl adenoviral cDNA)
5 mcl supertaq buffer
0.4 mcl dNTP
2 × 1 mcl oligonucleotide primers
variable volumes supertaq
variable volumes added magnesium
the balance to 50 mcl made up of H_2O (usually c 30 mcl)

Reaction tubes were placed in a thermal cycler and subjected to 30 cycles of

92°C 1 minute	[separation]
variable temperatures 2 minutes	[annealing]
72°C 4 minutes	[extension]

followed by a final 15 minute extension at 72°C.

IX.3.iv PCR product identification

Because of the multiplicity of primer pairs used, and hence the multiplicity of fragments potentially generated per specimen, identification by any means other than by fragment size on gel electrophoresis would have been impractical.

Ten mcl of each of the PCR reaction products were combined with 2 mcl of loading buffer and loaded onto a single well of an ethidium-bromide stained 2% agarose gel. The gel was placed in a TAE bath and a voltage of 100 V was applied for 1 hour. Ethidium is taken up by the DNA molecules as they migrate through the gel, and fluoresces under ultraviolet illumination. The gel was inspected visually under ultraviolet illumination and a polaroid photograph obtained. Polaroid photography is slightly more sensitive in identifying very faint bands of DNA than the naked eye.

The size of the fragments obtained was determined by comparison with a commercially available DNA "ladder" ie a solution of DNA fragments of known size, which is loaded into the "marker" well of the gel, and run in the "marker" lane.

IX.4 Reaction optimisation

Initially reaction conditions were optimised for each individual oligonucleotide pair, using appropriate positive control specimens as follows :-

Oligonucleotide pair	Control(s)
OL26 - OL26	culture grown coxsackie A9 (clinical isolate)
	culture grown coxsackie B4 (clinical isolate)
	culture grown coxsackie B6 (clinical isolate)
A1 - A2	culture grown influenza A (clinical isolate)
B1 - B2	culture grown influenza B (clinical isolate)
PI 1/3-1 - PI 1/3-2	culture grown parainfluenza 1
	culture grown parainfluenza 3
	(both clinical isolates)
PI 2/4-1 - PI 2/4-2	culture grown parainfluenza 2
	culture grown parainfluenza 4
	(both clinical isolates)
Cor1 - Cor2	culture grown coronavirus 229E
	(clinical isolate)
N1 - N2	culture grown RSV (clinical isolate)
	RSV cDNA (University of Warwick)
Ad1 - Ad2	Adenovirus cDNA (University of Warwick)

HEp2, primary monkey kidney (abbreviated to PMK), and human embryonic fibroblast (abbreviated to HEF) cells, served as negative controls throughout all PCR reactions.

Supertaq is far more active than conventional Taq, but paradoxically seems to inhibit the reaction if present in too high a concentration (personal communication, RJ Eglin). Initial failure to PCR adenoviral cDNA was due to this problem. Ultralow concentrations of Supertaq proved to be necessary, as shown in table 31. To ensure the accuracy of the volumes of supertaq a 2 mcl pipette was used.

After establishing the correct concentration of Supertaq for the PCR reaction the positive control samples were run with varying magnesium concentrations, as shown in table 32.

Table 31 - Ultralow Supertaq, Variable [Mg^{2+}], adenoviral cDNA, Ad1 - Ad2, annealed at 55^0C

Lane	Supertaq/mcl	Mg^{2+}/mmol	Band
1	0.1	3	-
2	0.1	6.5	-
3	0.1	11.5	+
4	0.1	16.5	+
5	0.05	3	-
6	0.05	6.5	-
7	0.05	11.5	+
8	0.05	16.5	+
9	0.01	3	-
10	0.01	6.5	+
11	0.01	11.5	+
12	0.01	16.5	-

Figure 29 - Ultralow Supertaq, Variable [Mg^{2+}], adenoviral cDNA, Ad1 - Ad2, annealed at $55^{0}C$, lanes 1-6

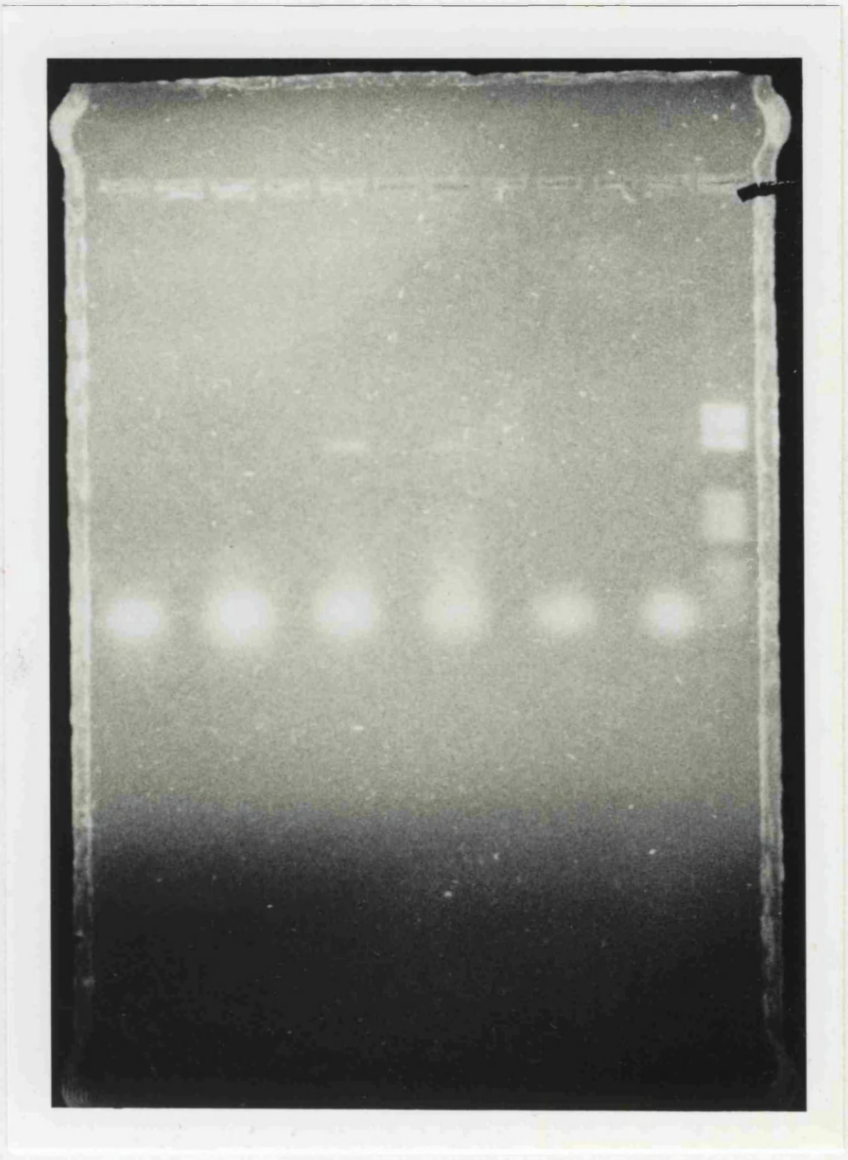


Figure 30 - Ultralow Supertaq, Variable [Mg^{2+}], adenoviral cDNA, Ad1 - Ad2, annealed at $55^{0}C$, lanes 7-12

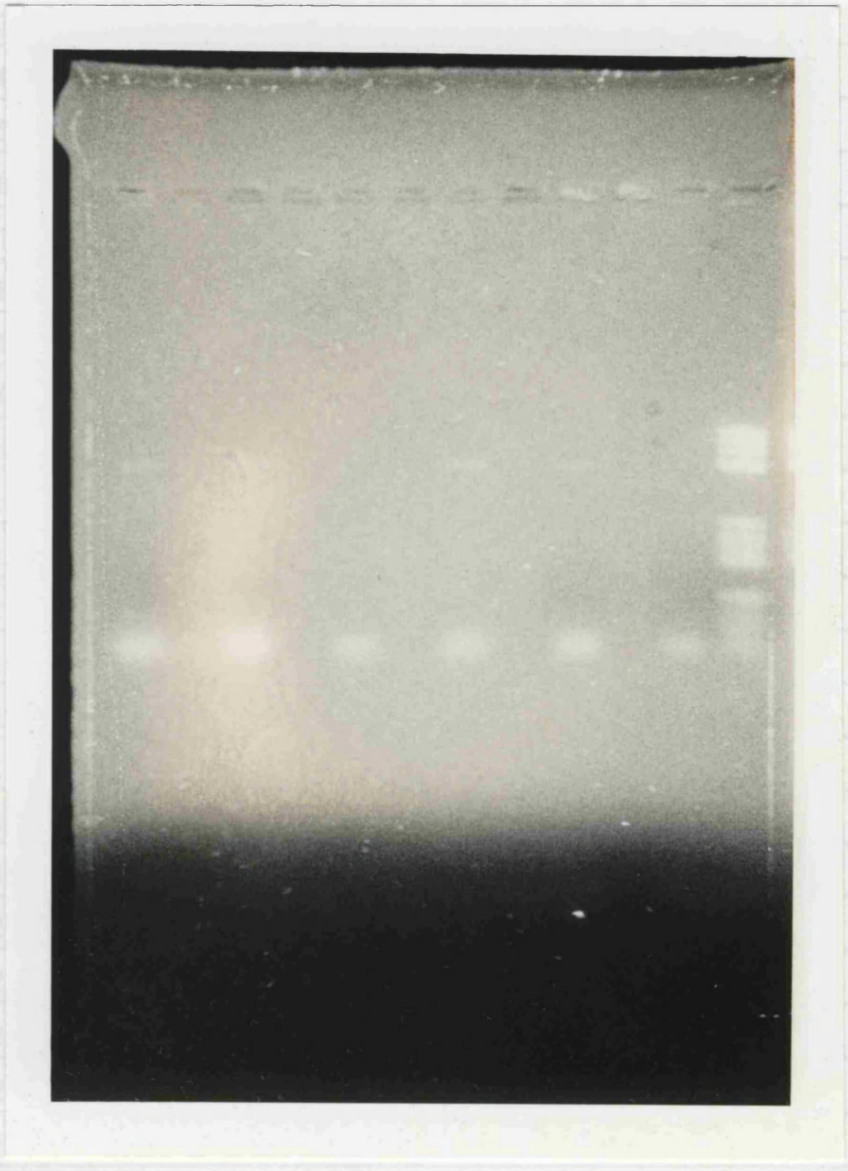


Table 32 - Positive control extractions, reverse transcription, and PCR with variable [Mg^{2+}], 0.05 mcl Supertaq/reaction

Lane	Control	[Mg^{2+}]/mmol	Band
1	A9	1.5	+++
2	A9	6.5	-
3	A9	11.5	++
4	B4	1.5	+++
5	B4	6.5	++
6	B4	11.5	+
7	B6	1.5	+++
8	B6	6.5	++
9	B6	11.5	+
10[1]	HEp2 + OL	1.5	-
11[1]	HEp2 + OL	6.5	-
12	Cor	1.5	+++
13	Cor	6.5	++
14	Cor	11.5	+
15	PI 1	1.5	-
16	PI 1	6.5	-
17	PI 1	11.5	-
18	PI 2	1.5	-
19	PI 2	6.5	-
20	PI 2	11.5	-
21	PI 3	1.5	-
22	PI 3	6.5	-
23	PI 3	11.5	-
24	PI 4	1.5	-
25	PI 4	6.5	-
26	PI 4	11.5	-
27	RSV	1.5	++
28	RSV	6.5	++

29	RSV	11.5	++
30	RSV cDNA	1.5	++
31	RSV cDNA	6.5	++
32	RSV cDNA	11.5	++
33[2]	Ad cDNA	1.5	++
34	Flu A	1.5	+
35	Flu A	6.5	-
36	Flu A	11.5	-
37	Flu A'	1.5	+++
38	Flu A'	6.5	++
39	Flu A'	11.5	+
40	Flu B	1.5	++
41	Flu B	6.5	-
42	Flu B	11.5	-
43[1]	PMK + OL	1.5	-
44[1]	PMK + OL	6.5	-
45[1]	HEp2 + OL	11.5	-
46[1]	HEp2 + OL	11.5	-

1 Negative controls

2 Run to check integrity of PCR step

A9, B4, B6, FluA, Flu A', and negative controls were run in one batch in the thermal cycler with an annealing temperature of 50^{0}C. (Terms FluA and FluA' denote two separate RNA sources.)

PI 1-4, RSV and RSV cDNA were run in a separate batch with an annealing temperature of 55^{0}C, as primers for these viruses were generally longer, with higher Tm (vide supra).

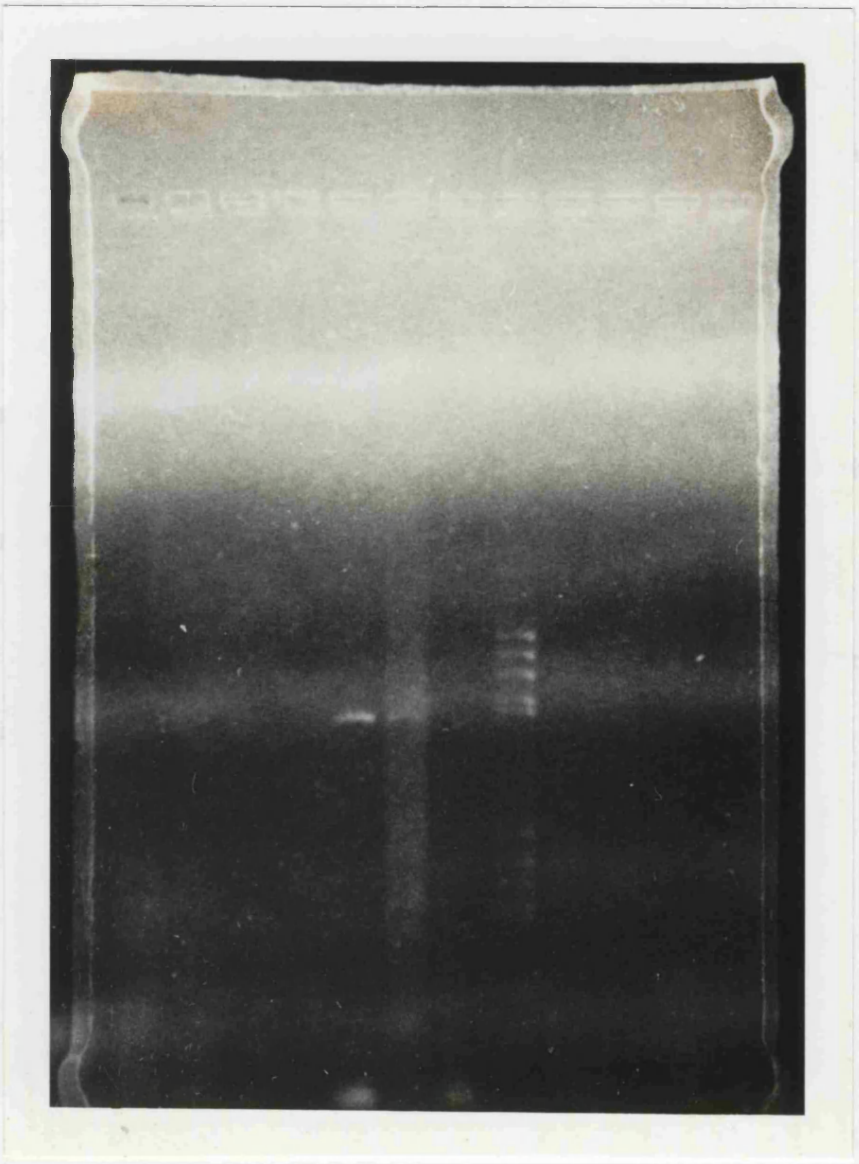
Hitherto, PCR was successful for positive control specimens with the exception of parainfluenza. The strongest bands were obtained with a magnesium concentration of 1.5 mmol. This was the concentration obtained using the Supertaq buffer supplied with the enzyme (15 mmol in 10× strength), with no extra magnesium added to the reaction.

The failure to PCR parainfluenza viruses required investigation. The Tm of PI 1/3-1,PI 1/3-2, Ad1 and Ad2 was noted to be particularly high, and so the protocol from the experiment shown in table 32 was repeated with positive control PI1, PI3 and adenovirus cDNA, and is shown in table 33.

Table 33 - RT-PCR for PI1, PI3, PCR for adenovirus cDNA, with varying [Mg^{2+}], annealing temperature 63^0C

Lane	Control	[Mg^{2+}]/mmol	Band
1	PI 1	1.5	-
2	PI 1	6.5	-
3	PI 1	11.5	-
5	Ad cDNA	1.5	++
6	Ad cDNA	6.5	+
7	Ad cDNA	11.5	-
9	PI 3	1.5	-
10	PI 3	6.5	-
11	PI 3	11.5	-

Figure 31 - RT-PCR for PI1, PI3, PCR for adenovirus cDNA, with varying [Mg^{2+}], annealing temperature $63^{0}C$



Raising the annealing temperature thus made no difference.

Several outstanding issues remained:-

1. The size of the coronavirus band was unexpected, but explicable on the basis of the lack of available sequence data. It was important to demonstrate that this band was generated reproducibly.

2. How important was the high Ad Tm, and hence the Ad annealing temperature ? Could the individual primer pairs be combined together and run at the same temperatures in the thermal cycler ?

3. Were the PI primer pairs not going to work, or was the source RNA defective ?

4. Were the primer pairs going to be compatible when run together as a multiplex PCR ?

To answer these questions several further experiments were run, with results shown in tables 34 - 39.

Table 34 - Positive controls, annealing temperature 55^{0}C, [Mg^{2+}] 1.5 mmol

Lane	Control	Primer pair(s)	Band
1	Ad cDNA	Ad	+
2	Cor cDNA	Cor	+
3	RSV cDNA	N	+
4	Flu A	A	++
5	Flu A'	A	++
6	Flu B	B	++
7	A9	OL	++
8	B4	OL	++
9	B6	OL	++
10	H_2O	OL + Cor + N	-
11	H_2O	PI 1/3 + Ad	-
12	H_2O	PI 1/3 + PI 2/4 + Ad	-

Coronavirus cDNA was used as the original coronavirus source RNA was scarce.

Conclusions

1. Adenovirus cDNA + Ad gave consistently stronger bands at annealing temperatures above 55^0C.

2. Primer pairs OL, N, Cor, A and B were all effective at an annealing temperature of 55^0C, and could therefore potentially be combined in a multiplex PCR reaction.

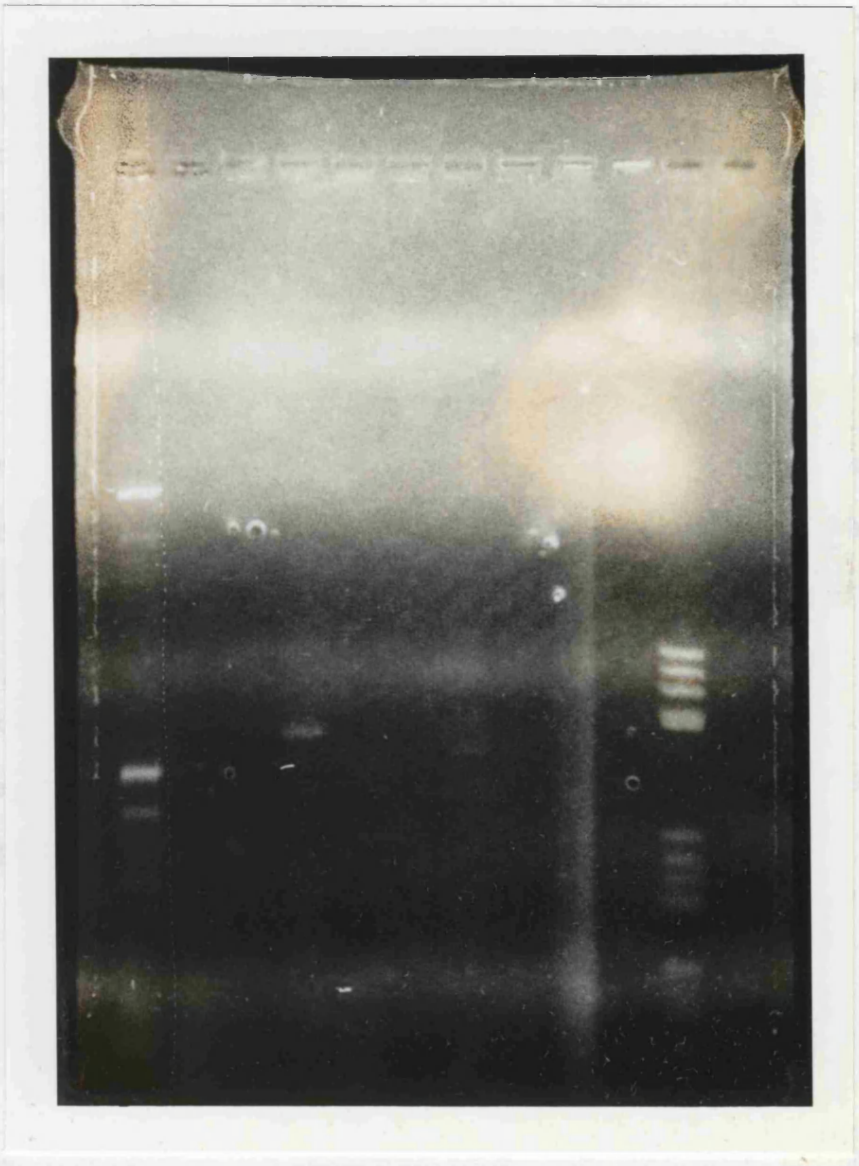
3. The addition together of 6 primers (3 pairs) in the same PCR reaction did not result in spontaneous priming.

Because of doubt about the integrity of the parainfluenza source RNA, freeze dried virus of each of the four serotypes was obtained from the central PHLS at Colindale. These were extracted and put through a PCR reaction with annealing at 55^0C. Adenovirus cDNA was put through a PCR reaction in a separate run with annealing at 63^0C.

Table 35 - Colindale PI 1-4 positive controls, annealing temperature 55^0C, adenovirus cDNA, annealing temperature 63^0C, $[Mg^{2+}]$ 1.5 mmol

Lane	Control	Primer pair	Band
1	PI 1	PI 1/3	++
2	PI 2	PI 2/4	-
4	PI 3	PI 1/3	+
5	PI 4	PI 2/4	-
7	Ad cDNA	Ad	+

Figure 32 - Colindale PI 1-4 positive controls, annealing temperature 55^0C, adenovirus cDNA, annealing temperature 63^0C, $[Mg^{2+}]$ 1.5 mmol



Conclusions

Four distinct bands were seen in lane 1. The strongest band was visualised at 350 bp. The two very large bands were almost certainly spurious. The only band seen in the PI 3 lane was at c400 bp. Consequently it was not possible to say whether any of these bands was significant. None were the predicted size for PI 1/3 (520), but 350 was the predicted size for PI 2/4.

The initial band in lane 5 degraded. Degradation of PCR products occasionally occurs during thermal cycling and is thought to be due to contamination with "environmental" DNAase. Degradation is apparent on gel electrophoresis by the bright "smeared" appearance. Repeat adenoviral PCR showed a strong band (not photographed), confirming that primer pair Ad preferred the higher annealing temperature (63^0C).

To ensure that the successful primer pairs primed specifically, the protocol was repeated with each of the three negative control specimens together with the successful primer pairs.

Table 36 - Varying primer pairs, [Mg^{2+}] 1.5 mmol, annealing temperature 50^0C for OL, Cor, A, B, 55^0C N, Ad

Lane	Primer pair	Control	Band
1	OL	HEp2	-
2	OL	PMK	-
3	OL	HEF	-
4	Cor	HEp2	-
5	Cor	PMK	-
6	Cor	HEF	-
7	A	HEp2	-
8	A	PMK	-
9	A	HEF	-
10	B	HEp2	-
11	B	PMK	-
12	B	HEF	-
13	N	HEp2	-
14	N	PMK	-
15	N	HEF	-
16	Ad	HEp2	-
17	Ad	PMK	-
18	Ad	HEF	-

Conclusion

Priming under these reaction conditions was therefore specific.

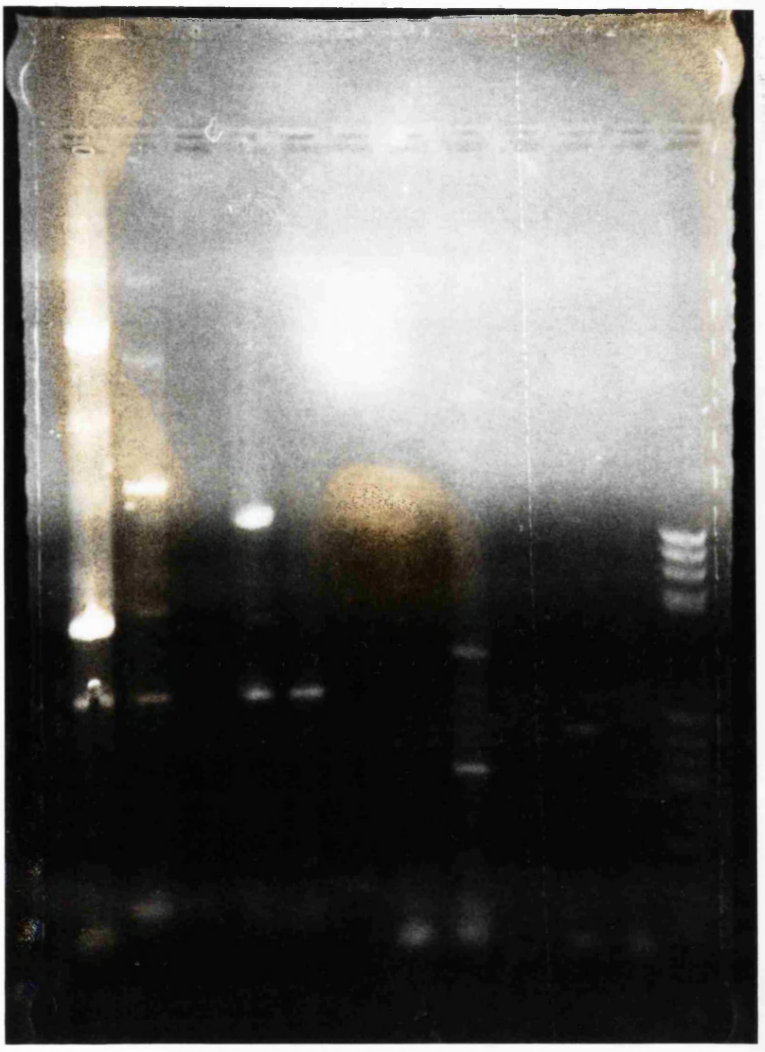
Doubt persisted about the integrity of the source parainfluenza virus RNA. There had been no positive bands with PI 2/4 whatsoever, and since previous culture-based prevalence studies have isolated these serotypes infrequently it was decided to drop the PI 2/4 primer pair at this stage. The PI 1/3 primer pair was retained for further investigation.

It is generally held that in multiplex PCR no more than three primer pairs should be used in any one reaction. Since the Ad primer pair required a higher annealing temperature than the other primer pairs, the final PCR protocol would consist of 3 separate PCR reactions for each clinical specimen, one for adenovirus and two for RNA viruses. Further development was aimed at this solution. The band sizes of all the five successfully PCR'd RNA viruses were different, so that the combination of primer pairs used was not influenced by identification on gel electrophoresis. An arbitrary decision was taken to run the OL, N, and Cor primer pairs together in one reaction and the PI 1/3, A, and B primer pairs together in a separate reaction. These combinations were tested with positive controls as follows.

Table 37 - RT-PCR of positive controls with multiple primer pairs, annealing temperature 55^0C, [Mg^{2+}] 1.5 mmol

Lane	Control	Primer pairs	Band
1	A9	OL, N, Cor	+
2	RSV	OL, N, Cor	+
4	Cor	OL, N, Cor	+
5	H_2O	OL, N, Cor	+
7	PI 1	PI 1/3, A, B	-
8	Flu A'	PI 1/3, A, B	+
10	Flu B	PI 1/3, A, B	+
11	H_2O	PI 1/3, A, B	-

Figure 33 - RT-PCR of positive controls with multiple primer pairs, annealing temperature 55^0C, [Mg^{2+}]



Conclusions

This gel illustrates two of the drawbacks to PCR methods. All of the first four lanes gave a positive RSV band, indicating contamination of one of the reaction constituents with RSV cDNA. This contamination was eventually traced to the Supertaq buffer, and did not recur with fresh buffer.

Lanes 1,2 and 6 illustrate the problem of the presence of DNA fragments other than those being sought. This problem of multiple band formation has been documented by previous workers (Ireland 1993). Some of these bands are larger than the entire genome of the virus being sought and are clearly spurious. Bands of this size do not represent a source of diagnostic confusion, but spurious bands of the approximate size of any of those being sought would lead to false positive diagnoses. One band occurs in lane 6, although at approximately 350 bp this does not "clash" with the fragments being sought. This phenomenon of multiple band formation had cropped up infrequently in previous experiments, particularly with A9, B4 and B6 controls together with the OL primer pair. However multiple band formation was much more prominent on the current gel, suggesting that the combination of six oligonucleotide primers together in the same PCR reaction increased the chances of spurious priming occurring.

Spurious band production probably originated in non-specific binding of oligonucleotides to source nucleic acid in the reverse transcription reaction. This problem

would be exacerbated by the necessity of having three primer pairs present in the reaction. A possible solution would be to use random priming of the reverse transcription reaction. Random primers are a mixture of randomly generated oligonucleotides, a small proportion of which will correspond to the specific oligonucleotide that would be used conventionally. This method is especially attractive in the context of looking for multiple viruses simultaneously, as it would allow just one reverse transcription reaction per clinical sample, which would mean less manipulation of each specimen. Unfortunately at this stage the supply of source coronavirus RNA was exhausted, reverse transcribed cDNA being used for coronavirus positive controls. Random priming was therefore attempted as follows.

Table 38 - Positive/negative control, reverse transcription with random or specific primers, PCR with specific primer mixes, annealing temperature 55^0C, [Mg^{2+}] 1.5 mmol

Lane	Control	Primer	Band
1	A9	OL, N, Cor	++
2	A9	random	++
3	A9	OL, N, Cor	+
4	A9	random	+
5	B4	OL, N, Cor	+
6	B4	random	+
7	B4	OL, N, Cor	+
8	B4	random	-
9[1]	HEp2	OL, N, Cor	-
10[1]	PMK	OL, N, Cor	-
11[1]	HEF	OL, N, Cor	-
12[2]	A9	OL, N, Cor	++
13	Flu A'	PI 1/3, A, B	+
14	Flu A'	random	++
15	Flu B	PI 1/3, A, B	+
16	Flu B	random	-
17	RSV	OL, N, Cor	+
18	RSV	random	+

1 negative control

2 lanes 13-18 - separate thermal cycling run from A9, B4. Lane 12 included as a further positive control for this run.

Conclusions

It was disappointing that influenza B apparently could not be randomly primed. This was unexpected, and the protocol was repeated with fresh RNA extractions, but with the same result. Consequently specific primers were used for the reverse transcription reaction.

Primer pairs were now combined into three sets for future use as follows, the original stock primers being retained as well.

Set 1	OL, N, Cor
Set 2	PI 1/3, A, B
Set 3	Ad

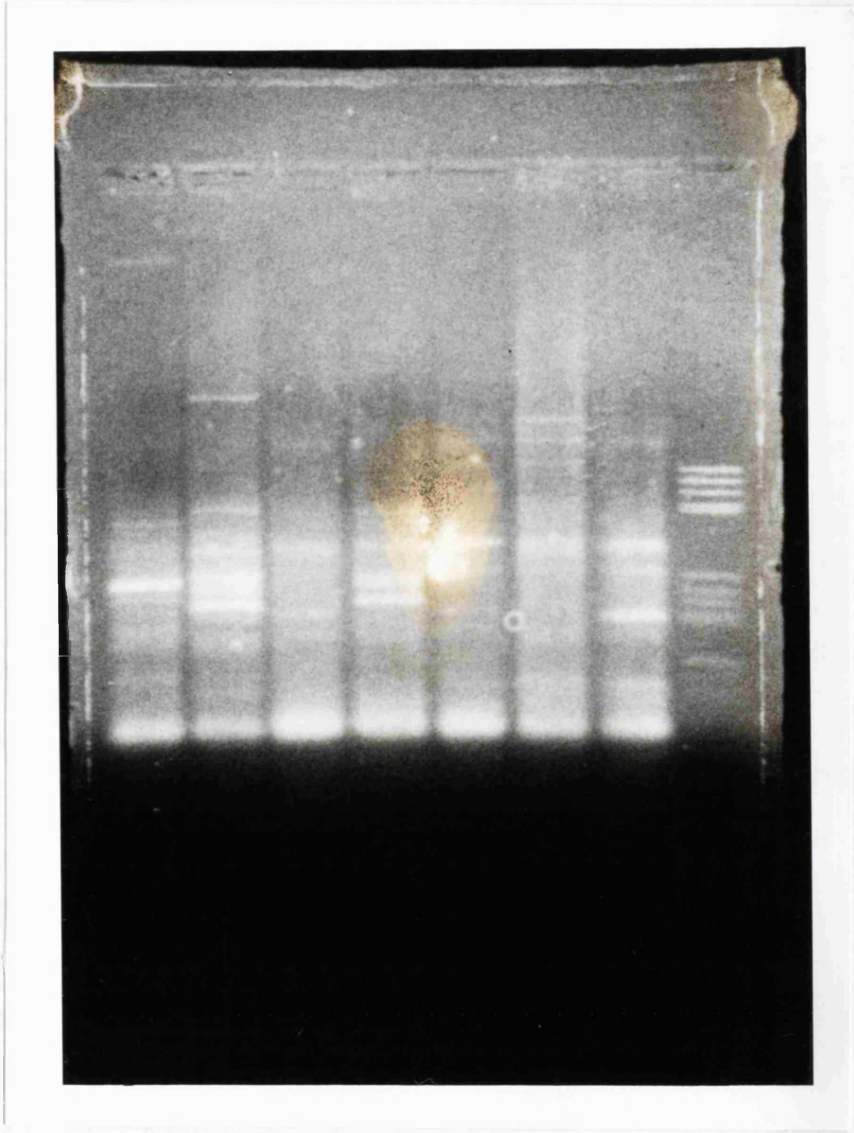
The working protocol at this stage was therefore to run three separate PCR reactions for each clinical sample, each using one of the primer sets, with the PCR products generated being run together in one lane of an agarose gel.

Table 39 - RT-PCR using sets 1,2,3, [Mg^{2+}] 1.5 mmol, annealing temperature 55^0C sets 1,2, 63^0C set 3 - controls

Lane	Control	Primer set	Band
1	A9	1	+
2	Cor	1	+
3	RSV	1	+
4	Flu A'	2	+
5	Flu B	2	+
6	PI 1/3	2	+
7	Ad cDNA	3	+
8	HEp2	1	-
9	PMK	2	-
10	HEF	3	-

The first batch of 30 clinical samples was then thawed and run using the above nucleic acid extraction method. The final pellet was resuspended in H_2O and divided into 3 aliquots. One aliquot was used straight away for adenoviral PCR (primer set 3), the other two underwent two separate reverse transcription reactions, one with primer set 1, and one with primer set 2. cDNA from each of these reverse transcription reactions was then used in the final PCR step. PCR products from all three PCR reactions were then pipetted into a single well for gel electrophoresis. Not all the gels were run, as the first two gels showed the presence of multiple bands.

Figure 34- RT-PCR using sets 1,2,3, [Mg^{2+}] 1.5 mmol, annealing temperature 55^0C sets 1,2, 63^0C set 3 - clinical specimens



Separating the products of the individual PCR reactions onto separate wells showed that the adenoviral PCR step had produced pristine negative results (but with a positive band in the adeno cDNA control lane). The PCR products from primer sets 1 and 2 both showed multiple banding. The complete absence of bands in the adenoviral PCR products again suggested strongly that the multiple bands were arising from mispriming during the reverse transcription step. One of the concerns throughout the whole study had been whether epithelial cells were being obtained reliably from the oropharynx with throat swabs. The multiple banding was reassuring evidence that nucleic acid, and presumably therefore epithelial cells, had indeed been recovered, and also had not denatured during storage, as it has been shown above that the primer mixes alone cannot generate DNA fragments. However the protocol clearly had to be modified to minimise this multiple banding if RT-PCR diagnosis was to be feasible.

It would be expected that most messenger RNA would have been denatured during storage, and certainly during the slow thawing that was allowed prior to RNA extraction. (Viral RNA is much more stable than eukaryote mRNA and is not destroyed by slow warming to room temperature.) Multiple band formation was therefore presumed to be initiated by non-specific binding of oligonucleotide primers to host nucleic acid, probably DNA, during the reverse transcription reaction. The theoretical answer to the problem was to remove as much of the host DNA as possible between the initial nucleic acid

extraction step and the subsequent reverse transcription by incubating with DNAase.

The protocol for the RNA viruses in outline was as follows:-

phenol/chloroform extraction
ethanol/acetate precipitation
resuspension of pellet in DNAase mix (and incubation)

phenol/chloroform extraction (to inactivate DNAase)
ethanol/acetate precipitation
resuspension of pellet in reverse transcription mix (and incubation)
cDNA to PCR reaction

The procedure for adenoviruses was much more straightforward, part of the initially resuspended nucleic acid pellet being added straight to the adenoviral PCR mix.

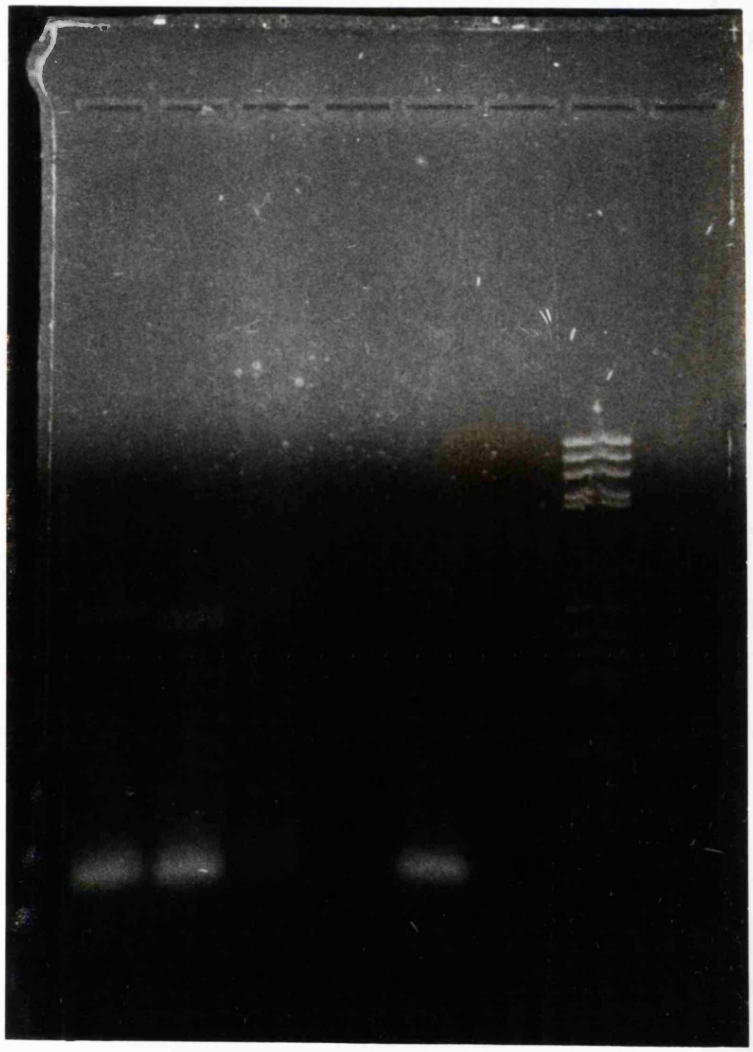
The addition of the DNAase step risked a certain loss of sensitivity because commercially available DNAase is occasionally contaminated with trace amounts of RNAase. A further drawback was the extra manipulation required, including a further organic acid extraction to remove DNAase before the reverse transcription step. This significantly extended the time required for sample processing, and increased the risk of specimen contamination.

The protocol was tried with 3 clinical specimens and the usual RNA positive controls.

Table 40 - Initial batch of clinical samples with DNAase protocol

Lane	RNA source	Primer set	Band
1	A9	1	+
2	RSV	1	+
3	Flu A'	2	+
4	Flu B	2	+
5	PI 3	2	-
6	clinical sample 1	1,2	+ Flu B
7	clinical sample 2	1,2	+ Flu B
8	clinical sample 3	1,2	+ RSV
9	HEp2	1,2	-
10	PMK	1,2	-
11	HEF	1,2	-

Figure 35 - Initial batch of clinical samples with DNAase protocol - lanes 6-11



The first three clinical samples subjected to DNAase after the initial nucleic acid extraction thus gave single bands of appropriate sizes. However further clinical samples continued to yield multiple bands, although the problem was less severe than prior to the addition of DNAase.

The final solution to this problem was therefore to run the reverse transcription and PCR reactions with primer sets A and B, following DNAase treatment. If bands of appropriate size were then generated, the sample was re-extracted with primers specific to the fragment being investigated. The clinical sample was then considered to be positive only if bands of appropriate size were generated with the specific primers.

IX.5 Final protocol

IX.5.i Nucleic Acid Extraction

Clinical samples (throat swab transport medium) were allowed to thaw slowly from -70^{0}C to room temperature. 300 mcl of the transport medium were then transferred to 1.5 ml reaction tubes containing:-

37.5 mcl (37.5 mcg) proteinase K

1.5 mcl (1.5 mcg) carrier tRNA

339 mcl 2× proteinase K buffer

The reaction tubes were then heated in a water bath at 37^{0}C for 30 minutes.

Samples were then extracted once with an equal volume of phenol/chloroform, the aqueous phase was retained and the

organic phase was discarded.

For nucleic acid precipitation 450 mcl of the aqueous phase was transferred to a fresh 2.5 ml reaction tube containing 50 mcl 3M sodium acetate and the contents were mixed before adding 1.5 ml 100% ethanol. The reaction tubes were then kept at -70^{0}C for thirty minutes. After centrifugation at 5000g for ten minutes the ethanol was discarded using a micropipette, great care being taken to avoid discarding the nucleic acid pellet (in practice this was always visible).

The pellet was washed once with 400 mcl 70% ethanol, without agitation, to remove residual solutes. After further centrifugation at 5000g for 5 minutes the ethanol was carefully discarded as described above and the washed pellet was air dried. The nucleic acid pellet was resuspended in 100 mcl water. 30 mcl were transferred directly to the adenoviral PCR reaction and the remaining 70 mcl to the DNAase reaction.

IX.5.ii DNAase reaction

Seventy mcl of resuspended nucleic acid pellet was transferred to fresh 1.5 ml reaction tubes containing :-

30 mcl DNAase I (total 0.5 mcg per reaction)

and the reaction tubes were heated at 37^{0}C in a water bath for one hour. The contents of the reaction tubes were then subjected to extraction with an equal volume (100 mcl) of

phenol/chloroform, the aqueous phase was retained and the organic phase was discarded.

For nucleic acid precipitation the aqueous phase (100 mcl) was transferred to a fresh 1.5 ml reaction tube containing 11 mcl 3M sodium acetate and the contents were mixed before adding 260 mcl 100% ethanol. After centrifugation at 5000g for ten minutes the ethanol was discarded using a micropipette, great care being taken to avoid discarding the nucleic acid pellet (in practice this was always visible).

The pellet was washed once with 150 mcl 70% ethanol, without agitation, to remove residual solutes. After further centrifugation at 5000g for 5 minutes the ethanol was carefully discarded as described above and the washed pellet was air dried. The nucleic acid pellet was resuspended in 50 mcl water. Twenty-five mcl were transferred to each reverse transcription reaction.

IX.5.iii Reverse transcription

Each reverse transcription reaction contained the following:-

1 mcl M-MLV reverse transcriptase (200 units)

1 mcl RNAguard RNAase inhibitor (40 units)

2 mcl DTT (10 mM)

10 mcl 5× buffer

1 mcl primer mix (set 1 or 2, 0.4 mM of <u>each</u> primer)

1 mcl dNTP (0.5 mM)

9 mcl water

25 mcl resuspended RNA (see above)

Reaction tubes were incubated at 37°C for one hour, in a total reaction volume of 50 mcl.

10 mcl of the resulting cDNA were then transferred to the appropriate PCR reaction (1 or 2).

IX.5.iv PCR reaction

Each PCR reaction contained the following:-

0.05 mcl Supertaq
0.4 mcl dNTP (0.2 mM)
5 mcl 10× Taq buffer (reaction Mg 1.5 mmol/l)
1 mcl primers (A,B or adeno, 0.4 mM of <u>each</u> primer)
10 mcl cDNA (A,B) or 30 mcl resuspended nucleic acid - (adenoviral PCR)
33.5 mcl water (A,B) or 13.5 mcl water (adenoviral PCR)
several drops of mineral oil, overlaid

Reaction tubes were placed in a thermal cycler and underwent 30 cycles of :-

92°C	1 minute	[separation]
55°C (A,B) 63°C (adeno)	2 minutes	[annealing]
72°C	4 minutes	[extension]

followed by a final 15 minute extension at 72°C.

IX.5.v Identification

IX.5.v.a Preparation of ethidium-stained 2% agarose gel

1 G agarose was added to 50 ml Tris-acetate (TAE) and microwaved at mark 2 for 2.5 minutes (resulting in a 2% gel). The mixture was then cooled to 60^0C and 25 mcl ethidium bromide added. The mixture was then poured into a plastic electrophoresis tray and loading wells made with a plastic insert. After cooling, the tray was immersed in 350 ml TAE in an electrophoresis bath.

IX.5.v.b PCR product identification

Each clinical sample had so far generated three 50 mcl sets of PCR reaction products. Ten mcl of each of the reaction products were combined with 6 mcl of loading buffer into a single well on the ethidium-bromide stained gel. Two mcl marker DNA (DNA molecular weight marker V, Boeringher Mannheim), 5 mcl distilled water and 3 mcl loading buffer were added to the well of the marker lane on each gel. A voltage of 100V was applied for 1 hour, until the marker dye was approximately 3/4 of the way to the end of the tray. The gel was then inspected visually under UV illumination and a polaroid photograph taken.

Despite the addition of the DNAase step, multiple banding still occurred to some extent. This meant that there was doubt as to the specificity of the bands generated.

Consequently, if bands were seen on the initial RT-PCR gel, the gel was rerun with the appropriate PCR products only eg with bands of c 277 bp size (potentially RSV), the PCR products of reaction 1 were run alone. If bands of the appropriate size were still seen the original clinical sample was re-extracted with virus-specific primers. Only if a band of the appropriate size was seen after RT-PCR with virus-specific primers was the clinical sample considered positive.

IX.5.vi Controls

One of the drawbacks of the PCR reaction is its potential for amplifying contaminating cDNA. One negative control for each primer mix was therefore included at the extraction stage. One positive control (A9) was also included at this stage to check the integrity of the extraction/reverse transcription procedures. Positive controls containing coronavirus, adenovirus, coxsackie (surrogate rhinovirus), RSV, influenza B and influenza A cDNA were included at the PCR stage to check the integrity of the PCR reaction.

In practice it was possible to perform thirty two extractions per day comprising twenty eight samples, one positive control and three negative controls.

Chapter X PCR diagnosis of respiratory viral infection

X.1 Incidence of respiratory viral infection

Introduction

PCR diagnosis can theoretically detect one copy of a viral genome. In the case of detection of hepatitis B and C viruses from serum, for example, this is an advantage, as the objective is simply to confirm the presence or absence of circulating virus. PCR diagnosis is now reasonably well established for these viruses. However in the case of respiratory viral infection the significance of detecting a single viral genome is not as clear cut. As yet there have been no studies relating PCR diagnosed respiratory viruses to the presence or absence of symptomatic clinical infection. In addition, it is known that adenovirus can remain latent for some weeks following clinical infection in the upper respiratory tract [Fox 1977], but latency has not been demonstrated for other respiratory viruses.

In practice however, PCR diagnosis is probably not sensitive enough to detect one viral genome. <u>In vitro</u> sensitivity studies have usually indicated a lower detection limit of $10^{2.5}$ infected cells [Gama 1989]. A positive PCR diagnosis is therefore likely to represent genuine infection.

Methods

Throat swabs and sputum (where appropriate) were stored for later PCR analysis starting on November 1st 1991. However logistical considerations prohibited the examination of sputum. PCR analysis was therefore confined to throat swab material. Throat swabs were inoculated into standard viral transport medium, and despatched to the laboratory as described in section VII.3, viral isolation methodology. Throat swabs were initially subjected to viral isolation procedures. Remaining transport medium, with the intact swab, was then stored at -70°C. Storage began in November 1991. This protocol was followed for all participants in the community study, and for all CF patients presenting early with symptoms (see chapter VIII). Throat swabs obtained from seventeen of the non-CF subjects in the community-based study when they were clinically well served as "negative clinical controls", analogous to swabs from CF patients treated with intravenous antibiotics in the absence of exacerbation. Lack of -70°C storage space meant that specimens from CF patients presenting with exacerbations were stored only between November 1st 1991 and June 30th 1992 (seven months).

Throat swabs were subjected to PCR analysis in batches of 28, with one positive control extraction and three negative control extractions, with further positive and negative controls for the PCR reaction, as described in chapter IX. The investigator (the author) was not blinded to the name of the subject or the date, but was blinded to the

clinical circumstances at the time of collection, as PCR was performed 12-18 months after sample collection.

Not all throat swabs were available for PCR analysis, as in some cases the material was consumed during the viral isolation step. The proportions of positive diagnoses among the various categories were calculated with the whole sample as the denominator, on an "intention to diagnose" basis.

Results

A total of 207 throat swabs was subjected to the PCR protocol in 8 separate runs. In all 8 runs the positive and negative laboratory controls were identified appropriately on gel electrophoresis. Thus in each case the integrity of the extraction, reverse transcription, and PCR reactions was confirmed, with no evidence of contamination with source RNA or DNA, or with cDNA or PCR products.

X.1.i CF patients treated between November 1st 1991 and June 30th 1992.

There were 128 treatments in this eight month period. Of these, throat swabs were available for PCR analysis in 88 (69%). Four of the twelve seroconversions documented in the two years took place in this period. There were nine positive PCR diagnoses in this group. PCR was negative in all four of the treatments associated with seroconversion. RSV was the virus found most frequently with PCR. No rhinoviruses,

adenoviruses or coronaviruses were detected. Details of the positive diagnoses are given in table 41.

Eighty-two of the treatments were classified as exacerbations, thirty-two were non-exacerbations, and fourteen were unclassifiable. One of the positive PCR diagnoses occurred in an unclassifiable treatment. Combined serology and PCR detected eleven respiratory viral infections associated with exacerbations (11/82 = 13%) and one infection not associated with exacerbation (1/32 = 4%). The difference between these proportions was not significant (Fisher's exact test $p = 0.11$). As with serological diagnosis, roughly half of the PCR positive treatments were not associated with symptoms suggestive of non-bacterial infection.

Table 41 - Positive respiratory viral diagnoses - CF treatments

Organism	Positive serology	Positive PCR	Exacerbation	SNBI
adenovirus	yes	no	yes	no
adenovirus	yes	no	yes	no
influenza A	yes	1	yes	no
influenza A	yes	no	yes	no
influenza B	no	yes	yes	yes
paraflu 1/3	2	yes	yes	yes
paraflu 1/3 + RSV	2 no	yes	yes	yes
RSV	no	yes	yes	yes
RSV	no	yes	yes	yes
RSV	no	yes	no	no
RSV	no	yes	yes	no
RSV	no	yes	not classifiable	no
RSV	no	yes	yes	yes

1 PCR not performed in this case

2 serology not performed for parainfluenza viruses

X.1.ii CF patients presenting early with symptoms of SNBI

Thirty-nine of the sixty throat swabs collected in this group were available for PCR analysis. PCR was positive in three cases, all for RSV. The two seroconversions documented were to adenovirus (1) and CMV (1). The combined PCR and serology approach therefore identified five respiratory viral infections in sixty episodes (8%). Thus collecting throat swabs at an early stage in symptomatic illness did not influence the rate of positive diagnosis with PCR.

X.1.iii Community study

Eighty-one of 113 throat swabs (72%) were available for PCR analysis. Seven new infections were found with PCR, and PCR was also positive for influenza A in one subject and for RSV in one subject who both subsequently seroconverted to the respective virus. Sixteen of 127 (13%) symptomatic episodes were positive with serology and PCR. None of the seventeen "negative clinical control swabs" was positive. The difference between these ratios was not significant ($p = 0.22$, Fisher's exact test). Details of the nine PCR positive episodes are shown in table 42. The rate of positive viral diagnosis in the various groups is summarised in table 43.

Table 42 - Details of symptomatic episodes with positive PCR diagnosis - community-based study.

organism	month	category	exacerbation	SNBI
coronavirus	November	asthma contact	NA	yes
influenza A	January	CF contact	NA	yes
influenza B	November	CF contact	NA	yes
RSV	January	CF patient	yes	yes
RSV	March	bronchiectasis patient	yes	yes
RSV	April	non-CF bronchiectasis patient	NA	yes
RSV	November	CF patient	yes	yes
RSV	December	CF contact	NA	yes
RSV	December	CF patient	yes	yes

NA - not applicable

Table 43 - Rate of positive diagnosis of respiratory viral infection with PCR and serology according to patient group

	total diagnoses	rate of diagnosis/ patient/year
CF patient	4	0.17
CF contact	5	0.13
non-CF bronchiectasis patient	4	0.29
non-CF bronchiectasis contact	0	0
asthma patient	1	0.06
asthma contact	2	0.12

X.1.iv Discussion

This is the first study to attempt identification of RSV, adenoviruses, parainfluenza, and influenza viruses directly from clinical material with PCR methods. In addition, this is the first study which has attempted to identify all seven known respiratory viruses simultaneously with PCR methods. In this study PCR products were identified by finding fragments of the expected length on agarose gel electrophoresis. Ideally these would be confirmed with direct sequencing or probe hybridisation. It was decided at the outset of development of the PCR method that application of these methods would be impractical given the large number of specimens to be processed and the need to identify seven different viral sequences. PCR methodology for the detection of hepatitis B and C viruses is now well established and usually employs band size matching for identification. The non-specific binding of oligonucleotide primers at the reverse transcription step and the consequent multiple banding on agarose gel electrophoresis presented particular problems with specificity in this study. For example, were the fragments approximating to 277 bp really derived from RSV, or merely the result of non-specific priming ? The only sure way to know would have been to sequence them, or use probe hybridisation. Many of the candidate bands were clearly spurious, as they arose with the "wrong" set of primers, eg bands of 277 bp (compatible with RSV) arising with the primer combination of {PI 1/3 - Flu A - Flu B}. Further spurious

bands were rejected after re-extraction and reverse transcription with the appropriate single primer pair failed to reproduce bands of the appropriate size. This rigorous approach meant that the remaining (positively diagnosed) bands were highly likely to represent genuine viral sequences. Confirmation that the bands identified were specific for the viruses sought was provided by four findings. Firstly, one influenza A and one RSV seroconversion were positive with PCR. Secondly, one CF patient developed PCR-positive RSV infection after the household contact had developed PCR- and serology- positive RSV infection. Thirdly, only one PCR-positive diagnosis was not associated with a pulmonary exacerbation or SNBI. Finally, in the year-long community-based study, eight of the nine positive PCR diagnoses occurred during the winter months November through March.

The overall rate of PCR-positive diagnosis was low (18% of all symptom episodes). There can be no doubt as to the integrity of the extraction, reverse transcription and PCR methods, as all the laboratory controls gave the appropriate result. The reverse transcription and PCR reactions are unlikely to have been adversely affected by the presence of inhibitors in the throat swab material, as virtually all samples generated bands. Throat swab material was stored for one to two years before PCR analysis, and some RNA degradation must have occurred during this time period. However, oligonucleotide pairs themselves are incapable of generating spurious bands (chapter IX), and therefore there

must have been intact nucleic acid in these samples. No spurious priming at all occurred with the adenovirus oligonucleotide pair, final confirmation that spurious priming was taking place in the reverse transcription reaction. The intact nucleic acid in the throat swabs must therefore have included intact RNA. Since viral RNA is generally more stable than eukaryote RNA, it is unlikely that much degradation of viral RNA took place. The presence of nucleic acid in the samples implies that the throat swab material collected must have been adequate for the purposes of viral isolation ie it included epithelial cells.

Most of the bands observed, and all of the bands which were positively identified, were faint, which illustrates the point that PCR is not an "all or nothing" reaction, but is semi-quantitative. In the time available for development of the technique it was not possible to perform in vitro assays of sensitivity, but sensitivity has been previously described for the rhinovirus and influenza oligonucleotide pairs [Gama 1989; Claas 1992], although the applicability of such sensitivity data, which is derived from laboratory passaged virus progressively diluted in buffer, to in vivo wild virus obtained in physiological secretions, must be doubtful. Compared to standard extraction and reverse transcription methods there must have been some reduction in sensitivity by the need to divide the extracted nucleic acid into three aliquots, and subsequently by the need to perform a second extraction on the RNA viruses following incubation with DNAase, but it was not possible to quantify this.

Seroconversion to influenza, RSV, and adenovirus occurred eleven times in the population investigated with PCR. Four seroconversions were in patients treated for exacerbations of CF (two adenoviral, two influenza A). None of these seroconversions was PCR-positive. This could have been due to late presentation for treatment after the onset of viral infection, although the overall PCR positive rate did not seem to be influenced by ensuring early sample collection following symptoms. In the community-based study only two of the nine seroconversions were PCR-positive (one influenza A and one RSV). The study protocol ensured that the absence of PCR positive diagnoses in this group was not due to late presentation. Overall, PCR seemed to be more sensitive in the detection of RSV infection, but less sensitive in the detection of influenza A. One influenza B infection was diagnosed with PCR, none with serology. There was only one seroconversion to adenovirus, which was asymptomatic. No adenoviruses were identified with PCR. Consequently it is not possible to comment on the relative sensitivities of the techniques for the identification of adenovirus. Of the four viruses that could be detected with serology, eight symptomatic episodes were diagnosed with serology, and eight with PCR. This multiplex PCR method was therefore no more sensitive than serology, but offered an alternative method of diagnosis, with an overall increase in sensitivity when combined with serology.

The main reason for using PCR was to provide an alternative to viral isolation in diagnosing infections for

which serology was not available viz rhinovirus, coronavirus and parainfluenza virus. No rhinoviruses were identified with PCR in this study. The rhinovirus oligonucleotides employed in this study were the same as those used in the study of Johnston et al [1993]. This pair of oligonucleotides detects all picornaviruses (rhinoviruses and coxsackie viruses). In their study they identified picornavirus in 50% of asthma exacerbations in children aged 9-11. Two thirds of these were thought to be rhinoviruses. The patient population in the present study were all of adult age, and thus represent a different population. The rate of symptomatic infection in Johnston et al's study was 2.7/patient/year, more than twice the rate in the present study. In Johnston et al's study no techniques to limit spurious priming were employed, and some of the positive diagnoses probably represent false positives. Their study employed nasal aspirates rather than throat swabs as used in this study. Despite these differences it is hard to fully explain such a wide discrepancy between the two studies. Nicholson et al [1993] used semi-nested PCR for rhinovirus in their study of adults with asthma. The clinical samples collected were nose and throat swabs. They identified rhinovirus in 33% of all clinical specimens, and 23% of symptomatic episodes. The semi-nested secondary PCR technique is more sensitive and specific than a single PCR reaction, and it is possible that secondary PCR is required to achieve adequate sensitivity in an adult population. No data were provided on the relative proportions of positive samples arising from throat and nasal swabs. It is possible that the

majority of their positive samples were nasal swabs.

Doubts about the integrity of the parainfluenza RNA sources during PCR optimisation meant that inclusion of the PI 1/3 oligonucleotide pair in the processing of the clinical samples was somewhat speculative. However two bands of the expected length were identified in the clinical samples, suggesting that this oligonucleotide pair were priming parainfluenza RNA, and that the problems during optimisation were indeed due to inadequate sources of viral RNA. One of the parainfluenza infections occurred together with RSV infection.

Combined serology and PCR identified thirteen viral infections in the treated CF patients. Eleven were associated with exacerbations, one with a treatment classified as a non-exacerbation, and one with a treatment that was not classifiable. Although these numbers are suggestive of an association, statistical significance was not reached, probably due to the small numbers of infections identified.

Although respiratory viral infections appeared to be associated with exacerbations of CF, asthma and bronchiectasis, they were not associated with symptoms suggestive of SNBI - only half of seroconversions and half of PCR positives were associated with SNBI. Although viral infections were identified in all three patient groups in association with exacerbations unaccompanied by SNBI, no asymptomatic infections were identified in the normal household contacts. This implies that respiratory viral infections that would normally cause typically "viral"

symptoms in normals can instead cause predominantly respiratory symptoms in patients with underlying respiratory disease.

In summary, PCR diagnosis was no more sensitive than serology in detecting influenza A and adenovirus infection, but appeared to be more sensitive than serology in detecting RSV infection. The overall rate of diagnosis was disappointingly low. This may reflect a genuine absence of respiratory viruses in the populations studied or absence from the oropharynx as opposed to the nasopharynx, but may also reflect a lack of sensitivity of this PCR system. Secondary, nested, PCR techniques need to be developed to enhance the sensitivity and specificity of PCR in the diagnosis of respiratory viral infection.

X.2 Incidence of respiratory viral infection in adult patients with cystic fibrosis in relation to disease severity

Introduction

Six of the fourteen studies of viral infection in cystic fibrosis reviewed in chapter II contained data linking infection rate to disease severity. This data is briefly reviewed here. Petersen et al (II.3) found the highest incidence of RSV, their most commonly isolated virus, in the group of patients in transition to chronic pseudomonal colonisation, rather than in the groups who were already

colonised, or not at all colonised, with *pseudomonas*. In terms of disease severity, this group would be expected to occupy the middle ground between the other two groups. Efthimiou et al (II.4) found more frequent seroconversions in their "deteriorated" group, as opposed to their "stable" group. The deteriorated group had poorer baseline pulmonary function. Wang et al (II.5) found that clinical indices were more severe in patients with CF who had more than 1.67 respiratory viral infections per year. Stroobant (II.6) found more seroconversions in children colonised with *pseudomonas* than in children not colonised. Ramsey et al (II.7) found more respiratory viral infections in patients not colonised with *pseudomonas*, and rate of infection was not associated with Shwachman-Kulczyki score or FVC. Hordvik et al (II.9) found no relation between initial disease severity and the time to nadir PEFR in symptomatic respiratory infections.

Methods

Individual regression analysis of percent predicted FEV1 and percent predicted FVC versus time was performed for each of the sixty patients in the cohort of patients with CF that was followed for two years. The regression was performed with data generated within the two year period of the study, and the mid-point of the regression line (ie the predicted value after one year) was taken to represent the average value for the period of the study. Average values were then compared between the 14 patients of the cohort with a confirmed viral

infection and the 46 patients with no confirmed infections. The number of intravenous treatments undertaken was also compared between the two groups.

Results

Some of the data was not normally distributed, and the Mann-Whitney U test was therefore used for statistical comparison. Results are summarised in table 44. Average percent predicted FVC over the two year study period was significantly lower in the virally-infected group, and the difference in average percent predicted FEV1 approached statistical significance. Intravenous antibiotic treatment was significantly more frequent in the virally-infected group.

Table 44 - Median (range) of average percent predicted spirometry over two years, and median (range) of number of treatments over two years, according to presence or absence of viral infection

	confirmed viral infection	no viral infection	significance level (Mann-Whitney)
percent predicted FEV1	38 (27 - 66)	58 (42 - 77)	p = 0.062
percent predicted FVC	71 (53 - 86)	91 (70 - 115)	p = 0.024
number of treatments	7 (2 - 10)	3 (0 - 13)	p = 0.0023

Discussion

Although three of the previous studies of viral infection in cystic fibrosis have suggested that patients with poorer clinical status are more prone to viral infection, none have attempted to control for ascertainment bias. Patients with poorer respiratory function are likely to present more frequently for intravenous antibiotic treatment, as the symptomatic consequences of respiratory exacerbation are likely to be more severe in patients with poorer baseline pulmonary function. Such patients are likely to be investigated for respiratory viral infection more frequently, which could account for the greater frequency of confirmed viral infection. In this study patients with confirmed viral infection had poorer pulmonary function, and did indeed present much more frequently for intravenous antibiotic treatment. Thus it is hard to know whether the increased frequency of confirmed viral infection in this group is genuine.

Approximately half of the diagnoses of viral infection in this cohort were made with serology. All patients in the cohort were tested for seroconversion at least every six months, with only one asymptomatic seroconversion. This makes confounding by more frequent sampling less likely, and a genuinely greater predisposition to viral infection in patients with poor pulmonary function more likely. A bacteriological parallel would be the greater predisposition to infection with *Burkholderia cepacia* in patients with poor

pulmonary function. The link between defective CFTR and subsequent bacteriological colonisation of the respiratory tract is not known at present. In particular, the sequence of initial colonisation with *S aureus* and subsequent colonisation with *pseudomonas* species can be explained only by postulating that the staphylococcal colonisation in some way alters the intrapulmonary milieu to favour pseudomonal colonisation. It is conceivable that prolonged colonisation with *pseudomonas* could render the respiratory tract more prone to viral infection.

Chapter XI - Clinical effects of SNBI and confirmed viral infections

XI.1 Introduction

At the outset of this study the basic hypothesis to be tested was that respiratory viral and atypical bacterial infections cause significant morbidity in adult patients with CF. Since no atypical bacterial infections were identified, the remaining hypothesis is that respiratory viruses alone cause significant morbidity. Using spirometry as the main measure of outcome, it has already been shown that respiratory viruses cannot have a major long-term impact on a CF population through the precipitation of exacerbations, as pulmonary function is recoverable with adequate treatment (chapter VII). However it is still possible that exacerbations associated with respiratory viral infection may be more severe, and that such infections might lead to an accelerated decline in pulmonary function. Although it has been shown that there is no relationship between confirmed respiratory viral infection and suggestive symptoms (ie SNBI), in everyday clinical practice viral infection is usually diagnosed on the basis of symptoms. Therefore it is still germane to examine the relationship between SNBI and severity of symptoms.

XI.2 Severity of exacerbations - SNBI and confirmed respiratory viral infection

Methods

The analysis was confined to the cohort that completed the two year study. Measures of severity were compared between those exacerbations (not treatments) associated with SNBI and those not associated with SNBI, and between exacerbations associated with confirmed respiratory viral infection and those not associated with confirmed respiratory viral infection. (The PCR system was only applied to specimens obtained during seven months of the two year study. Consequently, some of the exacerbations outwith this period classed as "non-viral" would be expected to have had a viral aetiology.) Patients served as their own controls - patients with no SNBI or no confirmed infections were not included in the analysis.

The outcome measures used were :- percentage change from baseline of FEV1 and FVC (determined using the method described in chapter VII), white cell count, plasma viscosity, and CRP, all determined at the start of treatment of the exacerbation. In addition, the total length of treatment was determined. All the above measures were recorded before classification according to exacerbation/ non-exacerbation and SNBI/non-SNBI, and before positive viral diagnosis. Patients in whom exacerbations occurred with and without associated SNBI were identified, and their

exacerbations included in the analysis. Subsequently, patients in whom exacerbations occurred associated with confirmed viral infection were identified, and all their exacerbations included. Finally, pulmonary function at the end of treatment was compared with pre-exacerbation baseline, separately in exacerbations with and without associated confirmed viral infections.

Statistical comparison was made using the general linear model (non-orthogonal ANOVA) with each measure of severity in turn as the response variable, presence or absence of SNBI as the dependent variable, and repeated treatments for each patient as a factor nested within presence or absence of SNBI.

Results

Exacerbations both with and without associated SNBI occurred in 33 patients. The total number of exacerbations included was 209, of which 99 were associated with SNBI. The results are summarised in table 45.

Exacerbations both with and without associated confirmed viral infection occurred in 14 patients. The total number of exacerbations included was 95, of which 16 were associated with confirmed viral infection. Results are summarised in tables 46 and 47.

Table 45 - Adjusted mean (SD) of measures of severity of exacerbation according to presence or absence of SNBI

	SNBI	no SNBI	significance level
dFEV1 (%)	-19.8 (1.8)	-17.3 (1.6)	p = 0.30
dFVC (%)	-18.3 (2.1)	-18.1 (1.9)	p = 0.94
WCC/10^9/l	11.47 (0.38)	10.63 (0.34)	p = 0.10
PV/mpas	1.85 (0.02)	1.81 (0.02)	p = 0.12
CRP/mg/dl	36.2 (4.6)	28.5 (4.2)	p = 0.22
length of treatment/ days	13.3 (0.9)	13.1 (0.9)	p = 0.85

Table 46 - Adjusted mean (SD) of measures of severity of exacerbation according to presence or absence of confirmed viral infection

	viral infection	no viral infection	significance level
dFEV1 (%)	-26.3 (4.0)	-17.2 (2.1)	p = 0.047
dFVC (%)	-24.6 (3.9)	-18.7 (2.0)	p = 0.18
WCC/10^9/l	11.57 (0.71)	11.43 (0.36)	p = 0.86
PV/mpas	1.88 (0.03)	1.82 (0.02)	p = 0.089
CRP/mg/dl	53.2 (7.5)	31.6 (3.8)	p = 0.012
length of treatment/ days	14.1 (1.3)	12.2 (0.7)	p = 0.20

Table 47 - Adjusted mean (SD) of pulmonary function before and after treatment of exacerbations with and without associated confirmed viral infection

	pre-treatment	post-treatment	significance level
FEV1/l confirmed viral infection	1.92 (0.05)	1.70 (0.05)	p = 0.041
FEV1/l no confirmed viral infection	1.86 (0.04)	1.83 (0.04)	p = 0.61
FVC/l confirmed viral infection	3.19 (0.10)	3.19 (0.10)	p = 0.96
FVC/l no confirmed viral infection	3.23 (0.06)	3.23 (0.06)	p = 0.99

Discussion

Pulmonary function and inflammatory markers at the outset of treatment, and overall length of treatment, were identical in exacerbations with and without SNBI. This may be because the majority of SNBI are not in fact due to viral infection, merely representing exacerbations with suggestive symptoms. The change in FEV1 from baseline (dFEV1) was significantly greater in exacerbations associated with confirmed infections than in those without confirmed infection. The difference between exacerbations with and without confirmed infection in change in FVC from baseline (dFVC) was of a similar magnitude, but failed to reach statistical significance. There was a trend for longer length of treatment in exacerbations with confirmed viral infection (14 vs 12 days), but this was not statistically significant.

Inflammatory markers in confirmed viral infection behaved unexpectedly. It was shown in chapter VI that plasma viscosity closely parallels pulmonary function, with no additional acute changes with exacerbation. C-reactive protein on the other hand is markedly more elevated in exacerbations than can be accounted for simply by decreased pulmonary function. White cell count shows a less marked specific increase in exacerbations. In exacerbations with confirmed viral infection plasma viscosity did indeed parallel dFEV1 and dFVC, and CRP was greater in exacerbations with confirmed viral infection. The increase in c-reactive protein implies that acute phase reactants increase in

keeping with the greater severity of exacerbation observed. White cell count, however, was identical in exacerbations with and without viral infection. This implies that viral infection suppresses the increase in circulating white cells that usually accompanies an exacerbation of cystic fibrosis.

At the cessation of treatment, a statistically significant deficit in FEV1 remained compared to pre-exacerbation baseline. However there was no deficit in FVC. This contrasts with the findings of Hordvik [1989], Pribble, [1990] and Smyth [1995], who did not find deficits in pulmonary function following exacerbations associated with viral infection. In the subpopulation in this study with confirmed viral infection there was no deficit in FEV1 following treatment in the exacerbations without associated viral infection. The effect is therefore specific to the viral infection, and not due to a proneness to post-treatment deficit in FEV1 arising randomly in this particular patient group. The decision to discontinue treatment of an exacerbation was made according to several criteria:- subjective improvement on the part of the patient, no further improvement in clinical score (a numerical measure of clinical improvement), no further increase in weight gain, and a perceived return to baseline of pulmonary function. In the case of the exacerbations with confirmed viral infection, the first three criteria and the return to normal of FVC presumably outweighed the deficit in FEV1 in the decision to discontinue treatment. The decision to stop treatment before the deficit in FEV1 was corrected cannot have been biased by

knowledge of viral aetiology, as this was not known at the time. Nor can it have been biased by the presence of SNBI, as only half the confirmed viral infections were associated with SNBI. It is conceivable that more prolonged treatment of the exacerbation might have corrected the deficit in FEV1.

XI.3 Long-term effect of viral infection on pulmonary function

Introduction

Although a deficit in FEV1 persisted on the cessation of treatment of pulmonary exacerbations, viral infection would be of most significance if there were long-term consequences for pulmonary function.

Methods

Linear regression of percent predicted FEV1 and percent predicted FVC was performed individually for each patient with a single documented viral infection, both for the year preceding and for the year following the infection. The mid-point of the regression slopes was considered to be representative of pulmonary function in the relevant year, and these values were compared before and after viral infection. In addition, the rate of deterioration of pulmonary function was compared in the years before and after

viral infection. The data were not normally distributed, and comparisons were therefore made using the Mann-Whitney test.

Results

Eleven patients with one confirmed viral infection had sufficient data available for regression analysis of both years. Results are summarised in tables 48 and 49.

Table 48 - Percent predicted pulmonary function before and after viral infection - absolute values

	pre-infection	post-infection	significance level (Mann-Whitney)
percent predicted FEV1	43	38	0.55
percent predicted FVC	82	78	0.51

Table 49 - Percent predicted pulmonary function before and after viral infection - regression coefficients

	pre-infection	post-infection	significance level (Mann-Whitney)
percent predicted FEV1 percent/year	-0.84	-3.1	1.00
percent predicted FVC percent/year	2.6	-7.5	0.51

Discussion

In this group of twelve patients there was a trend for lower absolute values of pulmonary function in the year following viral infection, but this did not reach statistical significance. Because pulmonary function would be expected to decline anyway during this period, it is more important to examine the rate of decline. There was a slight increase in FVC in the year preceding infection, with a decrease in the year following infection. Percent predicted FEV1 was virtually unchanged in the year preceding infection, but decreased in the year following infection. Again, the differences were not significant, but the statistical power must be very low, and it is possible that they may be real. If so, the magnitude of the change was certainly clinically significant. This would be in keeping with the case reports of Conway et al [1992], in which acute severe deterioration in pulmonary function was observed in three patients in association with serological evidence of influenza A infection, although no cases of severe deterioration were observed in this study.

It was shown above (XI.2) that there was a residual deficit in FEV1 immediately following treatment of exacerbations associated with respiratory viral infection. Inspection of the pulmonary function data in the year following infection showed that FEV1 returned to pre-exacerbation baseline in nine of the twelve patients, after a median (range) of 81 (9 - 313) days. The failure of FEV1 to

return to baseline in three patients is probably explained by their having the fastest rates of decline in pulmonary function prior to infection. Thus no long-term adverse effects of respiratory viral infection on pulmonary function have been demonstrated. The pattern of deterioration in pulmonary function associated with respiratory viral infection is therefore likely to be that of pattern 1 in figure 26, with exaggerated dips at the time of exacerbation, and in the case of FEV1 a delayed return to the baseline rate of deterioration.

Chapter XII Viral-bacterial interaction in adults with cystic fibrosis

XII.1 Introduction

It was shown in chapter XI that exacerbations associated with respiratory viral infection result in more severe changes in pulmonary function and CRP than are observed in exacerbations not associated with such infections. Is this a direct effect of viral infection, or does respiratory viral infection facilitate overgrowth of colonising pseudomonal organisms with a secondary inflammatory response directed against the bacterial component of infection ? The only study to examine laboratory parameters in naturally-acquired respiratory viral infection suggested that CRP was usually normal, in contrast to the raised levels seen in bacterial lower respiratory infections [McCarthy 1978]. If this observation is generalisable, the disproportionately raised CRP levels seen in association with respiratory viral infection in this study suggest that there may indeed be a bacterial component. On the other hand, the expected increase in white cell count was suppressed in exacerbations associated with respiratory viral infection, although this could have been due to a direct viral effect. Johansen and Hoiby [1992] found that disproportionately more patients developed chronic pseudomonal colonisation in the winter months as compared with the summer months, as determined by a defined increase in anti-pseudomonal serum precipitins, and

the same group have previously suggested that RSV co-infection results in an increase of precipitins [Petersen et al 1981]. None of the other studies of respiratory viral infection in adult patients with cystic fibrosis has attempted to address the issue.

It was shown in chapter VII that the coefficient of variation of quantitative pseudomonal colony determination was inadequate. Indirect methods therefore had to be employed. Anti-pseudomonal serum precipitins have been used but are only semi-quantitative. Specific anti-pseudomonal antibody ELISA is fully quantitative and is probably the best indirect marker of pseudomonal colonisation [Brett 1992]. A rise in antibody titre occurs consistently with initial colonisation, and may be more sensitive than conventional culture techniques [Brett 1987, Kronborg 1992].

In a cross-sectional study of young adult patients already colonised with pseudomonas Hoiby et al [1977] showed that the number of serum precipitins observed correlates with clinical status. However there are no data relating anti-pseudomonal ELISA to clinical status in adults, and no longitudinal data with either method. The first task therefore was to confirm the applicability of the method in adult patients already colonised with *P. aeruginosa*, and to confirm a correlation between ELISA score and clinical status.

XII.2 Evaluation of anti-pseudomonas antibody ELISA in adult patients with cystic fibrosis

Methods

Anti-pseudomonal antibodies were measured using the ELISA method first described by Brett et al [1986]. *P aeruginosa* serotypes 1, 3, 6, 9, 10, 11 and one untypable strain, were used, as these are the most commonly isolated serotypes in the Leeds cystic fibrosis unit. Washed cells were bound and fixed to microtitre plates. A pool of serum of known high IgG titre was used as a reference standard. Clinical samples and reference standard samples were incubated for 75 minutes after being added to the wells. Absorbance was measured with a Titretek plate reader after incubation with horseradish peroxidase conjugated goat antihuman IgG. The upper limit of normal is 200 units and the maximum score 12 000.

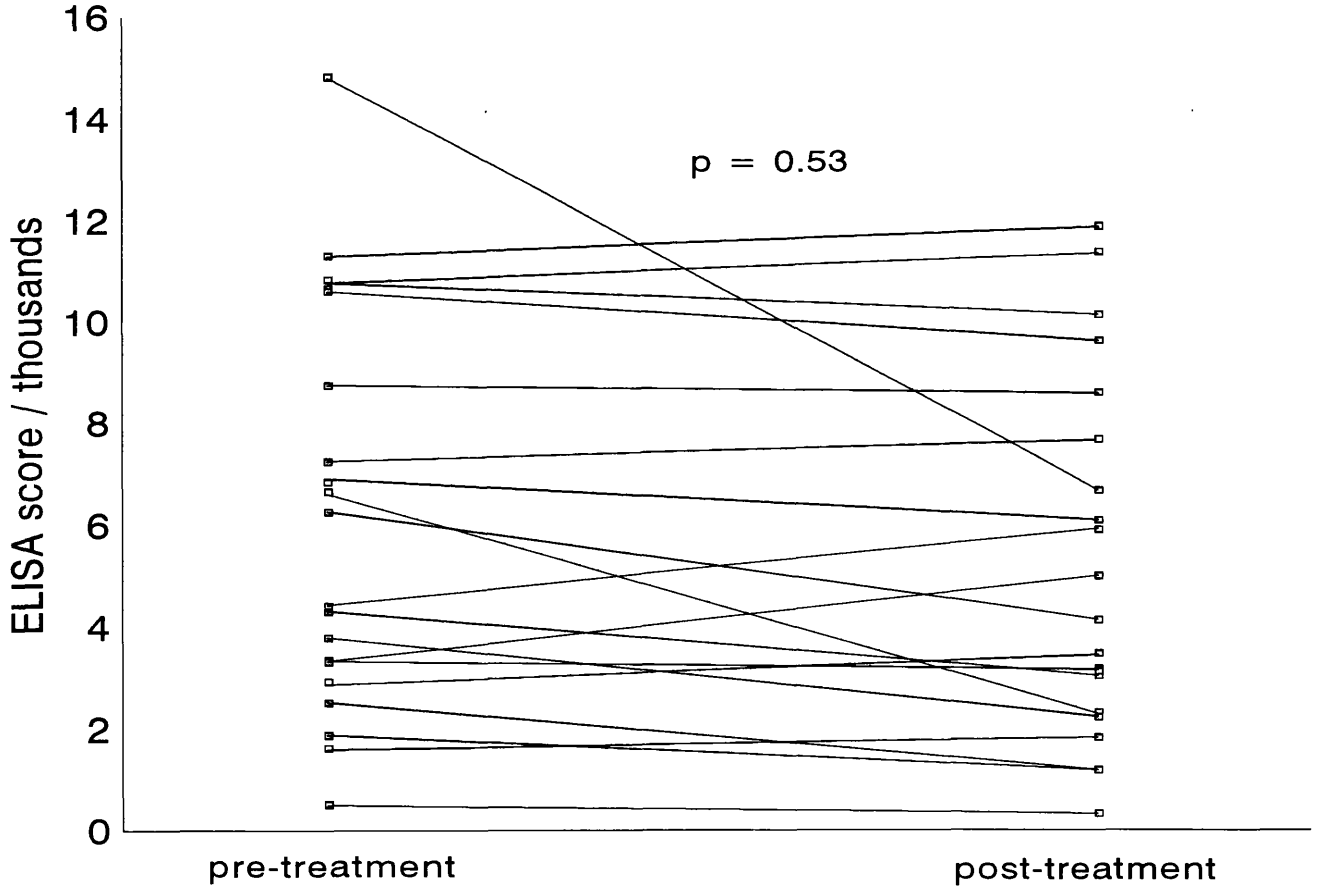
Given the absence of longitudinal data in the literature it was not known whether there would be any acute change in ELISA score with treatment of pulmonary exacerbations. ELISA scores were therefore determined at the start and end of all courses of intravenous antibiotic treatment. In addition, scores were measured in an out-patient setting in patients presenting infrequently for intravenous antibiotic treatment, with the aim of recording a score at least every six months. Twenty pulmonary exacerbations in twenty patients were selected at random, and scores before and after treatment

compared. Fifty three patients were then selected for analysis of longitudinal data on the basis of their pulmonary function, with the aim of including a broad range of disease severity. Regression analysis was carried out individually for each patient for percent predicted FEV1 and ELISA score for all the available data. The predicted value for the temporal mid-point was then taken to represent the average value during follow up for that patient. Percent predicted FEV1 and ELISA score were then compared with Pearson correlation, and divided into approximate tertiles on the basis of their ELISA score. Pulmonary function in each tertile was then compared with one way analysis of variance.

Results

In the twenty exacerbations selected at random, mean (SD) ELISA score was 6133 (3942) before treatment and 5386 (3504) after treatment, $p = 0.53$ (paired T-test). Individual changes in score are shown in figure 36.

Figure 36 - Anti-pseudomonal IgG ELISA scores, pre- and post- treatment



The 53 patients selected for longitudinal analysis were followed for a median of 740 days, range 306-1020. Two patients grew *P aeruginosa* on less than 50% of cultures, and on this basis were not considered to be chronically colonised. These two patients were excluded from further statistical analysis. In the remaining 51 patients median percent predicted FEV1 (as determined from regression) was 55, range 20 to 109. Likewise median ELISA score was 3896, range 175 to 9814. The Spearman correlation coefficient for percent predicted FEV1 vs ELISA score was -0.64, $p < 0.001$).

The regression equation was

$$\text{percent predicted FEV1} = 81.5 - 0.00526 \text{ ELISA score}$$

Dividing ELISA scores into approximate tertiles using round numbers of ELISA units as arbitrary dividing points gave a statistically significant one way analysis of variance for percent predicted FEV1 (see table 50).

Table 50 - Mean (SD) percent predicted FEV1 and numbers per group according to performance group, as determined by ELISA score tertile

ELISA score	< 3000 "good performance"	3000 - 5000 "mid performance"	> 5000 "poor performance"
percent predicted FEV1	75.2 (20.6)	58.1 (20.9)	39.4 (12.0)
number of patients	18	19	15

Oneway ANOVA $p < 0.0001$

Discussion

This is the first longitudinal study to evaluate pseudomonas-specific antibodies as markers of the host immune response. ELISA scores were identical before and after treatment of pulmonary exacerbations. The ELISA score is therefore not a short-term marker of inflammation. This is presumably because the ELISA detects IgG. The regression analysis showed a reasonable correlation between ELISA score and percent predicted FEV1. It was possible to divide the study population into performance groups on the basis of the ELISA score. ELISA score can thus serve as a useful long-term clinical marker of the immune response to *Pseudomonas aeruginosa*, much as glycosylated haemoglobin serves as a long-term marker of diabetic control.

Interestingly, the equation suggests that percent predicted FEV1 is already reduced to 80% before the ELISA score becomes elevated. This may reflect staphylococcal/haemophilus-induced damage before the onset of pseudomonal colonisation.

Of the two patients excluded from the analysis, the predominant pathogen in repeated sputum cultures was *S Aureus* in one, and *Burkholderia cepacia* in the other. ELISA score was 477, and percent predicted FEV1 54 in the first patient. ELISA score was 1393 and percent predicted FEV1 29 in the second. Both these values of percent predicted FEV1 were lower than would be predicted by the ELISA score, as judged by the three performance groups. Both patients had ELISA

scores which would have placed them in the good performance group, but values of percent predicted FEV1 in the mid performance group (patient one) and the poor performance group (patient 2). Although this "negative control" data is limited to two patients it does provide some evidence for the clinical specificity of the ELISA score for *P aeruginosa*. It is possible that ELISA score could be a useful indicator of the predominant pathogen where two or more pathogens are regularly cultured from sputum.

Figure 37 shows the most aggressive rise in ELISA score seen in the study population, with a concomitant sharp decline in percent predicted FEV1. For comparison, the relatively flat curve of total IgG determined by conventional means is also shown. Because of the wide short-term variation in pulmonary function it is difficult in clinical practice to determine whether a significant deterioration in pulmonary function is occurring. Visual inspection of the time courses of individual patients suggested that a deterioration in pulmonary function was always accompanied by a rise in ELISA score, and in some patients the increase in ELISA score appeared to predate the decline in pulmonary function by up to one year (figure 38 is one example). A significant rise in ELISA score might therefore serve as an aid in determining whether significant deterioration is occurring, or perhaps going to occur in the future, but there are insufficient data to confirm this with formal statistical analysis. This issue requires further evaluation.

Figure 37 - Example of acute rise in ELISA score, with fall in percent predicted FEV1, in patient with cystic fibrosis

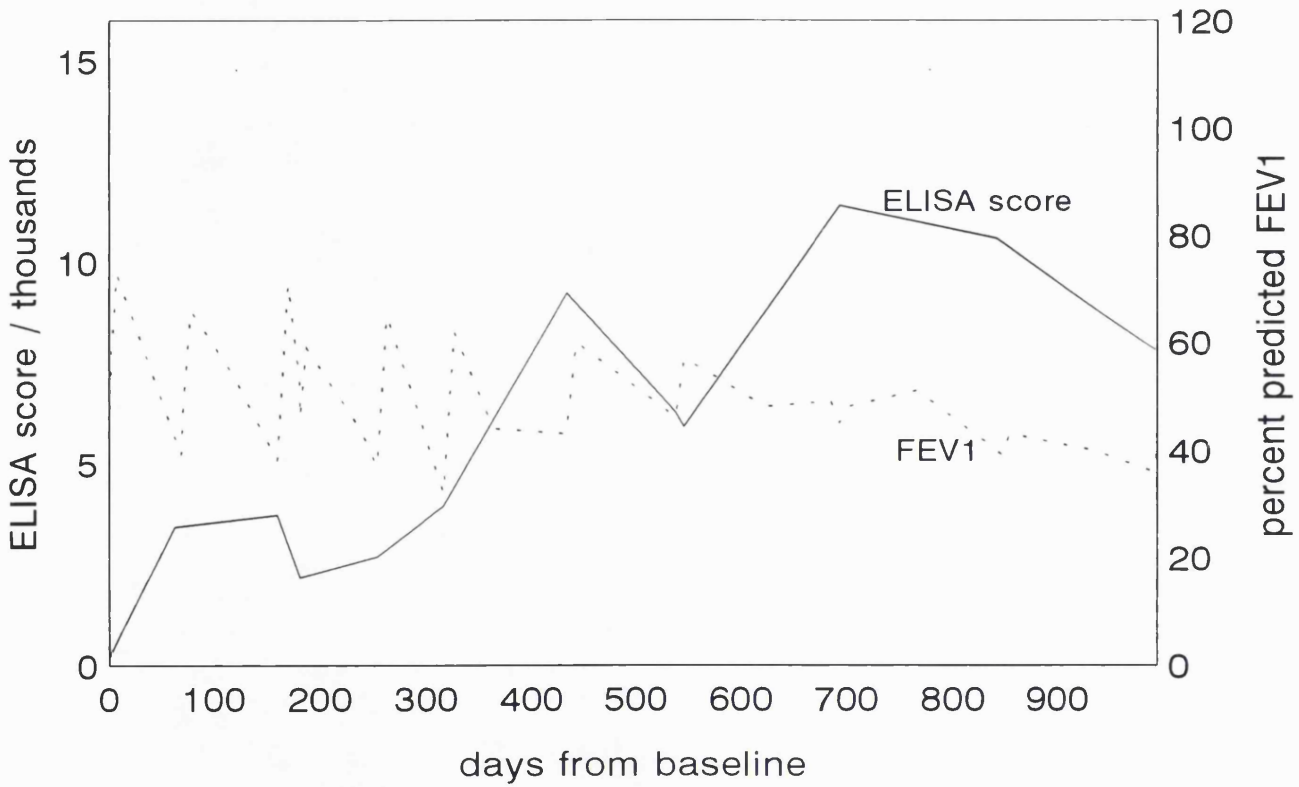
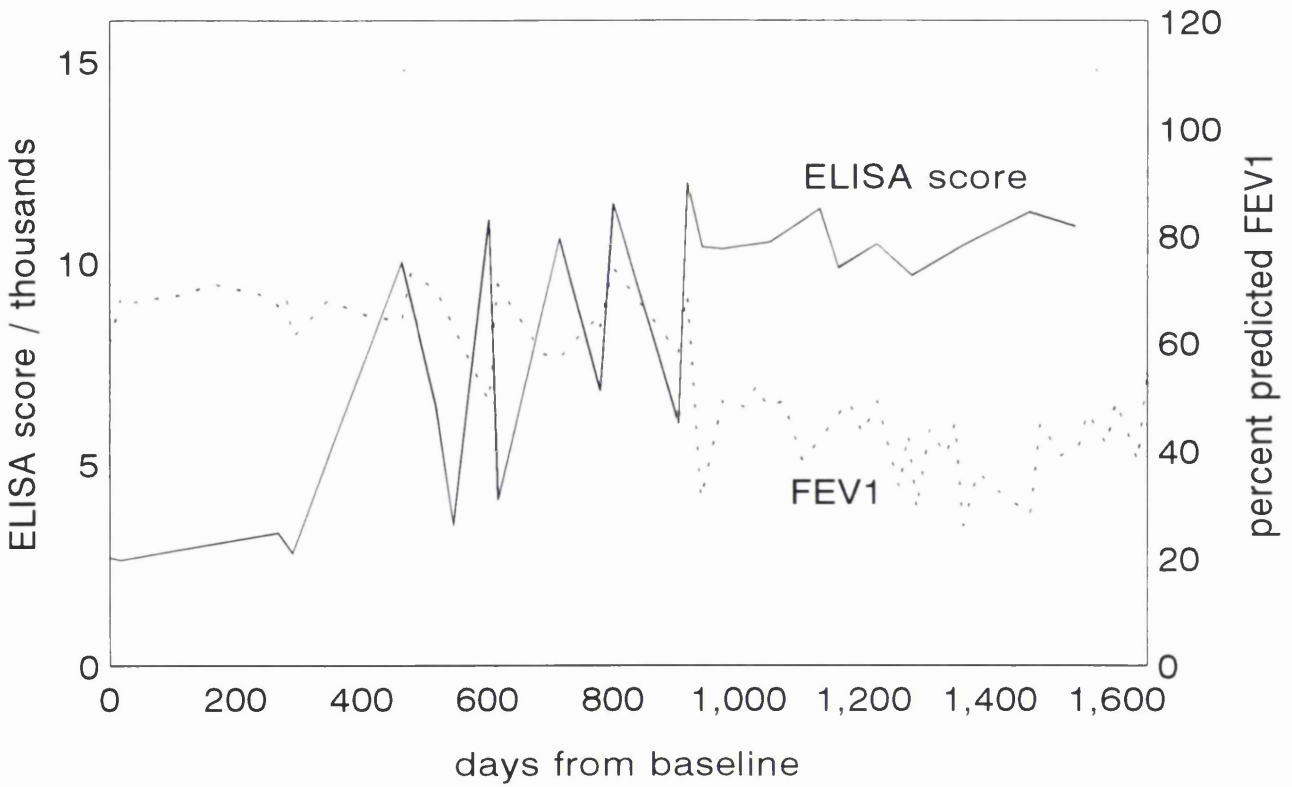


Figure 38 - Example of rise in ELISA score predating fall in FEV1 in a patient with cystic fibrosis



XII.3 Anti-pseudomonas antibody ELISA in patients with respiratory viral infection

Introduction

Since anti-pseudomonal antibody ELISA is a measure of chronic, and not acute, antibody response, it was not possible to investigate viral-bacterial interaction in the acute setting. However the ELISA provided an opportunity to investigate longer term production of antibody in response to viral infection.

Methods

Mean ELISA score in the year before respiratory viral infection was compared with the mean score in the year after infection in the members of the study cohort who had proven respiratory viral infections.

Results

ELISA scores were available for the year before and the year after respiratory viral infection in the same eleven patients whose long-term pulmonary function was analysed in chapter XI. Mean ELISA score was greater in the year following infection, 6657 vs 4332 ($p = 0.043$).

Discussion

Anti-pseudomonal ELISA score correlates inversely with pulmonary function, and is therefore a useful long-term clinical marker. Caution must be used in interpreting the association as causal however. ELISA scores are presumed to reflect the strength of the pseudomonas-specific host inflammatory response. This in turn may be a reflection of overall intrapulmonary pseudomonal load. However an increased ELISA score could just represent an epiphenomenon. If ELISA scores do genuinely reflect pathologically significant long-term changes in intrapulmonary inflammation, an increase in ELISA score would be expected to predate a fall in pulmonary function. In this study percent predicted FEV1 and FVC were 5% and 4% lower respectively following respiratory viral infection, but the difference was not statistically significant. In contrast, ELISA scores were significantly greater following viral infection. This provides confirmation of the original suggestion of Petersen et al [1981]. From the regression equation of XII.2 a fall of 14% in percent predicted FEV1 would be expected for the observed increase of 2325 ELISA units, rather than the 5% fall observed. Thus it may be that rather than causing significant adverse effects acutely, viral infection causes long-term upregulation of the specific anti-pseudomonal inflammatory response, with a delayed effect on pulmonary function. Further follow up with repeated ELISA scores and pulmonary function would be required to confirm this finding.

The finding that respiratory viruses appear to cause long-term upregulation of the inflammatory response to *pseudomonas* carries worrying implications for the delivery of gene therapy with viral vectors. Adenoviral vectors require repeated administration to deliver DNA, and such repeated administration has been shown to induce an adenoviral antibody response [Yang 1995]. In addition to concern that the antibody response may limit DNA transfection, it is possible that repeated administration of the viral vector may be detrimental through inflammatory upregulation. Liposomal delivery systems are less immunogenic, and should therefore be safer in this respect. Monitoring of anti-pseudomonas ELISA scores could be a valuable adjunct to safety studies of DNA vectors.

Upregulation of the inflammatory response could either be a direct effect mediated by any of the immunological mechanisms discussed in IV.2, or could follow if viral infection facilitates an increased intrapulmonary pseudomonal load. In the absence of a reliable method of measurement of bacterial load it is not possible to distinguish between direct and indirect upregulation, but given the specificity of the antibody assay it is likely that increased bacterial load plays at least some part. It is then interesting to speculate whether intervention with aggressive anti-pseudomonal antibiotic therapy following a rise in ELISA score, whether or not it occurs after viral infection, could be effective in preventing the expected decline in pulmonary function. It may be that this is an exception to the

inference of VII.10 that regular "asymptomatic treatments" are unlikely to confer long-term benefit. Further work is required to address this issue.

Chapter XIII - Summary of positive findings and further research

XIII.1 Summary of positive findings

Regression of the three routinely used peripheral markers of inflammation, white cell count, plasma viscosity and c-reactive protein, against percent predicted FEV1 and percent predicted FVC, showed that all three increase in a quadratic fashion with increasing disease severity (decreasing pulmonary function). However the markers behaved differently in the context of intravenous antibiotic treatment of pulmonary disease. The pre- and post- treatment regression curves for plasma viscosity showed complete overlap over the whole range of pulmonary function. The 95% confidence limits of the pre- and post- treatment regression curves diverged over most of the range of pulmonary function for both white cell count and crp. Thus for these two markers there is an added anti-inflammatory effect of antibiotic treatment over and above that which can be accounted for by the change in pulmonary function. In both cases only 20% of the observed changes could be accounted for by the expected change with improvement in pulmonary function. This added effect may be due to a) an enhanced level of the markers at the outset of treatment, with suppression towards baseline levels with treatment, b) to a "normally" elevated level at the outset of treatment, with suppression to less than baseline levels with treatment, or c) to a combination of the

two. An enhanced level of inflammatory markers at the outset of treatment would lend support to the concept of exacerbations as discreet entities, possibly with discreet precipitants, rather than simply random variations from the baseline rate of deterioration of pulmonary function.

In practical terms, plasma viscosity provides no more information than pulmonary function. White cell count and crp could have a role in monitoring the response to treatment, and in defining the term "exacerbation". Changes in white cell count were small, mean $3.0 \times 10^9/l$, approximately 25% of the upper limit of normal. Changes in crp were greater, mean 12.5 mg/dl, roughly 100% of the upper limit of normal, and should therefore be much easier to interpret in a clinical context. Unfortunately neither marker was especially sensitive. The regression equation predicted elevated pre-treatment white cell counts only at or below percent predicted FEV1 of 48%. Pre-treatment crp was predicted to be elevated at percent predicted FEV1 of 81%, but the broad spread of data meant that 38% of patients with FEV1 lower than this value did not show elevated crp levels. Thus the clinical utility of both white cell count and crp is limited, although both are useful epidemiological markers of inflammation.

Since the bulk of pulmonary damage in cystic fibrosis is thought to be mediated by host neutrophil elastase, intuitively measurement of peripheral neutrophil elastase complexed to α-1-antitrypsin (NEATC) is likely to be the most accurate marker of inflammation. The assay itself is easy to

perform, but samples must be collected on ice, centrifuged, and stored as soon as possible to minimise leakage of elastase from neutrophils following collection. A preliminary study of NEATC showed it to be elevated in each of thirty patients both pre- and post- treatment, while showing statistically significant suppression with treatment. In contrast crp was normal in 33% of pre-treatment cases and white cell count in 43% of pre-treatment cases in this study. NEATC is therefore more sensitive than these two "conventional" inflammatory markers, and may be able to serve as a marker of both acute and chronic inflammation.

In seeking a practical definition of "pulmonary exacerbation", changes in pulmonary function from baseline were first considered. Both FEV1 and FVC changed from baseline at the time of intravenous antibiotic treatment by similar proportions, but the range of differences in individuals varied widely. Assuming a deterioration from baseline of 10% of the initial value of FEV1 or FVC to be significant, using FEV1 or FVC alone gave a sensitivity of approximately 90% compared to using both FEV1 and FVC. A Bland-Altman analysis showed that the limits of agreement between the proportional changes in FEV1 and FVC were wide. Consequently both FEV1 and FVC need to be included in a definition of exacerbation based on pulmonary function.

A definition based on a deterioration from baseline of FEV1 or FVC of at least 10% was then compared with a definition based on a deterioration in two or more lower respiratory tract symptoms, derived from a structured

questionnaire. Symptomatic deterioration was significantly more frequent than significant changes in spirometry (76% vs 66%), suggesting that a symptom-based approach to the definition of exacerbation may be more sensitive than one based on pulmonary function. However, a symptom-based definition is not simply a more sensitive version of a spirometric definition, since although symptoms deteriorated in the absence of a change in pulmonary function in 16% of the treatment courses, there was no change in symptoms in 6% of treatments in which there was a pulmonary deterioration. This is unfortunate, as it would be convenient to be able to dispense with calculations of serial pulmonary function. However the sensitivity of symptom-based definition compared with a combined symptom and pulmonary function definition is 92%, and it could be argued that this is adequate.

The pulmonary function and symptom definitions behaved similarly when a severely affected subgroup, defined as having post-treatment percent predicted FVC of 45% or less, was examined. The sensitivity of FEV1 and FVC alone was 89% compared with FEV1 and FVC together. However, limits of agreement were much wider, as expected, in this group, emphasising the need for the inclusion of both FEV1 and FVC in the definition. The symptom-based definition was 96% sensitive compared with a combined symptom and pulmonary function definition.

Measurement of peripheral markers of inflammation could conceivably provide a more convenient method of defining exacerbation. However, when compared with the combined

pulmonary function and symptom definition, plasma viscosity was elevated in only 72% of exacerbations, white cell count in only 47%, and c-reactive protein in only 61%. The "conventional" inflammatory markers are therefore too insensitive to be useful although there could be a role for NEATC, which was elevated in all the intravenous antibiotic treatment courses examined.

It is assumed that most exacerbations are accompanied by an increase in the intrapulmonary load of *pseudomonas*. At present it is not possible to directly quantify total bacterial load. The surrogate, quantitative culture of sputum, proved to have a coefficient of variation too high (log-based, 22%) to be clinically useful.

Therefore of the four potential methods of defining pulmonary exacerbation, only serial pulmonary function and deterioration in two or more lower respiratory tract symptoms were practical. The exact definition of pulmonary exacerbation is arbitrary, and must ultimately be arrived at by international consensus. The sensitivity of 92% of a symptom-based definition compared to one comprising both symptoms and pulmonary function may be considered to be adequate. However, for this thesis, the combined symptom and pulmonary function definition was adopted.

Using this definition of exacerbation, there was no correlation between the number of exacerbations and the rate of decline in pulmonary function over two years, as determined by linear regression. This was not simply due to the symptom-based definition being too sensitive, as there

was no relation between the number of exacerbations when defined solely in terms of pulmonary function and the rate of loss of pulmonary function. In addition, pre-exacerbation pulmonary function was identical to pulmonary function following treatment of the exacerbation. These two results show that, providing they are adequately treated, the bulk of pulmonary exacerbations do not have significant long-term effects on pulmonary function. This result means that frequent co-infection with respiratory viruses and atypical bacteria cannot cause long-term pulmonary harm, but does not rule out a detrimental effect of sporadic infection.

Criteria for symptoms considered to be suggestive of "non-bacterial infection" (ie infection with respiratory viruses or atypical bacteria), were defined at the outset of the study. Each patient with cystic fibrosis was specifically asked about such symptoms at the start of a course of intravenous antibiotic treatment. An episode associated with suggestive symptoms was abbreviated to "SNBI". SNBI were highly associated with pulmonary exacerbations. Only one SNBI occurred in a total of 58 treatments in which there was no exacerbation. Because of concern about overlap of the symptom criteria for the definition of SNBI and exacerbation, the data were reanalysed using only pulmonary function criteria to define exacerbation. The association remained highly statistically significant, although 29 of 117 (25%) treatments which were not exacerbations were then associated with SNBI. SNBI may simply be epiphenomena, rather than indicators of co-infection.

Respiratory viruses are presumed to predominate in the winter months. The seasonal distribution of SNBI and pulmonary exacerbations was therefore examined in the cohort of 60 patients who were followed for the whole two year study period. A significant excess (21) of SNBI were documented in the winter months (defined as October through March), although there were still 40 SNBI in the summer months. There was a similar excess (18) of exacerbations in the winter months, but because of the greater number of exacerbations compared to SNBI this was not statistically significant. It is unlikely that all 101 SNBI were due to co-infection with respiratory viruses or atypical bacteria. Probably, at least 18 exacerbations in the winter months were associated with non-bacterial co-infection, with a small number in the summer months, with an additional, equal, number in the winter months.

Eighteen percent of the study cohort had demonstrable antibodies against CMV at the outset of the study. Half of these became antibody negative at some point in the two year period. Seroconversion to CMV occurred in three patients, a rate of 2.5% of the population per year. In each case seroconversion was associated with a respiratory exacerbation. There were no viral isolations in the two year study period. In addition to the 3 seroconversions to CMV, 9 further seroconversions occurred, 4 to adenovirus, 2 to influenza A, 2 to RSV and 1 to influenza B. Ten of the twelve seroconversions occurred in the cohort of 60 patients followed for the whole two years of the study, a

seroconversion rate of 0.083 per patient per year. No seroconversions to *mycoplasma*, *chlamydia*, or *legionella* occurred. One adenoviral seroconversion was asymptomatic. The remaining 11 were all associated with respiratory exacerbations, although only five met the criteria for suggestive symptoms (SNBI).

The lack of viral isolation was not due to late presentation following the initial infectious episode, as there were still no viral cultures from 60 throat swabs taken from 39 patients which were sent to the laboratory within 48 hours of the onset of symptoms.

A one year comparison of SNBI and confirmed viral and atypical infection was undertaken in 23 patients with CF, 17 patients with asthma, and 14 patients with non-CF bronchiectasis, with their household contacts acting as healthy controls. Three quarters of episodes reported by the patients in each category were associated with SNBI. In all three groups the patients reported SNBI roughly twice as frequently as their household contacts. The contacts reported 0.6 SNBI/person/year. This is likely to approximate to the true rate of infection in both contacts and patients, with the excess in the patients representing atypical symptoms arising from the underlying condition.

Uptake of influenza vaccination was 23% in the first year, and 15% in the second year of the study, as judged by seroconversion. For the purposes of the study, most of the patients were unprotected against influenza.

Since direct antigen methods are no more sensitive than

culture methods it was decided to use a PCR-based system. A multiplex PCR system worked well with laboratory controls, but was swamped by spurious priming when applied to clinical samples. The addition of a DNAase incubation step following organic acid extraction of nucleic acid reduced the problem, but separate extraction, reverse transcription, and PCR with specific primers was required to confirm positives.

There were ten positive PCR diagnoses in intravenous antibiotic treatments in an eight month period from November to June, with RSV the most frequently isolated organism. No coronaviruses, rhinoviruses or adenoviruses were found in this group. Nine viral infections were associated with exacerbations (not statistically significant). Combined serology and PCR identified viral infection in 15% of exacerbations, and 4% of treatments (one) in which there was no exacerbation. Again, the difference was not statistically significant. Combined serology and PCR identified viral infection in 8% of samples in which patients were specifically asked to present as soon as possible following the onset of SNBI. In the community study, 13% of symptomatic episodes were positive with serology and PCR. None of the swabs from asymptomatic subjects was PCR positive, although again the difference was not statistically significant. The only coronavirus identified was found in the community-based study. Overall, PCR was no more sensitive than serology, but provided an alternative method of diagnosis. Combined serology and PCR almost doubled the sensitivity of each technique used alone. As with serology, approximately half

the PCR diagnoses were associated with SNBI.

In the patients with cystic fibrosis with confirmed respiratory viral infection, percent predicted FVC was significantly lower, and there was a trend for lower percent predicted FEV1 which approached statistical significance, as determined by regression analysis over the two year study period. The number of treatments (and hence the frequency of sampling) was also greater in the group with confirmed viral infection. The finding of poorer pulmonary function in the group with confirmed infection might therefore have arisen through confounding by more frequent sampling, but the almost complete absence of asymptomatic seroconversion suggests that this was not the case.

Pulmonary function and inflammatory markers at the outset of treatment, and overall length of treatment, were identical in exacerbations with and without SNBI (patients acting as their own controls). The change from baseline FEV1 was significantly greater in exacerbations with confirmed viral infection. The change from baseline FVC was also greater by a similar amount, but was not statistically significant. Plasma viscosity and CRP were both greater in exacerbations with confirmed viral infection, but white cell count was identical. Viral infection therefore suppresses the increase in neutrophils expected with exacerbation. There was a trend for longer treatment courses in exacerbations with confirmed viral infection, but this was not statistically significant.

Following cessation of treatment, percent predicted FVC

reverted to pre-treatment levels, but a deficit in percent predicted FEV1 persisted. This deficit eventually corrected itself after a median of 81 days in 9 of the 12 patients. Absolute values of percent predicted pulmonary function were lower, and their rate of decline higher, in the year following viral infection. However, neither was statistically significant. With such a small sample size the statistical power must be extremely low, and it is possible that these effects are real.

Anti-pseudomonal IgG, as determined by ELISA, did not change acutely with administration of intravenous antibiotics. Longitudinal analysis in 53 patients showed a good correlation between ELISA score and percent predicted pulmonary function. ELISA scores were significantly greater in the year following infection with respiratory viruses, than in the preceding year. Increases in ELISA score might be expected to predate the deterioration in pulmonary function. Although the observed change in ELISA score predicted a fall of 14% in percent predicted FEV1, only a 5% fall was observed. Thus it may be that rather than causing significant adverse effects acutely, viral infection causes long-term upregulation of the specific anti-pseudomonal inflammatory response, with a delayed effect on pulmonary function.

XIII.2 Further Research

Conventionally measured inflammatory markers are clearly inadequate for clinical use, but have some utility in a research context. Neutrophil elastase is much more sensitive, and a larger study to evaluate its relation to pulmonary function, both chronically and in the context of exacerbations, is warranted. A larger study to document whether the specific anti-inflammatory effect of intravenous antibiotic treatment on white cell count, crp (and neutrophil elastase ?) arises through specifically elevated pre-treatment levels at the time of exacerbation would be helpful in distinguishing whether the bulk of exacerbations are specific entities, or arise through random variation in inflammation/pulmonary function. If most exacerbations are specific entities, the hunt for precipitating causes, including viruses, becomes worthwhile. Such a study would require continued collection of inflammatory markers at frequent, regular intervals, and would be difficult in an adult CF population.

A method which can quantify total pulmonary pseudomonal load is required, partly to evaluate the relationship between bacterial load and exacerbation, and also to examine viral-bacterial relationships in more detail. A possible method might be to measure serum or urine levels of the water soluble siderophores produced by *P aeruginosa*.

The great problem with all epidemiological studies of viral infection is with the viral diagnostic methods. Culture

methods, direct and indirect antigen methods, and serological methods are all probably insensitive, although in the absence of a gold standard it is impossible to know just how insensitive. Polymerase chain reaction techniques should theoretically be much more sensitive, although they have a definite lower detection limit. Although the PCR system developed here worked reasonably well, no rhinoviruses and only one coronavirus were identified. A less cumbersome system with greater sensitivity and specificity is required. This could most easily be achieved by subjecting the initial PCR products to a secondary PCR reaction, which would amplify specific fragments further, and cut out non-specific fragments. Ideally this would be combined with a simpler method of nucleic acid extraction than organic acid extraction and ethanol precipitation, and a reverse transcription reaction primed by random oligonucleotides. Such a system ought to be feasible. With such a system, further epidemiological studies in patients with various respiratory conditions and in the normal population could be undertaken.

Further longitudinal analysis of anti-pseudomonal IgG ELISA scores vs pulmonary function is required to further investigate the exciting possibility that rising scores may predate deteriorations in pulmonary function, perhaps yielding a window during which treatment could be intensified in an effort to prevent or delay the deterioration.

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Appendix - symptom questionnaire proforma

Patients were asked specifically about the following symptoms, and the appropriate entry ringed.

1. Cough

current	nil	occasional	frequent	constant
usual	nil	occasional	frequent	constant

2. Sputum volume

current	nil	<10	20	50	100	>100	ml/day
usual	nil	<10	20	50	100	>100	ml/day

3. Sputum colour

current	white	yellow	light green	dark green	brown
usual	white	yellow	light green	dark green	brown

4. Dyspnoea

current	none	moderate exercise	light exercise	rest
usual	none	moderate exercise	light exercise	rest

4. a) Dyspnoea on exercise

current yes no

usual yes no

if yes state distance able to walk current

usual

distance able to run current

usual

5. Wheeze

current none occasional frequent constant

usual none occasional frequent constant

6. Fatigue

current none moderate exercise light exercise rest

usual none moderate exercise light exercise rest

7. Weight loss

a) yes how much .....

over how long .....

b) no