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Chronic Infection with Chlamydia Pneumoniae in COPD and Lung Cancer

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Utan tvivel är man inte klok

Tage Danielsson
Samlade tankar från roten

ABSTRACT

Lung cancer and chronic obstructive pulmonary disease (COPD) are major causes of morbidity and mortality throughout the world. Smoking is the main cause for both diseases, but other factors seem to contribute. *Chlamydia pneumoniae* (Cpn) is an obligate intracellular bacterium with a unique biphasic replicative cycle associated with respiratory infections. The ability to cause chronic infections is characteristic for bacteria of the family *Chlamydiaceae*. Persistent elevated IgA antibodies to Cpn is used as a marker for chronic Cpn infection. The aims of the thesis were to study the prevalence of chronic Cpn infection in patients with lung cancer (I), to study the prevalence of chronic Cpn infection in relation to lung function in patients without lung cancer (II), to study a new treatment regimen in patients with longstanding airway and/or pharyngeal symptoms and chronic Cpn infection (III), and to detect Cpn in cytopsin preparations from bronchoalveolar lavage (BAL) fluid and in lung tissue from patients with COPD (IV). In studies I-II we investigated patients who underwent bronchoscopy due to longstanding airway symptoms and/or pathological chest X-rays.

Study I: 136 men and 74 women with lung cancer (LC) were included. Currently smoking or ex-smoking consecutively collected blood donors and 70-year olds from a population study were used as control groups. Blood specimens for specific Cpn antibodies were analysed using the microimmunofluorescence (MIF) technique. The prevalence of Cpn IgG antibody titres of $\geq 1/512$ was 57% in male LC patients compared to 27% in male 70 year olds and 17% in male blood donors. The prevalence of Cpn IgA antibody titres $\geq 1/64$ was 69% for male LC patients compared to 25% and 20% for respective control groups. The difference between male patients and controls was statistically significant. For female LC patients a statistically significant difference was found in prevalence only regarding IgA antibodies; 57% compared to 30% and 9% for the control groups.

Study II: 199 patients for whom spirometry and paired serum samples were available were included. Thirty patients fulfilled criteria for COPD. Antibodies in acute and convalescent sera were analysed by MIF. Chronic Cpn infection (defined as stable IgA titre $\geq 1/64$) was present in 85 patients. IgA titres increased with age in both COPD and non-COPD patients, but were higher in the COPD group independent of age. Chronic infection was associated with smoking and higher age, but no

difference was observed between genders. A statistically significant association was observed between chronic Cpn infection and COPD. This remained after correction for smoking.

Study III: 103 patients were treated with azithromycin 500 mg daily for five days, repeated 3 times with a 23 days interval, or placebo. Patients were examined 4 months and one year after completed treatment. A general improvement of symptoms and less hawking was found in patients treated with azithromycin compared to placebo after 4 months, but there was no sustained difference one year after completed treatment. The antibody titres remained stable, and there was no influence on lung function.

Study IV: Cytospin preparations of BAL fluid from 14 COPD patients, 10 healthy smokers and 7 non-smokers were studied using a direct immunofluorescence technique for detection of Cpn. Lung tissue from 24 patients with emphysema were tested using immunohistochemistry (IHC) for Cpn. Serum samples were available for all patients undergoing BAL and in 11 of the emphysema patients. Elementary body like structures were detected in cytospin slides from 29% of COPD patients, 10% of healthy smokers and 14% of non-smokers. Cpn was detected in lung tissue from 8% of patients with advanced emphysema. COPD patients demonstrated a tendency to have specific Cpn IgA $\geq 1/64$ to a larger extent than the control groups, but no correlation was found between detection of Cpn and antibody titres.

Conclusions: An association was found between serological signs of chronic Cpn infection and COPD and lung cancer. Cpn was detected in the respiratory tract in a minority of the patients, but there was no correlation between the presence of organisms and antibodies. Azithromycin treatment resulted in transient effect on symptoms, without affecting the antibody titres or lung function.

LIST OF PAPERS

- I Hirsh Koyi, Eva Brandén, Judy Gnarpe, Håkan Gnarpe, Bertil Steen. An association between chronic infection with *Chlamydia pneumoniae* and lung cancer. A prospective 2-year study. *APMIS* 2001;109:572-580
- II Eva Brandén, Hirsh Koyi, Judy Gnarpe, Håkan Gnarpe, Göran Tornling. Chronic *Chlamydia pneumoniae* infection is a risk factor for the development of COPD. *Respiratory Medicine* 2005;99:20-26
- III Eva Brandén, Hirsh Koyi, Judy Gnarpe, Håkan Gnarpe, Göran Tornling. Intermittent Azithromycin treatment for respiratory symptoms in patients with chronic *Chlamydia pneumoniae* infection. *Scand J Infect Dis* 2004;36:811-816
- IV Eva Brandén, Judy Gnarpe, Håkan Gnarpe, Gunnar Hillerdal, Lotta Orre, C Magnus Sköld, Magnus Löfdahl, Hirsh Koyi, Göran Tornling. Detection of *Chlamydia pneumoniae* on cytopsin preparations from bronchoalveolar lavage in COPD patients and in lung tissue from advanced emphysema. Manuscript

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LIST OF ABBREVIATIONS

AEC	Alveolar epithelial cell
ATP	Adenosine triphosphate
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
CMV	Cytomegal virus
COPD	Chronic obstructive pulmonary disease
Cpn	Chlamydia pneumoniae
CT	Computer tomography
DFA	Direct fluorescence antibody
DLCO	Carbon monoxide diffusion capacity
EB	Elementary body
ETS	Environmental tobacco smoke
FEV ₁	Forced expiratory volume in one second
FVC	Forced vital capacity
GOLD	Global initiative for chronic Obstructive Lung Disease
HPV	Human papilloma virus
HRCT	High resolution computer tomography
Hsp	Heat shock protein
IFN- γ	Interferon γ
IgA-M	Immunoglobulin A-M
IHC	Immunohistochemistry
IL1-10	Interleukin 1-10
LVRS	Lung volume reduction surgery
MIF	Microimmunofluorescence
MMP-9	Matrix metalloproteinase -9
NSCLC	Non small cell lung cancer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PY	Pack years
RB	Reticulate body
SCLC	Small cell lung cancer
SEM	Scanning electron microscopy
T _{1/2}	Half time for elimination
TEM	Transmission electron microscopy
Th2	T-helper lymphocyte, type 2
TNF- α	Tumour necrosis factor- α
UV	Ultraviolet

INTRODUCTION

Chlamydia pneumoniae (Cpn) is an obligate intracellular bacterium with a unique biphasic replicative cycle (Figure 1). It can cause acute upper and lower respiratory tract infections such as otitis, sinusitis, pharyngitis, bronchitis, and pneumonia, and is also involved in exacerbations of COPD (Blasi et al. 1993; Mogulkoc et al. 1999). Most individuals experience two or three Cpn infections during a lifetime (Grayston et al. 1990). The ability to cause chronic infections is characteristic for bacteria of the family *Chlamydiaceae*. Chronic infection may follow acute, often asymptomatic primary infections. Studies *in vitro* have demonstrated a persistent inactive state with aberrant morphological forms of the bacterium, which can be reactivated if the environmental conditions change.

Chronic infection with Cpn has been associated with COPD (von Hertzen et al. 1996; Von Hertzen et al. 1997), asthma (Hahn et al. 1991; Hahn 1999), cardiovascular disease (Saikku et al. 1992 a); Kuo et al. 1993) and lung cancer (Jackson et al. 2000; Koyi et al. 2001). The aims of this thesis was to study the association between chronic Cpn infection and respiratory diseases such as COPD and lung cancer, and to evaluate a new strategy for treatment of patients with longstanding respiratory and/or pharyngeal symptoms and chronic Cpn infection.

GENERAL BACKGROUND

Chlamydia Pneumoniae

History

Chlamydia pneumoniae was first isolated in 1965, when it was cultured from the conjunctiva of a Taiwanese child participating in a trachoma vaccine trial. The isolate was labelled “TW-183” and stored for later study (Woolridge et al. 1966). In 1983 a bacterium with the same characteristics was isolated from the respiratory tract of a student and designated as “AR-39” (Grayston JT 1986). It was initially assumed that the bacterium was an atypical strain of *Chlamydia psittaci*, and it was given the nickname “TWAR” (the letters originating from the isolates TW –183 and AR-39). In 1985 the bacterium was found to be associated with a mild form of pneumonia (Saikku et al. 1985). It was defined as a separate species of Chlamydia in 1989 and renamed *Chlamydia pneumoniae* (Grayston JT 1989 a).

Taxonomy

The order Chlamydiales was initially designated as one family, Chlamydiaceae, and one genus, Chlamydia, which consisted of four species; *Chlamydia trachomatis* and *Chlamydia pneumoniae* (both human pathogens), *Chlamydia pecorum* (a cattle pathogen) and *Chlamydia psittaci* (a bird, animal and human pathogen). In 1999 a revision in nomenclature was suggested based on DNA homology. This was that the family Chlamydiaceae should be divided into two genera: Chlamydophila and Chlamydia. *Chlamydia pneumoniae* was to be renamed *Chlamydophila pneumoniae*, while *Chlamydia trachomatis* would remain within the genus Chlamydia (Everett et al. 1999). The new nomenclature has not been generally accepted (Schachter et al. 2001), and the name *Chlamydia pneumoniae* is still used in scientific publications and also in this thesis.

Microbiology

Cpn has two distinct forms separated by morphology and functionality (Figure 1). Infectious, metabolically inactive, elementary bodies (EBs) enter the host cell, which

can be infected by several EBs. The mechanism of attachment to and entry into the cells has as yet not been fully clarified. After binding to an undefined host membrane receptor, the EB is endocytosed and can be detected within a membrane-bound endosome that incorporates host cell phospholipids (sphingolipids) into the inclusion envelope. The EBs in the endosome differentiate into non-infectious metabolically active reproductive reticulate bodies (RBs). These RBs divide within the expanding endosome, and after a period of growth (two or more days after the host cell infection), they reorganise into new EBs that are released by the host cells (Byrne 1988). At this time the endosome contains RBs, mature EBs and intermediate forms. Release of organisms (new EBs) from host cells is either through host cell rupture, or by fusion of the inclusion and host cell membranes in a manner resembling exocytosis. The Chlamydiae are described as energy parasites, since they utilize adenosine triphosphate (ATP) and nutrient from the host cell for metabolism and replication. They are also incapable of de novo nucleotide synthesis and are thus dependent on host's nucleotide pool. The RBs are susceptible to some antibiotics, but the EBs are not due to a lack of metabolic activity. Only EBs survive outside the host cell, and it is not known how long EBs can persist in tissues.

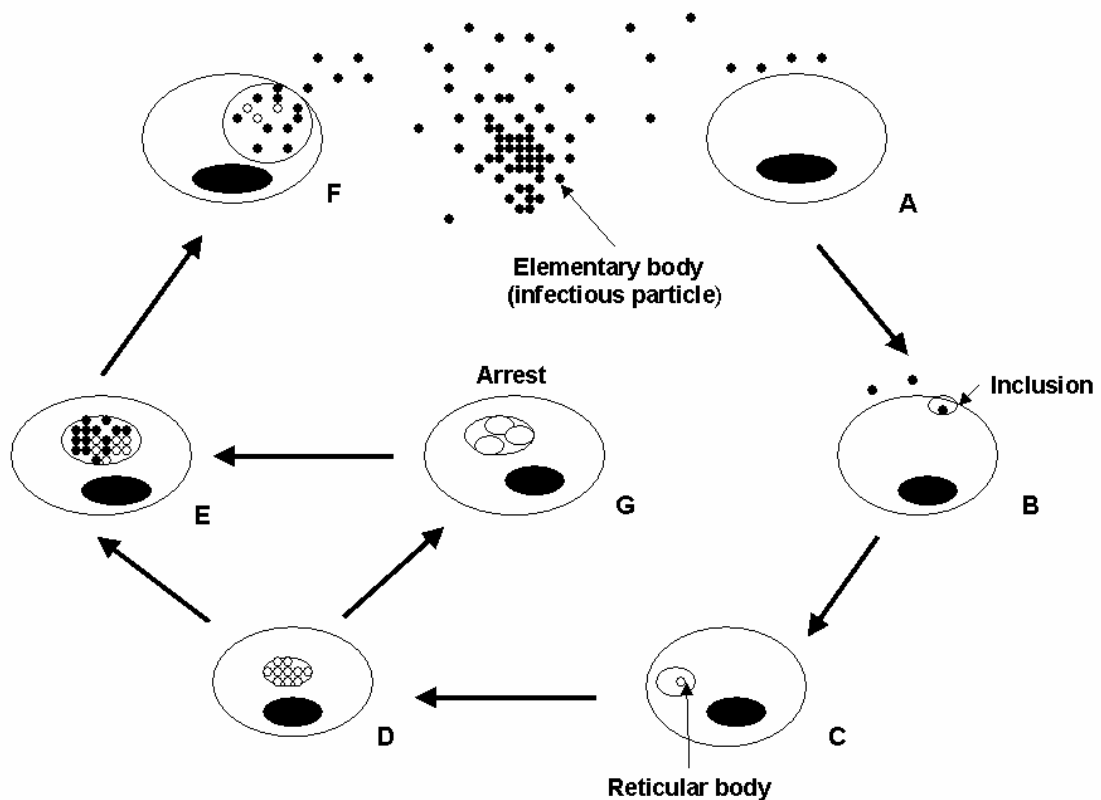


Figure 1. The developmental cycle of Chlamydia. Figure adapted from (Hahn et al. 2002).

A: EB attach to the host cell. **B:** Chlamydia modifies the endosome to escape the endocytic pathway and fusion with lysosomes. **C:** EB develops into the metabolic active RB. **D:** RB divide within the expanding endosome. **E:** After multiple divisions, the RBs reorganize into EBs. **F:** Release of infectious EBs. **G:** In states of low nutrient availability, IFN- γ mediated tryptophan starvation, treatment with antibiotics that fail to eradicate the infection or other stressful conditions, a persistent state may appear with non-dividing enlarged aberrant RBs. When the environmental stress is removed these aberrant RBs re-differentiate into infectious EB and begin a new cycle of replication.

For *Cpn* grown in cell culture the intracellular stage lasts for 72-96 hours, during which the bacteria stay inside the inclusion.

Persistent and chronic infection

All chlamydiae may cause persistent infection in their appropriate hosts. Intracellular RBs may enter a non-replicative and non-infectious stage of development and establish a persistent, long-term relationship with the infected cell. In laboratory cultures factors found to favour incomplete, persistent chlamydial infection include:

treatment with penicillin (an antibiotic not normally used for chlamydial infection), the depletion of host cell tryptophan (an amino acid essential for the production of chlamydial proteins) and the action of interferon gamma (IFN γ - one of the key factors in immunity to chlamydial infection). It seems likely that these aberrant chlamydiae can be transferred to new host cells during cell division (Richmond 1985). A continuous infection model has been described by Kutlin et al (Kutlin et al. 2001) for mimicking *in vivo* conditions. Persistent forms either do not replicate, or have reduced metabolic activity and are therefore often refractory to antibiotic treatment.

A mechanism for chronic infection where the chlamydia is growing and replicating is inhibition of host cell apoptosis. During chlamydial intracellular growth, maintenance of the host cell's integrity is essential not only for supplying nutrients, but also for shielding the intracellular organisms from the immune system. *In vitro* studies have shown that chlamydia infected cells are resistant to apoptosis induced by proapoptotic stimuli like the kinase inhibitor staurosporine, the DNA-damaging agent etoposid and for example TNF α . The anti-apoptotic activity exerted by the chlamydiae is dependent on chlamydial protein synthesis (Fan et al. 1998; Airene et al. 2002).

Immunological response to Cpn infection

Infections with Cpn result in the production of specific immunoglobulins that are detectable in serum as IgG, IgA and IgM antibodies. The immunological response is different in a primary infection as opposed to re-infection. In a primary infection with Cpn, it takes about 2-3 weeks before detectable levels of IgM appear; these antibodies are usually undetectable after 2-6 months. IgG antibodies take a longer time to appear, and it may take 6-8 weeks after the onset of illness before they reach high enough levels to be detected. IgA antibodies do not seem to occur in primary infection. In re-infection IgM may not be detectable, and the IgG increases rapidly within 1-2 weeks. The $T_{1/2}$ of IgG is approximately 23-28 days, but these antibodies may remain for several years. Low titres of specific IgG antibodies to Cpn are thus regarded as a marker for previous exposure to the bacterium, and high titres as suggestive for recent exposure (Grayston 1992). During re-infection short-lived ($T_{1/2}$ 5-7 days) IgA antibodies are produced (Tomasi et al. 1972). Due to the high prevalence of Cpn specific IgG antibodies among adults, the value of IgG antibodies

alone as a diagnostic marker for chronic infections is minimal (Grayston et al. 1990; Saikku 1992 b). Repeated analysis of sequential specimens is necessary to detect persistent elevated titres as a sign of chronic infection.

The persistence of elevated serum levels of short-lived IgA has been proposed as a marker for chronic, recurrent or active carrier state (Saikku et al. 1992 a).

A gender difference is found in the Cpn specific humoral response with more men having antibodies and higher titres of antibodies than women (Karvonen et al. 1994; Paltiel et al. 1995). No explanation for this phenomenon has been found so far.

Cell-mediated immunity to Cpn seems to participate in both recovery and immunopathology of infection. According to Airene (Airene et al. 2002), Cpn infected monocytes produce pro-inflammatory (IL-1, IL-6, TNF- α) and anti-inflammatory (IL-10) cytokines, and induce activation of antigen-specific cell-mediated responses, which promote the eradication of the organism. Cpn can remain metabolically active for several days in human monocytes *in vitro* (Airene et al. 1999), suggesting that the organism can interfere with the anti-chlamydial mechanism of the host cell. IL-10 is an important immunosuppressive regulator of cell mediated immunity and T-cell differentiation to Th2 cells (Daugelat et al. 1996; Curfs et al. 1997). Further, IL-10 is a powerful inhibitor of IFN- γ , which is needed for chlamydial eradication and activation of monocytes. When low concentrations of IFN γ are produced *in vitro*, elimination of chlamydia is hampered, leading to the development of aberrant non-productive forms resembling those found in chronic infections *in vivo* (Beatty et al. 1993). IL-10 is also involved in the resistance of Cpn-infected monocytes to apoptosis (Geng et al. 2000). Stress-response proteins, such as the heat shock proteins (hsp), are produced in greatly elevated amounts during a Cpn infection (LaVerda et al. 1999), and both *in vitro* and *in vivo* data suggests that they contributes to chronic inflammatory disease in humans (Kol et al. 1998).

Diagnosis

Diagnosis of a Cpn infection can be accomplished by isolation or detection of the organism and determination of specific antibodies by serological techniques. Culture of Cpn is done in eukaryotic cell lines, and is a time-consuming, labour intensive procedure not routinely done in laboratories. Non-culture methods such as polymerase chain reaction (PCR), direct fluorescence antibodies (DFA) and

immunohistochemistry (IHC) are widely used for bacterial detection. The recommended serologic technique is microimmunofluorescence (MIF).

Methods are described in detail later in this thesis.

Epidemiology

Results obtained from seroepidemiological studies have revealed that Cpn is a very common and widespread pathogen; 50% of adults in the general population have IgG antibody titres $\geq 1/16$ (Grayston 1989 b). The prevalence of Cpn antibodies increases rapidly between 5 and 20 years of age but only gradually thereafter (Grayston 1992), indicating that most primary infections occur in children and young adults. As no lifelong immunity develops after infection, re-infections may appear throughout life. The high prevalence of Cpn infection is supported by the results of Boman et al, who found that Cpn DNA was often found in peripheral blood mononuclear cells of even asymptomatic adults by PCR (Boman et al. 1998).

Transmission

Cpn is a primary human pathogen spread by droplets from person to person via the respiratory tract (Hyman et al. 1991; Theunissen et al. 1993). Outbreaks of infections have been reported in families, schools, among military trainees and in nursing homes. Epidemics may continue for several months even in close populations, indicating a low transmission rate and a long incubation period, which seems to be about three weeks (Grayston et al. 1990; Mordhorst CH 1992). Asymptomatic individuals may also be involved in the transmission.

Pathogenesis

Chlamydia pneumoniae EBs enter the respiratory tract, and can cause clinically evident or asymptomatic infections (Gnarpe et al. 1991; Gaydos et al. 1994). *In vivo* and *in vitro* studies have demonstrated that Cpn is capable of infecting airway epithelial, phagocytic, endothelial and smooth-muscle cells (Wong et al. 1992; Yang et al. 1994; Gaydos et al. 1996). Cpn has also been shown to cause ciliar dysfunction (Shemer-Avni et al. 1995), which might potentiate the ciliostatic effect of smoking and further contribute to an increased susceptibility in affected individuals. Co-infections with other pathogens are common (Kauppinen et al. 1995). Both humoral and cellular

immunity are involved in the immune response to chlamydia infections, but intracellular chlamydiae are adept at evading the immune defences, and the host is not always capable of eradicating the organism. Thus, prolonged often sub-clinical infections can occur. The host immune response can be protective by limiting chlamydial reproduction, but the same mechanisms may also contribute to the establishment of a persistent infection, resulting in damaging long-term consequences for the host. An example of this is *Chlamydia trachomatis* infections affecting the eyes, where patients with early stages of trachoma have mild symptoms and the organism is culturable. Repeated infections during a lifetime can lead to scarring and blindness, but it is rarely possible to isolate the organism from the diseased tissue after the acute infective stage (Beatty et al. 1994). The same is seen with genital *C. trachomatis*: asymptomatic infection can progress to irreversible scarring of the fallopian tubes and infertility, and detection of the chlamydiae in end stage disease is rare.

Treatment

Tetracycline and macrolides have been considered as drugs of choice for acute chlamydial infections. Block et al (Block et al. 1995) showed that in children with community-acquired pneumonia, Cpn was eradicated from the nasopharyngeal cultures in 79% of the children after clarithromycin treatment and in 86% after erythromycin treatment. However, when testing *in vitro* activities of azithromycin and doxycycline against 15 different isolates of Cpn, Gnarpe et al (Gnarpe et al. 1996) showed that all chlamydia strains regrew after four passages in cell culture after removal of the antibiotic, regardless of the initial drug concentration.

Several treatment studies have been performed in patients with chronic infections caused by Cpn and atherosclerotic cardiovascular disease, and in 2003 Grayston concluded that it is not yet known whether antibiotics have a role in the treatment of coronary heart disease (Grayston 2003). As far as we know there are no earlier studies reporting the treatment of chronic Cpn infection and longstanding lower respiratory tract symptoms.

Lung Cancer

Epidemiology

In the European Union there were over two million incident cases of cancer in 2004. Lung, colorectal and breast cancer represent the three most common incident forms, accounting for two-fifths of the total European cancer burden, with lung–cancer dominating among the cancer deaths (Boyle et al. 2005).

Etiology/Pathogenesis

Cigarette smoking is the major risk factor for lung cancer in both men and women, and the increasing incidence of lung cancer among women reflects their increased smoking habits. Host factors, inherited and/or acquired, may also contribute. Prior lung disease, such as chronic bronchitis and emphysema, are known to be independently associated with lung cancer (Littman et al. 2004).

Diagnosis

Airway symptoms such as cough, haemoptysis and dyspnoea, especially in a smoker, motivates chest X-ray, CT scan for staging (limited/extensive disease) and bronchoscopy for histopathological/cytological verification of suspected lung cancer. Lung cancer is divided into small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC). NSCLC includes adenocarcinoma, bronchioloalveolar carcinoma, squamous cell carcinoma and large cell carcinoma. Staging is important for determining the type of treatment. Lung cancer is often in advanced stage when symptoms occur. During 2002-2003, 42% of NSCLC and 61% of SCLC were in stage IV (distant metastases) at the time of diagnosis, according to the National Lung Cancer Registry in Sweden. A minority of the NSCLC were judged as resectable at diagnosis.

Treatment and Prognosis

Surgical resection is the treatment of choice for NSCLC, but only 20% of the patients are operated since the disease is often advanced when diagnosed. Further, despite careful selection of patients likely to benefit from surgery, all are not radically resected. Postoperative chemotherapy is now routine, and seems to improve the

prognosis (Arriagada et al. 2004). Stereotactic radiotherapy is a promising alternative for technically operable patients with low lung function. SCLC is treated in a curative attempt with chemotherapy combined with radiotherapy in case of limited disease. Patients with extensive NSCLC and SCLC and a good performance status are treated with palliative chemotherapy to prolong life and improve the quality of life. The prognosis depends on the disease stage at diagnosis and the type of cancer. Despite numerous medical and surgical advances during the past decades, the prognosis is still poor. Cure is rare, and the 5-year survival varies from 5 to 15% in various materials.

Infections and cancer

Chronic infection and inflammation have long been recognized as a risk factor for a variety of human cancers (Ohshima et al. 1994; Liang et al. 2004; Stoicov et al. 2004).

Virus may induce cancer by exerting an oncogenic effect on cells. Cheng et al described the association of human papilloma virus (HPV) type 16/18 infection with lung cancer among non-smoking Taiwanese women (Cheng et al. 2001). HPV16 is also associated with cervix cancer (Moberg et al. 2005) and Hepatitis B with hepatocellular cancer (Liang et al. 2004). An association between SV 40 (Simian Virus 40), from contaminated polio vaccine, and cancer has been presented (Vilchez et al. 2003). SV 40 has been detected in malignant mesothelioma (Rizzo et al. 1999), but other studies (Hubner et al. 2002; Priftakis et al. 2002) have not been able to confirm their findings.

Bacteria have been linked to cancer by two mechanisms: induction of chronic inflammation and the production of carcinogenic bacterial metabolites. Anttila et al have demonstrated a link between past *Chlamydia trachomatis* infection and cervical squamous cell carcinoma (Anttila et al. 2001). The association of helicobacter pylori and stomach cancer is well known. Ekström et al found that 71% of noncardia adenocarcinomas were attributable to H. pylori in the general Swedish population (Ekstrom et al. 2001). The oncogenic activity of H. pylori seems to be modified by factors in the microorganism, the host (proinflammatory genotypes) and the environment.

COPD

Epidemiology

The World Health Organization predicts that by 2020 COPD will rise from its current ranking as the 12th most prevalent disease worldwide to the 5th (Lopez et al. 1998). The true actual prevalence seems to be underestimated, since symptoms may be sparse, and patients often neglect their symptoms of cough and breathlessness. Lundbäck et al estimated that around 50% of elderly smokers developed COPD (Lundback et al. 2003).

Etiology/Pathogenesis

Smoking is the most important risk factor for COPD. Chemicals found in cigarette smoke are ciliostatic and have been found to be chemotactic for human neutrophils (Totti et al. 1984). Other environmental factors seem to contribute, and host factors, inherited and/or acquired, have also been suggested.

COPD includes signs of “small airways disease” and/or emphysema to a varying degree. The pathogenic mechanisms are likely to differ between these two components of COPD, since “small airways disease” includes cell proliferation, and emphysema is characterised by degradation of connective tissue. “Small airways disease” is characterised by obstructive bronchiolitis with infiltration of neutrophils, lymphocytes and macrophages, thickening of the airway wall (fibrosis), and increased muscle mass. A study examining surgical specimens obtained from two groups of smokers, one asymptomatic group with normal lung function and one group with symptoms of chronic bronchitis and mild chronic airflow limitation, showed that the smooth muscle mass was increased in the smokers with airflow limitation (Saetta 1999).

Emphysema is defined by the permanent abnormal enlargement of the respiratory airspaces due to destruction of the alveolar walls. The histopathological features are peribronchial destruction of alveolar attachments, airway collapse, and enlargement of air spaces distal to the terminal bronchioles. Imbalance between proteinases and antiproteinases are important in the pathogenesis of emphysema (Barnes 2000). Matrix metalloproteinases may generate chemotactic peptides that promote recruitment of macrophages to the parenchyma and airways.

Definition/Diagnosis

COPD is a disease characterized by airflow limitation that is not fully reversible. Classification of COPD by severity according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (National Institutes of Health 2001) has been used in this thesis (Table 1).

Table 1: Definition of COPD severity according to GOLD (National Institutes of Health 2001)

2003 Definition	2001 Definition	Criteria
Stage 0 At risk	Stage 0 At risk	<ul style="list-style-type: none"> • Normal spirometry but chronic symptoms (cough, sputum production)
Stage I Mild COPD	Stage I Mild COPD	<ul style="list-style-type: none"> • $FEV_1/FVC < 70\%$ • $FEV_1 \geq 80\%$ predicted • With or without chronic symptoms
Stage II Moderate COPD	Stage IIA Moderate COPD	<ul style="list-style-type: none"> • $FEV_1/FVC < 70\%$ • $50\% \leq FEV_1 < 80\%$ predicted • With or without chronic symptoms
Stage III Severe COPD	Stage IIB Moderate COPD	<ul style="list-style-type: none"> • $FEV_1/FVC < 70\%$ • $30\% \leq FEV_1 < 50\%$ predicted • With or without chronic symptoms (cough, sputum production) [or dyspnoea in OLD classification]
Stage IV Very severe COPD	Stage III Severe COPD	<ul style="list-style-type: none"> • $FEV_1/FVC < 70\%$ • $FEV_1 < 30\%$ predicted or • $FEV_1 < 50\%$ predicted plus chronic respiratory failure (or clinical signs of right heart failure in OLD classification) <p style="text-align: right;"><i>Lung function should be measured after bronchodilatation</i></p>

Treatment and prognosis

Smoking cessation reduces the rate of decline in lung function, and can give small improvement in lung function and a reduction of symptoms such as cough, phlegm and dyspnoea (Kanner et al. 1999). Unless COPD patients quit smoking, they may develop a rapid decline in lung function. However, in some patients the lung function continues to decline despite smoking cessation. No pharmacological intervention has

been shown to modify the progression of disease. Bronchodilators, such as β_2 -antagonists and anticholinergics, may improve symptoms in patients with COPD despite limited improvement in lung function. Inhaled steroids, in particular when combined with long-acting β_2 -antagonists, reduce the incidence of exacerbations among patients with severe COPD.

Nonpharmacological therapies, including exercise training, psychosocial support, coping strategies, nutrition and education, improve the quality of life and increase participation in everyday activities.

Lung Volume Reduction Surgery (LVRS) has been tried as a palliative treatment in advanced emphysema. By surgically removing of the most hyper-inflated emphysematous areas of the lungs, an improvement is seen in the mechanical efficiency of the inspiratory muscles. Effects on mortality, duration of benefit and the criteria for selection of patients for this procedure have been presented (Cooper et al. 1996; Fishman et al. 2003).

Transplantation may be chosen in carefully selected patients with severe emphysema.

Infections and COPD

The Vicious Circle Hypothesis describes how infections can lead to impaired mucociliary clearance which enhances bacterial colonization, resulting in airway epithelium injury by bacterial products. This airway epithelial injury may lead to a direct progression of COPD, and the bacterial colonization can cause an increased elastolytic activity because of the inflammatory response leading to a changed elastase-antielastase balance and progression of COPD (Figure 2).

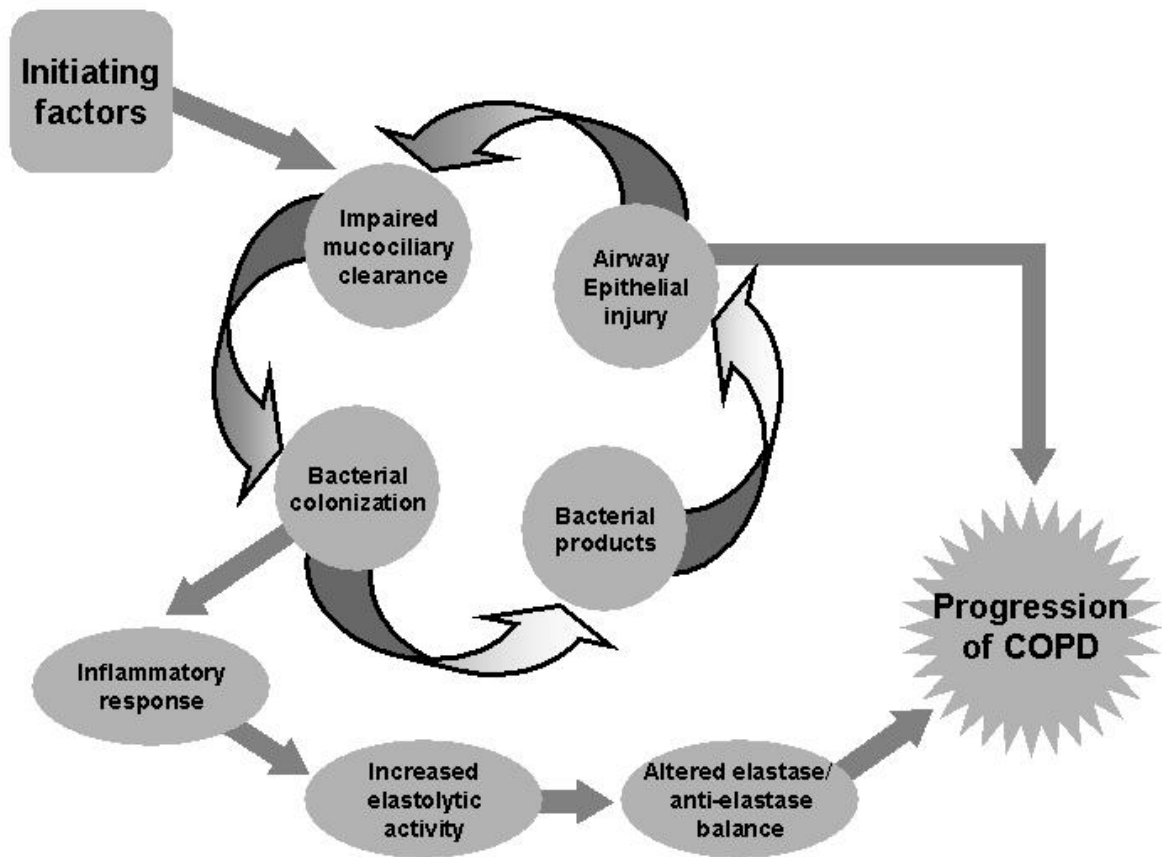


Figure 2. The Vicious Circle Hypothesis for development of COPD. Figure adapted from Sethi S (Sethi 2000).

AIMS OF THE THESIS

The aims of the thesis were to

- study the prevalence of chronic *Chlamydia pneumoniae* infection in patients with lung cancer
- study the prevalence of chronic *Chlamydia pneumoniae* infection in relation to lung function in patients without lung cancer
- study a new treatment regime in patients with longstanding airway and/or pharyngeal symptoms and chronic *Chlamydia pneumoniae* infection
- detect *Chlamydia pneumoniae* in cytospin preparations of bronchoalveolar lavage and in lung tissue from patients with COPD

MATERIAL AND METHODS

Objectives and Populations

To evaluate the prevalence of chronic Cpn infection in patients with respiratory diseases we first conducted two studies on patients referred to the Department of Respiratory Medicine at Gävle County Hospital between January 1997 and February 1999 because of longstanding airway symptoms and/or pathological chest X-rays leading to bronchoscopy. Gävle County hospital serves a population of about 290 000.

The first study was done on lung cancer patients (Paper I), and the second on patients without lung cancer (Paper II). The findings in study I and II inspired us to perform a treatment study on 103 patients with longstanding airway and/or pharyngeal symptoms and chronic Cpn infection (Paper III). We found an association between COPD and chronic Cpn infection, and then continued with a study to evaluate whether Cpn was detectable using DFA on cytospin specimens obtained from bronchoalveolar lavage (BAL) fluid in COPD patients. These patients were compared with healthy smokers and non-smoking controls. IHC for Cpn was also performed on lung tissue from patients with severe emphysema who had undergone LVRS at Karolinska Universitetssjukhuset January 1995 to May 2001 (Paper IV).

The Ethical Committee of the Medical Faculty, Uppsala University, Sweden approved the studies I-III, and the Ethical committee of Karolinska Institutet, Stockholm, Sweden, approved study IV. The Swedish Medical Products Agency approved study III.

All patients gave informed and written consent before inclusion into the studies.

Paper I

Patients: 210 patients (136 men) with lung cancer were investigated. Blood samples from these patients were used for evaluation of antibody titres to Cpn using MIF. Specimens were taken the day before bronchoscopy in 198 patients, and on new blood specimens taken after 3 months in 127 of the same patients. Throat swabs as well as samples taken from the bronchial mucosa with a cytobrush were analysed for

Cpn using PCR on samples taken the first year of the study. During the second year IHC on formalin-fixed paraffin-embedded biopsies obtained at bronchoscopy was done instead of PCR on cytobrush. Throat swabs for Cpn DNA analysis by PCR were taken from 173 patients. Seventy-four cytobrush specimens from the bronchial tree were analyzed for Cpn with PCR. Fifty bronchial biopsies were analysed with IHC.

One control group consisted of all consecutive blood donors presenting at the County Hospital during a period in the spring of 1998 (89 male and 31 female) and who were asked to complete a questionnaire concerning their current state of health, medication and smoking habits. Blood was obtained for analysis of antibodies to Cpn with MIF, and a throat swab for Cpn DNA analysis was done using PCR. Only smokers and ex-smokers were used for comparison with the cancer cases. As another control group we used a study on a randomly selected cohort of currently smoking or ex-smoking 70-year olds (97 men, 114 women).

Paper II

During the study period, 403 patients without lung cancer underwent bronchoscopy. Spirometry was not available in 70 subjects, and 125 were excluded, without knowledge of the actual lung function or antibody titres, due to abnormal chest X-ray findings that could influence the lung function (e.g. sarcoidosis, status post tuberculosis, pulmonary fibrosis, pneumonia, heart failure); patients with pulmonary emphysema were not excluded. The exclusion was done without knowledge of the actual lung function or antibody titres. Nine additional patients were excluded either because that serology specimens were not collected before bronchoscope (1), or convalescent sera were missing (8). Thus, 199 patients (110 women) remained for analysis. At bronchoscopy no intrabronchial findings were observed that could explain a reduction in lung function in any of the patients. Blood specimens were collected for evaluation of antibody titres to Cpn with MIF the day before bronchoscopy, and convalescent serum was obtained at a median of 97 days later. Throat swabs, as well as samples taken from the bronchial mucosa with a cytobrush, were analysed for Cpn using PCR for all samples taken during the first year, and IHC was used for formalin-fixed paraffin-embedded biopsies obtained at bronchoscopy during the second year. PCR tests were done on throat specimens in 191 patients and on samples collected from bronchial mucosa by cytobrush from 78 patients. Biopsies were excised from the bronchial mucous membrane of 85 patients.

Paper III

Serological signs of chronic infection with Cpn (IgA $\geq 1/64$ or/and IgG $\geq 1/1024$ in paired sera) were detected in 166 patients, who were informed about the planned study by telephone. A questionnaire concerning their symptoms was sent to those 152 who were willing to participate, but 49 were free from symptoms and were excluded from further investigations. The remaining 103 patients, who had longstanding airway and/or pharyngeal symptoms, were included in this prospective double-blind, randomised study comparing intermittent azithromycin 500mg daily for 5 days repeated 3 times with a 23 d interval and compared to placebo. Symptoms were evaluated following a questionnaire, and effects on antibody titres and lung function were assessed. Patients were examined 4 months and 1 year after completed treatment.

Paper IV

The detection rate was low for Cpn with PCR from throat swabs and cytobrush samples taken from the bronchial mucosa as well as with IHC on biopsies obtained at bronchoscopy in our previous studies. We therefore performed DFA on cytopspin preparations of BAL fluid from 14 patients with COPD, 10 healthy smokers and 7 non smokers. IHC for Cpn was performed on lung tissue from 24 patients with severe emphysema who had undergone LVRS. We also analysed archived serum samples for detection of specific Cpn antibody titres in all patients with cytopspin specimens and in 11 of the emphysema patients.

Laboratory Methods

Serology – microimmunofluorescence (MIF)

MIF is the serological testing method of choice for diagnosing acute Cpn infections, but a standardized approach in performing and interpreting the MIF is necessary (Dowell et al. 2001), and experience with the technique is important for consistent results. Purified EBs from *Chlamydia pneumoniae*, *trachomatis* and *psittaci* are fixed onto glass slides as distinct antigen dots. Standardized dilutions of patient sera are placed over the dots and incubated at 4 °C overnight for binding. Fluorescent labelled anti-human-antibodies are then incubated with slides prior to visualisation with UV light. If IgA or IgM are positive in screening tests, IgG has to be absorbed out of the

serum because it has a high binding affinity and may bind unspecifically to the antigen, leading to false positive readings.

In Paper I-IV Cpn antibodies were analysed by microimmunofluorescence (MIF) at an accredited clinical microbiology laboratory using a previously standardised and optimised technique and reagents (Gnarpe et al. 2000; Gnarpe et al. 2000). Sera were diluted 1:32 with phosphate-buffered saline (PBS) solution, pH 7.4, and tested for IgG, IgA and IgM antibodies using 21-well antigen slides with elementary bodies of *Chlamydia psittaci*, *Chlamydia trachomatis* and *Chlamydia pneumoniae* in each test well (LabSystems Oy; Helsinki, Finland).

Sera found to be positive in a 1/64 screening dilution for IgG were re-diluted and tested in doubling dilutions to endpoint. Sera positive in a 1/32 screening dilution for IgA or IgM were absorbed using GullSorb (Gull Laboratories Ltd, USA) to remove IgG, and then tested in doubling dilutions. Serum dilutions were incubated with antigens for 14-16 h at 4 °C. Slides were washed thoroughly in three changes of PBS solution (pH 7.4), then incubated with fluorescein isothiocyanate-conjugated rabbit antihuman IgG, IgA or IgM (Dakopatts; Glostrup, Denmark) at 37 °C for 30 min. Control sera with specified high and low titres were used on each testing occasion, and tests were accepted only if the titres were within one dilution step of the predetermined mean titres for the respective quality control sera. An experienced microbiologist, without knowledge of patient identities or diagnoses, read all tests with a Zeiss UV microscope (Zeiss; Jena, Germany) using a 40× oil immersion lens and a 10× ocular lens (total magnification 400×). All sera were separated from erythrocytes upon arrival at the laboratory, and frozen at -20 °C until tested. Repeat blood specimens were always analysed in parallel with earlier specimens on the same antigen slide to increase the ease of comparison of titre level assignments for all specimens from the same patients. Endpoint titration was done in all cases.

Polymerase chain reaction (PCR)

The PCR is a nucleic acid-based amplification technique. PCR detects a specific nucleotide sequence, which can then be visualised using agarose gel electrophoresis. The method is fast, accurate and detects the presence of the organism, but it does not differentiate active from inactive or dead bacteria. PCR is not dependent on replication, which might be an advantage as it could detect

nonreplicating organisms in persistent infections. Contamination is a major problem with the PCR method, and it is important to adhere to a strictly controlled procedure in the laboratory employing separate rooms for each step of the procedure. At least one, but preferably two negative controls are needed in each run to rule out accidental contamination. Taq polymerase enzyme inhibitors, for example haemoglobin, may cause a false negative PCR result, and appropriate positive controls must therefore be included in every run, preferable with every specimen. It is essential that the method used for preparation of DNA from clinical samples is optimised.

In Paper I-III we used specimens taken from the posterior wall of oropharynx with a CTA swab (*Chlamydia trachomatis* aluminum, Biohospital AB, Kopparberg, Sweden) as described earlier (Falck et al. 1997). Swabs were immersed in 2 SP (sucrose-phosphate buffer), stored in the refrigerator, and transported to the laboratory the same day. The specimens were either frozen at -70°C or immediately processed at the laboratory. Samples were prepared for PCR using a previously described method (Gnarpe et al. 1995). PCR was done according to Campbell et al (Campbell et al. 1992) using HL1 and HR1 as primers on all samples. All samples were run in duplicate for each PCR method; one of the replicates was spiked with Cpn DNA equivalent to 20 elementary bodies to test for inhibitors of the Taq polymerase enzyme. When inhibitors were found, specimens were diluted 1:100 with sterile deionised water to reduce the effect of the inhibitors, then retested.

PCR was also performed on cytobrush specimens taken from bronchial mucosa. All analyses were done by two microbiologists with extensive experience with the methods but without knowledge of the patient identities or diagnoses.

Immunohistochemistry (IHC)

A specific Cpn monoclonal antibody which shows reactivity to the major outer membrane protein of Cpn was used to overlay a tissue sample. This antigen is not shared by other chlamydia species. The EB form of the bacterium is not visible using the IHC technique, and only large inclusions containing antigen can be safely detected (Naas et al. 1999).

In Paper I and II IHC was done on small bronchial biopsies during the second year of the study and in study IV on lung tissue. Biopsies obtained at bronchoscopy and lung tissue were formalin-fixed, embedded in paraffin and cut at 4 microns of thickness.

The sections of paraffin embedded tissue fixed to glass microscope slides were deparaffinized by immersion in xylene and hydrated through a series of alcohols with decreasing concentrations. Sections were overlaid with 0.5% pepsin (Sigma Chemical Co, St. Louis, Mo) to open up the cells and expose the Chlamydia to the antibody, and then incubated for 15 minutes at 37 °C. The IHC procedure used in Paper I-II was according to an earlier report (Naas et al. 1999). A specific Cpn monoclonal antibody generously donated by Dr Kenneth Persson, Malmö, Sweden, was used after the specificity of the antibody had been ascertained. No cross-reactions were found in control samples containing *Chlamydia trachomatis* or *Chlamydia psittaci*. The monoclonal antibody used was found to react in an identical manner to RR-402, a specific Cpn monoclonal obtainable commercially from DAKOPATTS, Denmark. PBS, pH 7.4, fetal calf serum, mouse serum and a monoclonal antibody to CMV were used as negative controls. A positive control was constructed by embedding cells from a culture infected with Cpn in paraffin, and treating it the same way as the patient tissue sections. This was done when the method was set up, and subsequently, stored known positive patient tissues were used as positive controls for staining.

In Paper IV the IHC method is the same as in Paper I-II except that the monoclonal was from a commercial source for study IV.

After washing with PBS-Tween 20 (PBST) at pH 7.6 twice for 5 minutes, peroxidase blocking reagent (Dako Corporation, Carpinteria CA) was overlaid and slides incubated for 15 minutes at room temperature in a moist chamber. Washing was done twice for 5 minutes in PBST and universal blocking solution (Dako Diagnostics Canada Inc, Mississauga, ON) was overlaid and incubated for 15 min at room temperature. Slides were gently drained and a 1:5 dilution of mouse monoclonal antibody to *Chlamydia pneumoniae*, RR-402 (Dakocytomation Ltd, Ely, UK) in antibody diluting buffer (DAKO Diagnostics Canada Inc., Mississauga, ON) was added. Slides were incubated overnight at 4 °C then washed twice with PBST. StreptABComplex/HRP Duet, Mouse/Rabbit (DAKO A/S, Denmark) was used for the streptavidin/biotin reaction using a biotinylated goat antibody to mouse and rabbit immunoglobulin, streptavidin and a biotinylated horseradish Peroxidase. Finally, DAKO AEC Substrate System (DAKO CORPORATION, Carpinteria, CA) was used for visualization of the red coloured Chlamydia antigen in the tissue sections.

Tissue sections obtained earlier from patients with lung cancer and proven Cpn infection were used as positive controls. For negative controls antibody to mouse IgG3 (NeoMarkers, Fremont, CA), the same class of antibody as the RR-402 monoclonal, was used.

In Paper I-II one microbiologist with extensive experience with the IHC method made the analysis without knowledge of the patient identities or diagnoses, in Paper IV only the identity was blinded.

Immunofluorescence

In Paper IV, Direct Fluorescence Antibody (DFA) was done on cytospin preparations of BAL fluid. Cytospin specimens on glass microscope slides were fixed in cold acetone for 15 minutes and air dried. 25 microliters of a monoclonal to Cpn (Imagen™ Chlamydia pneumoniae, DAKO Ltd, Cambs UK) were overlaid on each specimen and slides incubated at 37 °C for 30 min in a moist chamber. Positive controls were slides provided in the Imagen kit containing Cpn infected cells. Negative controls were Cpn infected cells incubated with a fluorescein isothiocyanate labelled adenovirus monoclonal. Washing with gentle agitation was done with PBS pH 7.5 for 5 minutes, after which the slides were air dried, mounted, coverslipped and immediately examined using an Olympus UV microscope at 500×. The presence of bright apple-green fluorescing round bodies concomitant in size with elementary bodies were reported as possible positive findings. The same microbiologist who made the IHC also performed the FDA analysis without knowledge of the patient identities or diagnoses.

Lung function

Lung function was determined by dynamic spirometers (Paper I-III: Vitalograph Compact II, Vitalograph Ltd, Buckingham, UK; Paper IV before bronchoscopy and BAL: Vitalograph Compact, Vitalograph Ltd, Buckingham, UK; Paper IV before LVRS: Gould 2400, Gould Electronics, The Netherlands). The procedures were performed in a standardised manner, and the results for forced vital capacity (FVC), vital capacity (VC) and forced expiratory volume in one second (FEV₁) were expressed as percentage of predicted, in Paper II-III (Berglund et al. 1963) and in Paper IV

(Quanjer PH 1983; Quanjer PH 1993). Reversibility of airways obstruction was tested by measuring FEV₁ before and 10 min after inhalation of bronchodilators and was calculated as improvement of FEV₁, both in percentage of baseline (paper IV) and of predicted FEV₁ (paper II-IV). Bronchodilators for reversibility testing were 0,8 mg salbutamol (Ventoline diskhaler; GlaxoSmithKline) in Paper I-III, 1 mg terbutaline (Bricanyl Turbuhaler; AstraZeneca) before bronchoscopy in Paper IV, or nebulisation of 5 mg salbutamol (Ventoline; GlaxoSmithKline) and 0.25 mg ipratropium bromide (Atrovent; Boehringer Ingelheim) before LVRS in Paper IV.

Bronchoscopy and handling of BAL fluid

In Paper IV, bronchoscopy was performed on an outpatient basis after overnight fasting. BAL was performed by wedging the bronchoscope in a subsegment of the middle lobe. Five aliquots of 50ml of sterile PBS at 37 °C were instilled and gently suctioned back with a negative pressure of -40 –50mm Hg. Dwell time was kept to a minimum as recommended by the European Respiratory Society (ERS) task force (Haslam P 1999). BAL was interrupted if the recovery, after 150-200ml of instilled fluid, was <35ml or if persistent cough or desaturation occurred (<90% oxygen saturation despite appropriate oxygen supplement). The fluid was collected in a silicone treated bottle kept on ice, which was transported to the laboratory immediately. The BAL fluid was filtered through a Dacron membrane (Millipore, Cork, Ireland) and centrifuged onto slides at 400 x g for 10 min at 4 °C and frozen in -80 °.

Questionnaire

In Paper III, patients were interviewed at their first visit following a detailed questionnaire regarding detailed medical history, medication, smoking habits, cough (presence and intensity), mucus (amount and colour), pharyngeal symptoms and use of antibiotics during the last 3 months. At their second and third visit a similar questionnaire was used, which now included questions about any changes in their general condition and if the amounts of mucus had changed since the start of treatment. Patients were also asked if they had needed any other antibiotics after the course of study medication.

RESULTS

Chronic infection with *Chlamydia pneumoniae* and lung cancer. Paper I

The difference between male lung cancer patients and controls in prevalence of specific Cpn IgG antibody titres of $\geq 1/512$ and Cpn IgA antibody titres $\geq 1/64$ was statistically highly significant. A significant difference in prevalence of specific Cpn IgA antibody titres $\geq 1/64$ was found when comparing female patients with controls. Very high specific Cpn antibody titres (IgG $\geq 1/2048$, IgA $\geq 1/512$) were significantly more prevalent in male lung cancer patients compared with controls. In women with lung cancer very high IgA levels, but not IgG, were significantly more prevalent. The detection rate of Cpn with PCR in throat and cytobrush specimen and with IHC on mucosal biopsies was low and not correlated to elevated specific antibody titres.

Since high titres of specific Cpn antibodies were more common in the lung cancer patients compared with controls, the results suggest that it is a possibility that lung cancer could be induced by chronic Cpn infection.

Chronic infection with *Chlamydia pneumoniae* and COPD. Paper II

The specific IgA titres increased with age in both COPD and non-COPD patients, and showed a tendency to be higher in the COPD group independent of age. Chronic Cpn infection, defined as persistent elevated serum antibody titres of IgA $\geq 1/64$, was present in 85 of 199 patients, and was associated with smoking and higher age, but no gender difference was observed. Thirty patients had COPD, defined as FEV₁/FVC <70% without any features of asthma as judged from the medical history or reversibility test (FEV₁ reversibility <15%). 18 patients were defined as Stage 0-II, 7 Stage III and 2 Stage IV according to the GOLD 2003 criteria. A statistically significant association, remaining after correction for smoking, was observed between chronic Cpn infection and COPD. The detection rate of Cpn was low and not correlated to elevated specific antibody titres.

The results suggest that chronic Cpn infection may be an independent risk factor for the development of COPD.

Effect of intermittent azithromycin treatment. Paper III

Evaluation of symptoms showed general improvement and less hawking in patients treated with azithromycin compared to placebo after 4 months, but there was no sustained difference one year after completed treatment. The antibody titres remained stable, and there was no influence on lung function. Adverse events, primarily gastrointestinal, were more frequently reported with azithromycin than placebo.

In conclusion, azithromycin was found to be effective for reduction of respiratory symptoms in patients with chronic Cpn infection; however prolonged intermittent treatment with high doses did not eradicate the chronic infection.

Detection of Cpn in cytospin and lung tissue. Paper IV

Elementary body like structures were detected in cytospin slides from 4 of 14 COPD patients, 1 of 10 healthy smokers and 1 of 7 never-smokers. These elementary body like structures could not be confirmed as Cpn due to the fact that we had archival specimens of which there were a limited number of slides. There was no correlation between high antibody titres and detection of the suspected elementary bodies. COPD patients had specific IgA $\geq 1/64$ to a larger extent than the control groups, but the difference was not significant in this small group.

Cpn was detected in lung tissue in 2 of 24 patients with advanced emphysema, one never smoking woman with α_1 -antitrypsin deficiency and one ex-smoking man. Serology on these two patients was not available.

In conclusion, this study supported a relationship between Cpn infection and COPD by serology. The methodology used for detection of Cpn in cytospin preparations requires further evaluation and confirmatory procedures are necessary.

DISCUSSION

Lung cancer

Smoking is the major cause of lung cancer, but also never smokers may get lung cancer. Only a small proportion of smokers develop lung cancer, and other factors such as environmental and/or host factors may contribute. In individuals diagnosed with lung cancer, lung function may be affected by the cancer making the diagnosis of COPD problematic, and prospective studies are important. Recently, Littman et al presented a large prospective study showing that smokers with a history of chronic bronchitis/emphysema may be at higher risk of developing lung cancer, independent of their smoking history (Littman et al. 2004 a). In our study we found significantly higher specific IgG and IgA titres among male lung cancer patients than in age matched controls. Female lung cancer patients had lower titres than male lung cancer, although still significantly higher IgA than controls. A difference was found between males and females regarding smoking history; the average pack-years (PY) of cigarette smoking was 28 for the women and 34 for the men, and 17% of the women and 4% of the men were never smokers. Specific Cpn IgG antibodies are common in adults and have been interpreted as a sign of earlier exposition, but IgA antibodies are not usually found in healthy individuals. 9/46 male and 2/22 female blood donors in this study had IgA $\geq 1/64$ in single blood samples which may reflect subclinical infection, which can result in an increase of IgA which then rapidly declines because of the short $T_{1/2}$ of IgA. However, IgA titres that persist for months and years are indicative of chronic infection. One shortcoming of the study is that repeated measurements of titres not were performed on the blood donors.

There are a few reports on lung cancer and Cpn. The first in 1997 by Laurila et al (Laurila et al. 1997) reported serological evidence of an association between Cpn and lung cancer. After the publication of our paper in 2001, three more articles were published on chronic Cpn infection and lung cancer. Littman et al (Littman et al. 2004 b) found that individuals with IgA $\geq 1/16$ had 1.2 times the risk of lung cancer compared to those with lower titres, and there was a significant trend ($p=0.007$) of increasing odds ratios with increasing IgA titres, primarily due to an odds ratio of 2.8 associated with titres $\geq 1/256$. Lung cancer risk associated with IgA titres $\geq 1/16$ was strongest among former smokers. Anttila et al (Anttila et al. 2003) studied the risk of female early-onset lung cancer and Cpn infection, and found the disease to be

associated with Cpn specific immune complexes and both IgG and IgA antibodies, but there was no statistically significant difference between the histological cancer subgroups associated with Cpn IgG antibodies. One strength in this study was that the mean time between the serum specimens and the diagnosis of cancer was 9.1 years (range 1.6-16.7), adding to the discussion about “what comes first”. Kocazeybeks study supports the hypothesis that chronic Cpn infection increases the risk of lung carcinoma (Kocazeybek 2003). In 2000 Jackson et al found that an IgA $\geq 1/16$ was independently associated with risk for lung cancer among subjects <60years old, but not among older subjects (Jackson et al. 2000).

A causal relationship between Cpn and lung cancer is possible as Cpn stimulates the release of inflammatory mediators into tissue. TNF- α , interleukin-1 β , IL-8 have all been found to be released *in vitro* (Gaydos 2000). Chronic infections may cause genetic damage. IL-8 might act as a promotor of tumour growth for human lung carcinoma through its angiogenic properties (Arenberg et al. 1996; Chen et al. 2003). Cpn can impair or even block apoptosis of infected cells (Fan et al. 1998) by induction of IL-10 (Geng et al. 2000), resulting in chronic infection and an increased risk for of malignant transformation of infected cells.

COPD

We found a significant association between chronic Cpn infection and COPD, which remained after correction for smoking. Results obtained from the study of this selected population, patients where bronchoscopy was indicated because of longstanding airway symptoms, cannot however be extrapolated to a general population. The number of patients in this study was too few to allow multivariate analysis of parameters. The study included an attempt to identify the bacterium by taking samples during bronchoscopy, but the results did not show any correlation between serology and PCR/IHC.

The detection of Cpn by PCR on throat samples and cytobrush specimens in our material was low. Von Hertzen et al (Von Hertzen et al. 1997) detected Cpn DNA using PCR in 59% of spontaneous sputa from patients with severe COPD in contrast to 11% on induced sputa in a study of patients with less advanced COPD (Smieja et al. 2002). Von Hertzen et al found discrepant results between serum antibodies and presence of Cpn in sputum. In the later study (Smieja et al. 2002) nothing is stated

about specific Cpn antibodies in serum. A study presented by Falck et al confirmed an association between chronic Cpn infection as defined by high prevalence of antibodies, especially IgA and decreased lung function (Falck et al. 2002). Detection of Cpn was not done in this cohort and it is not known whether the presence of Cpn in tissues correlates with decreased lung function as well in this patient group.

Cpn is known to produce persistent infections *in vitro* (Beatty et al. 1994) and chronic lung infection in *in vivo* animal models (Malinverni et al. 1995), which support the possible association with chronic lung disease. When Cpn is cultured in human bronchial cells, basic fibroblast growth factor is produced which could contribute to subepithelial fibrosis in small airways (Rodel et al. 2000). When Cpn is cultured in human alveolar macrophages there is a production of cytokines associated with inflammation (Redecke et al. 1998). Rupp et al (Rupp et al. 2004) stated that one way that chronic Cpn infection of AEC type II could contribute to the progress of lung destruction in COPD patients may be that chlamydial infection of these cells might stimulate the release of surfactant protein-A (Wissel et al. 2003) which has been shown to trigger the secretion of MMP-9 from alveolar macrophages (Vazquez de Lara et al. 2003).

Cpn seems to have the ability to cause COPD, but this hypothesis remains to be proven and large prospective studies are necessary.

Treatment

Treatment studies of chronic Cpn infection have been focused on the suspected association of this bacterium with cardiovascular disease and the presence of the bacterium in atherosclerotic arteries. There is so far sparse data on the treatment of chronic Cpn infection and lower respiratory tract symptoms. The requirement for successful treatment of chronic Cpn infection may be the ability to reactivate the latent forms of the bacterium into the actively metabolising forms that are susceptible to antibiotic treatment.

Reactivation from persistent infection to acute infection has been described in mice following immunosuppression (Malinverni et al. 1995). A combination treatment with azithromycin and rifampicin in mice with pneumonitis was studied by Bin et al (Bin et al. 2000). Combination treatment was found to be superior for the treatment of infection, and this may be due to the observation that rifampicin is a glucocorticoid

receptor ligand with the ability to transactivate the receptor (Calleja et al. 1998). Rifampicin could act as an immunosuppressive agent, allowing for the reactivation of infection, and thus allowing azithromycin to act on replicating chlamydiae, eventually suppressing the inflammatory reactivity to infection. A combination therapy with azithromycin and rifampicin might therefore have been more successful. As far as we know this strategy has not been studied in humans.

Azithromycin may exert anti-inflammatory activities as reviewed by Parchure (Parchure et al. 2002). In a recent small double blind study comparing azithromycin (500mg once daily for three days) with placebo to patients with moderate and severe COPD, it was shown that azithromycin was able to inhibit some markers of the inflammatory response (Parnham et al. 2005).

To evaluate the effect of treatment is problematic, since antibody titres decrease slowly and Cpn is difficult to detect. Before treatment only 5 of 59 were tested positive for Cpn DNA with PCR from throat samples in our study (data not shown), though the patients had symptoms and elevated titres. Monitoring symptoms could be one manner in which to test for antibiotic effectiveness, but the data is only qualitative and co-infections with other organisms can interfere with the evaluation. The antibody titres and lung function were stable throughout our study.

Detection

When detected in the airways it is not likely that Cpn is an innocent bystander because of the intracellular nature of the infection and the earlier described immunological reactions. We wanted to repeat the studies of Theegarten and Wu concerning Cpn detection in lung tissue from 24 LVRS patients by using IHC. Theegarten (Theegarten et al. 2000) studied fixed lung tissue from 12 of 120 LVRS patients (12 with normal α_1 -antitrypsin levels) by SEM, 10 for TEM and 4 were analysed with immunofluorescence microscopy; 3 with α_1 -antitrypsin deficiency were investigated using SEM and TEM. SEM analysis demonstrated spherical bodies resembling the aberrant Cpn seen in atherosclerotic lesions in all patients. Wu (Wu et al. 2000) performed IHC for Cpn in archival tissue from 37 patients who had undergone lobectomy for bronchial carcinoma, including 16 patients with COPD and 21 with normal lung function. The samples were from sites remote from the carcinoma. Tissues from all subjects showed Cpn, but COPD patients had more

positive cells per field. In a healthy control group of younger individuals, who had died by accidents, Cpn was not found as often in lung tissue as in the individuals with lung disease. No serum samples were available to evaluate the presence chronic infection. The concomitant lung cancer present in Wu's patients might have influenced his results, and Wu also discusses the difficulties surrounding the detection of Cpn by PCR in formalin-fixed, paraffin embedded tissue and suggest that IHC could be a more sensitive method. On the other hand Mygind et al (Mygind et al. 2001) found good correlation between real-time quantitative PCR for identification and quantification of Cpn and IHC in paraffin-embedded formalin-fixed lung tissues in experimentally infected mice. He concludes his paper by commenting that care should be taken when interpreting PCR results from paraffin-embedded formalin-fixed samples, and parameters such as age of the section and the DNA purification method should be considered. Our sparse findings might be explained by the fact that our samples for analysis were taken from only one part of the affected lung, and in other studies Cpn has been found to appear in patchy distributions. Another important consideration could be that in end stage emphysema there is little inflammation and fewer cells that can easily harbour the bacterium. Nothing is stated about actual lung function in the article of Theegarten, thus the indications for LVRS might be different. The age of specimens from the emphysema patients may not have influenced our results as Cpn have been detected in archive specimens with IHC.

To evaluate if Cpn was detectable in small airways in less advanced COPD we tested cytopsin preparations of BAL by Direct Fluorescence Antibody, but the results were not convincing. Rupp et al (Rupp et al. 2004) found that target cells differ in acute and chronic chlamydial infection and that the alveolar epithelial cells (AEC) type II are target cells in chronic infection. In BAL the majority of cells are alveolar macrophages which might explain our low detection rate.

CONCLUSIONS

The thesis has demonstrated that

- Chronic *Chlamydia pneumoniae* infection defined by serology was associated with both COPD and lung cancer, but longitudinal studies are needed to confirm a causal relationship.
- Treatment for long standing respiratory symptoms with intermittent high doses azithromycin in patients with serological signs of chronic *Chlamydia pneumoniae* infection gave a transient symptomatic improvement, but did not affect the chronic infection, and development of new treatment strategies is important.
- *Chlamydia pneumoniae* could be detected in the respiratory tract in a limited number of patients with COPD, but the occurrence was not correlated with serological signs of chronic infection, and further development of methods for *Chlamydia pneumoniae* detection is needed.

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