ORIGINAL ARTICLE

Chlamydia pneumoniae and chronic bronchitis: association with severity and bacterial clearance following treatment

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Background: A study was undertaken to evaluate *Chlamydia pneumoniae* chronic infection, other respiratory infections, and functional impairment in patients with chronic bronchitis (stage 1) and to examine chronic *C pneumoniae* infection, rate of acute exacerbations of chronic bronchitis, and rate of *C pneumoniae* eradication following antibiotic treatment (stage 2).

Methods: In the stage 1 study respiratory specimens from 42 patients with steady state chronic bronchitis were analysed for Gram staining, sputum culture, and *C pneumoniae* DNA detection by nested touchdown polymerase chain reaction (PCR). On the basis of the results of stage 1, a second population of 141 consecutive patients with steady state mild to moderate chronic bronchitis ($FEV_1 \geqslant 50\%$ predicted) was studied. On admission, at regular intervals, and at exacerbation all patients underwent serological testing for *C pneumoniae* (microimmunofluorescence) and a nested touchdown PCR to detect *C pneumoniae* DNA was performed on peripheral blood mononuclear cells (PBMCs). Patients were assessed over a 12 month period. Information regarding the previous 12 months was taken from medical records.

Results: Chronic colonisation of the sputum with *C pneumoniae* was significantly associated with lower FEV_1 and greater airway bacterial colonisation. On admission to the stage 2 study, 80 patients were PCR negative and 61 were PCR positive. Over the 2 years a mean (SD) of 1.43 (1.32) acute exacerbations occurred in PCR negative patients and 2.03 (1.21) in PCR positive patients ($p<0.01$). During the 12 month follow up period 34 PCR positive patients had acute exacerbations and were treated with azithromycin for 6 weeks. Serological evidence of acute *C pneumoniae* reinfection/reactivation was found in two of the 34 patients. The rate of *C pneumoniae* DNA clearance from blood following treatment was 29% at follow up.

Conclusion: Chronic colonisation with *C pneumoniae* is associated with a higher rate of exacerbations of chronic bronchitis. Long term treatment is required to obtain clearance of the organism from the blood.

Chlamydia pneumoniae has been recognised as a cause of respiratory tract infections and is considered the most common non-viral intracellular human respiratory pathogen.[1] It is involved in a wide spectrum of respiratory infections of the upper respiratory tract (pharyngitis, sinusitis and otitis) and lower respiratory tract (acute bronchitis, exacerbations of chronic bronchitis and asthma, community acquired pneumonia) in both immunocompetent and immunocompromised hosts.[2]

Chronic *C pneumoniae* infection has been found to be common in chronic bronchitis and could contribute to disease progression by a toxic effect on bronchial epithelial cells, impairing ciliary function, and increasing chronic inflammation via proinflammatory cytokine production. Several studies have provided evidence of this involvement. Blasi *et al* reported a significantly higher prevalence of the IgG antibody fraction in patients with chronic obstructive pulmonary disease (COPD) than in controls, particularly in those aged over 50 years.[3] Moreover, they found an increase in both specific IgG prevalence and geometric mean titre with age, suggesting chronic infection. Another study in hospitalised and non-hospitalised patients with COPD showed a significantly higher IgA seroprevalence in COPD patients than in control subjects.[4] The authors also found a correlation between the geometric mean titre of IgA and COPD severity that persisted after controlling for smoking. Some years later the same group studied patients with COPD enrolled in a separate geographical area.[5] They determined four different markers of *C pneumoniae* infection: (1) serum antibodies, (2) sputum antibodies (secretory IgA), (3) sputum polymerase chain reaction (PCR), and (4) circulating immunocomplexes. The authors found a gradient of marker positive patients which increased from mild to severe COPD. The overall prevalence of chronic infection was 71% in patients with severe COPD and 46% in those with mild COPD.

A hypothetical model for the *C pneumoniae*/chronic bronchitis interaction was discussed in a recent review.[6] In this model involvement of *C pneumoniae* in patients with COPD is characterised by raised serum antibodies and the presence of secretory IgA in sputum, both associated with a Th2 type immune response that may lead to chronic infection.

C pneumoniae infection induces secretion of tumour necrosis factor (TNF)-α by monocytes,[7] a cytokine involved in the endothelial and epithelial expression of IL-1 and adhesion molecules.[8] TNF-α production is induced by *C pneumoniae* heat shock protein 60 (HSP60) in a concentration and time dependent fashion.[9] HSP60 also induces production of matrix metalloproteinases (MMPs) by macrophages, particularly MMP-9.[9] Recent observations have contributed to growing evidence of the importance of macrophages and MMPs in the pathogenesis of emphysema.[10 11]

In addition, *C pneumoniae* infection activates nuclear factor-κB (NF-κB) which is thought to control the transcriptional initiation of inflammatory genes.[12 13] The genes encoding

Table 1 Association between *C pneumoniae* PCR sputum positivity and pathogens on sputum culture in 42 patients with steady state chronic bronchitis (stage 1)

	C pneumoniae +ve (16 patients/69 sputum samples)	*C pneumoniae* –ve (26 patients/125 sputum samples)
No pathogen	24	86
H influenzae	18	15
S pneumoniae	17	14
M catarrhalis	17	6
Enterobacteriacae	9	5
S aureus	6	1
Pseudomonas spp	4	6
Fungi	1	0
Total number of isolated bacteria*	72†	47†

*More than one pathogen isolated from 20 and six sputum samples obtained from *C pneumoniae* PCR positive and negative patients, respectively.
†p<0.05 isolated pathogens/sputum samples (*C pneumoniae* PCR positive *v* negative patients).

proinflammatory cytokines such as TNF-α and IL-1β, chemokines including IL-8, RANTES, and macrophage chemotactic protein-3, adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, and inflammatory enzymes such as COX-2 have all been shown to depend on NF-κB for their expression. A recent study showed that the level of NF-κB activity is highly correlated with the degree of lung dysfunction and that the kinetics of NF-κB activity are strongly related to the course of the disease in an animal model of asthma.[14]

Preliminary data on chronic infection with *C pneumoniae* in patients with COPD and its interaction with host cells indicate that this agent may be responsible for the *modulation* of the natural history of chronic bronchitis and emphysema. However, a recent paper questioned the influence of *C pneumoniae* infection—defined by serological analysis—on the decline in lung function.[15]

We examined the association between *C pneumoniae* infection, degree of airflow limitation, and rate of acute exacerbations using a combined diagnostic approach for the identification of chronic *C pneumoniae* infection.

METHODS

The study was performed in two stages. During the first part (stage 1) we examined the association between *C pneumoniae* infection and baseline severity of chronic bronchitis. In the second part of the study (stage 2) we prospectively evaluated the rate of acute exacerbations in *C pneumoniae* positive and negative patients with mild to moderate chronic bronchitis. Patients with suspected bronchiectasis were excluded from the study. Written informed consent was obtained from all patients and the study was approved by the ethical committee of the University of Milan.

Stage 1

Between 1996 and 1997 our pulmonary rehabilitation centre enrolled 42 patients (31 men) of mean (SD) age 62.2 (6.3) years (range 43–78) with steady state chronic bronchitis (mean forced expiratory volume in 1 second (FEV_1) 66.3 (19.8)% of predicted value), defined by the absence of an acute exacerbation during the previous 4 weeks.

Patients were assessed over a 12 month period. At least four sputum samples were obtained from each patient at 4 week intervals with the aid of chest physiotherapy under the supervision of trained nurses. Gram staining and culture were carried out on all sputum specimens. Validity of sputum samples was determined by the presence of ⩾25 polymorphonuclear leucocytes/field and <10 squamous epithelial cells/field. Culture was considered significant when a pathogen count of $\geqslant 10^6$ cfu/ml was obtained. Respiratory specimens were also analysed to detect *C pneumoniae* DNA by nested touchdown PCR.[16]

For each patient a serological test for *C pneumoniae* was performed using a microimmunofluorescence (MIF) test for IgG, IgM, and IgA (Labsystems, Helsinki, Finland). MIF results were classified as follows: past (chronic infection) pattern (⩾16 IgG <512, ⩾16 IgA <256); acute first infection (IgM ⩾16 associated with IgG ⩾512, IgA ⩾256, or fourfold increase in IgG or IgA titres); reinfection (IgG ⩾512, IgA ⩾256, or fourfold increase in IgG or IgA titres).

Lung function tests and blood gas analysis were performed in all patients on admission and at each successive visit.

Stage 2

A different population of 141 consecutive patients (100 men) of mean (SD) age 68.2 (7.5) years (range 47–81) with steady state mild to moderate chronic bronchitis (FEV_1 73.6 (15.3)% of predicted value) was subsequently studied. On admission all patients underwent a serological test for *C pneumoniae* using the MIF test as described above. In addition, *C pneumoniae* DNA detection was performed on peripheral blood mononuclear cells (PBMCs).[17] Patients were defined as *C pneumoniae* positive or *C pneumoniae* negative on the basis of the PCR results. Lung function tests and blood gas analysis were performed for all patients on admission. Patients were assessed over a 12 month follow up period. Information regarding the previous 12 month period was taken from medical records.

Acute exacerbations were defined by the Winnipeg I criteria.[18] During the observation period patients were contacted by telephone periodically and all subjects were instructed to report to the outpatient clinics if an exacerbation occurred. At the first acute exacerbation *C pneumoniae* DNA positive patients were treated with azithromycin for a total of 6 weeks (0.5 g daily for 3 days on 3 successive weeks followed by a single dose of 1 g/week for 3 weeks). At the onset of an exacerbation all patients performed sputum culture, MIF *C pneumoniae* serology, *C pneumoniae* DNA detection on PBMCs, blood gas analysis and lung function testing, all of which were repeated 1 week later. During the following 2 weeks the patients were contacted by telephone to ascertain compliance with treatment and adverse events. Successive visits were performed at weeks 4 and 7 (end of treatment). A first follow up examination was performed approximately 2 months (72 (9) days) after the end of treatment.

Analysis of data

Data were analysed using the SPSS statistical package. Homogeneity analysis was performed using the ANOVA F-test for parametric data and a χ^2 test for non-parametric data. Wilcoxon's rank sum test and median test were applied for comparison of exacerbations between PCR positive and negative groups.

Table 2 Association between *C pneumoniae* chronic infection and severity of lung function impairment in 42 patients with steady state chronic bronchitis (stage 1)

	FEV_1 ⩽35%	FEV_1 35–50%	FEV_1 >50%
C pneumoniae +ve (n=16)	8	4	4
C pneumoniae –ve (n=26)	2	8	16

FEV_1=forced expiratory volume in 1 second.
p<0.01.

Table 3 Correlation between *C pneumoniae* serological findings and PCR results on PBMCs in 141 patients with mild to moderate chronic bronchitis (stage 2)

	IgG ⩾1:16	IgG ⩾1:16 + IgA ⩾1:16	Negative
C pneumoniae PCR +ve (n=61)	17 (28%)	37 (61%)	7 (11%)
C pneumoniae PCR –ve (n=80)	32 (40%)	28 (35%)	20 (25%)

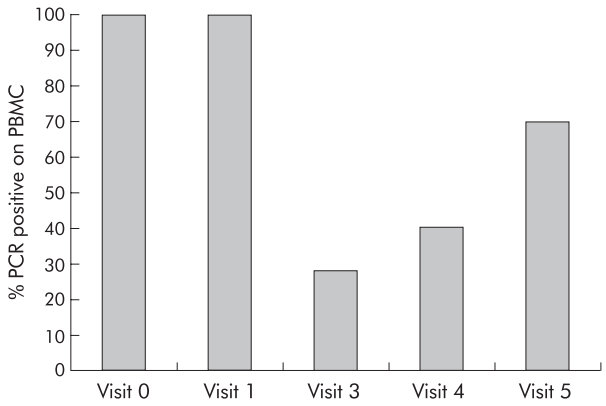

Figure 1 Percentage of *C pneumoniae* clearance from blood in 34 patients with chronic bronchitis treated with azithromycin for 6 weeks (visit 0 = screening, visit 1 = exacerbation, visit 3 = 4th week of treatment, visit 4 = end of treatment, visit 5 = follow up 72 (9) days after visit 4).

RESULTS

Stage 1

Of the 42 patients enrolled, those whose respiratory samples were *C pneumoniae* DNA PCR positive (n=16) had a significantly greater number of pathogens on sputum culture than PCR negative patients (n=26, table 1). In addition, PCR positivity for *C pneumoniae* was significantly more common in chronic bronchitis patients with greater functional impairment (table 2).

Stage 2

On admission 80 of the 141 patients were *C pneumoniae* PCR negative and 61 were PCR positive on PBMCs. No significant differences in age, sex, smoking habits, and FEV_1 were observed between PCR negative and positive subjects. Correlation between serological and PCR results is shown in table 3.

Over the 2 year observation period a mean (SD) of 1.43 (1.32) acute exacerbations occurred in PCR negative patients and 2.03 (1.21) in PCR positive patients (p=0.0021, Wilcoxon's rank sum test; p<0.01 median test (χ^2 = 7.947)). Thirty four of the 61 PCR positive patients with an exacerbation agreed to treatment with azithromycin for 6 weeks. Serological evidence of acute *C pneumoniae* reinfection/reactivation was found in two of the 34 patients. The geometric mean *C pneumoniae* IgG titre between admission and follow up decreased from 128 (4.8) to 77.5 (4.5), p<0.0001, and the geometric mean IgA titre between admission and follow up varied from 17 (2.25) to 16.5 (3.1), p=NS.

Clearance of *C pneumoniae* from the blood following treatment with azithromycin was achieved in 20 of the 34 patients (59%) at the end of treatment and in 10 (29%) at follow up, as assessed by PBMC PCR. Figure 1 shows variations in the rate of *C pneumoniae* PBMC PCR positivity during the study period.

DISCUSSION

In patients with COPD the relevance of chronic infection may be twofold: persistence of micro-organisms in the respiratory tract may facilitate access of different pathogens to the lower airways, and longstanding infection might trigger what is traditionally described as the vicious circle of chronic bronchitis. According to this hypothesis, infection may play an aetiological role in the development of the disease and act as a worsening factor. Epithelial damage, impairment of defence mechanisms, and an increase in the inflammatory and oxidative burden within the airways may all accelerate the functional and anatomical deterioration associated with the disease.

The possibility of chronic colonisation with *C pneumoniae* in patients with COPD, first suggested on the basis of serological evidence, has recently been further supported by electron microscopic and immunohistochemical identification of the organism in diseased airways.[19 20]

The first stage of this study indicates that patients with chronic bronchitis with chronic *C pneumoniae* infection have a higher rate of airway microbial colonisation. This may be because persistence of this intracellular organism in the airways favours ongoing inflammatory processes, dampens the immune response, and depresses mucociliary function.[12–13 21] Moreover, we found that the probability of chronic colonisation with *C pneumoniae* is significantly associated with lung function, as shown by the increased rate of *C pneumoniae* positivity in patients with more severe functional impairment.

In order to clarify whether chronic *C pneumoniae* infection may contribute to disease progression from mild to severe functional impairment, we performed a second stage study on patients with mild to moderate COPD. Data from the this study show that the presence of chronic *C pneumoniae* infection, as assessed by PCR on peripheral blood monocytes, in patients with chronic bronchitis is associated with a greater propensity to develop acute exacerbations. Our group has previously shown that the rate of *C pneumoniae* DNA identification is similar in biopsy specimens (vascular and bronchial) and PBMCs, which suggests that blood PCR may be a useful tool

for identifying patients with chronic *C pneumoniae* infection.[17 22]

C pneumoniae DNA positive patients who developed exacerbations were started on a prolonged (6 weeks) course of treatment with azithromycin. This antibiotic has a high level of intracellular accumulation and has been shown to be effective against *C pneumoniae*. Serial PCR testing on PBMCs from patients with an exacerbation of chronic bronchitis showed transient clearing of *C pneumoniae* from the blood. At the end of the 6 week treatment period clearance of *C pneumoniae* was observed in 20 of 34 patients (59%). Approximately 2 months later the rate of clearance had decreased to 29%. The persistence of *C pneumoniae* in the blood following treatment is not attributable to reinfection since no serological evidence consistent with acute infection was observed. We suggest that the treatment was insufficient to eradicate the organism completely and that the subsequent increase in the rate of infection may be due to new release of the organism into the bloodstream. On the other hand, we feel that the cases in which bacterial clearance occurred should be attributed to the effect of antibiotic treatment since spontaneous clearance seems unlikely, given that all of the 34 patients with *C pneumoniae* DNA detection in PBMCs on admission were persistently PCR positive on visit 1 after 111.8 (67.5) days.

As in previous studies,[17] our results show a low degree of correlation between *C pneumoniae* serology and PBMC PCR. A greater degree of correlation was observed combining IgG and IgA titres. Furthermore, following treatment we observed a significant fall in the geometric mean IgG titre but the IgA titre was unchanged. Considering the limited half life of IgA antibodies in the organism, persistent titres probably indicate ongoing *C pneumoniae* antigen release. Antibiotic treatment, delivered over a 6 week period, may therefore achieve bacterial clearance from the blood rather than systemic eradication. Whether the observed reduction in the bacterial burden is sufficient to alter the natural history of the disease deserves further investigation. On the other hand, as in other intracellular chronic infections such as *Mycobacterium tuberculosis*, complete eradication probably requires prolonged treatment periods and/or combination therapy.

One of the limitations of the present study is associated with the methodological problems regarding standardisation of both the diagnostic techniques (MIF and PCR) used for detection of *C pneumoniae*.[23 24] A meeting was recently convened by the Centers for Disease Control and Prevention and the Laboratory Centre for Disease Control to provide recommendations for standardised approaches to *C pneumoniae* diagnostic testing.[25] MIF is recommended as the only reliable serological test available in commercial kits, and standardised definitions for "acute" and "past" infection are proposed. These definitions are in line with those used throughout our study. With regard to PCR techniques, although no assay has been commercially standardised or cleared by the FDA, the above mentioned paper identifies four PCR protocols (including the nested touchdown PCR used in our study) that have been validated for sensitivity and specificity in two or more outside laboratories, possess a level of detection of $\leqslant 1$ inclusion forming units, and whose specificity has been tested against other *Chlamydia* species as well as other prokaryotic and eukaryotic DNA. Furthermore, in this study the researchers conducting PCR assays and interpreting the results were blinded to patient status (sputum culture positivity, functional impairment, exacerbation rate) and to the results of MIF testing. In a previous study our group performed PCR testing twice in all patients and in 28 of 30 cases the results were in agreement in terms of *C pneumoniae* DNA detection, indicating that the technique is highly reproducible.[26] In addition, a recent paper has shown that detection of *C pneumoniae* in circulating monocytes by PCR is reproducible and correlates with the results of other detection techniques such as immunoelectron microscopy and culture.[27]

Peripheral blood mononuclear cell PCR for *C pneumoniae* is probably a useful tool in identifying chronic *C pneumoniae* infection and in monitoring bacterial clearance following treatment. In addition, IgA titres may be usefully combined with PCR results in defining chronic *C pneumoniae* infection.

The results of our study indicate a possible role for *C pneumoniae* chronic infection in disease progression in patients with COPD. Further confirmation in large scale trials is needed.

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