HOST RESPONSE TO INFECTION

Immune responses to Chlamydia pneumoniae in twins in relation to gender and smoking

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This study investigated whether gender or smoking has an impact on immune responses to Chlamydia pneumoniae in generally healthy adults. A total of 129 twins (46 twin pairs and 37 single twins) from the Finnish Twin Cohort who had previously reported the highest discordance for smoking with their co-twins participated. C. pneumoniae-specific serum IgA and IgG antibody levels were measured by the micro-immunofluorescence test (micro-IF) at admission and 3 months later if the IgA level in the first sample was elevated. Cell-mediated immune (CMI) responses to C. pneumoniae and control antigens from heparinised blood samples were assessed by the lymphoproliferation (LP) assay. When all the subjects were pooled and analysed by gender and smoking status, marked differences in the humoral immune response between the genders were observed, irrespective of smoking status. When twin pairs solely were analysed, significantly elevated IgA antibody levels suggestive of persistent infection were found among the currently or formerly smoking men compared to their non-smoking co-twins. The CMI response showed a reciprocal trend with respect to humoral immunity. In conclusion, specific antibody levels were found to be higher in men than in women irrespective of smoking status, although smoking may further enhance the humoral response and depress the CMI response in men.

Introduction

The characteristic feature of all chlamydial species is their tendency to establish a long-lasting parasitic relationship with the host. Recent studies have indicated a possible association between persistent Chlamydia pneumoniae infection and several chronic diseases of major importance for public health, including coronary heart disease (CHD) [1–3], adult-onset asthma [4] and chronic obstructive pulmonary disease (COPD) [5, 6]. The significance of smoking as a major risk factor for COPD and CHD is indisputable, whereas the role of smoking as a confounder of C. pneumoniae infection has remained controversial [1–3, 7]. The question of whether smoking and the presence of C. pneumoniae antibodies are independent variables or whether there are interactions between them has not been thoroughly elucidated.

It is well recognised that smokers are more susceptible to respiratory infections and malignancies of several sites than non-smokers [8, 9]. Smoking is associated with alterations in both local and systemic immune responses, including changes in the number and function of T cells, natural killer (NK) cells, alveolar macrophages and B cells [10, 11]. Alveolar macrophages from smokers have been found to be largely functionally incompetent in terms of mediating lymphoproliferation (LP) to antigens and mitogens [12]. Moreover, smoking has been shown to increase the plasma cortisol level in a dose-related manner [13] and enhance IL-4 production from mononuclear cells [14], both of which factors are associated with the Th2-type immune response [15, 16]. As the cell-mediated immune (CMI) response has been found to be crucial in the resolution of chlamydial infections [17, 18], it is possible that the altered cytokine proportions in the lung micro-environment may lead to a predominance of
the Th2-type response and further promote the establishment of chronic chlamydial infection.

Although the effects of smoking on the different categories of immune response have been widely studied, the possible smoking-associated changes in C. pneumoniae-specific humoral and CMI responses have not been elucidated. It is not known whether smoking is a contributory factor in the establishment of chronic infection. In particular, studies of cell-mediated immunity to C. pneumoniae have been lacking. The aim of this preliminary study was to investigate whether smoking affects C. pneumoniae-specific humoral or CMI responses in generally healthy twins, and whether there are gender differences in these responses. A twin cohort was chosen as the study population because analysis of twin pairs was expected to increase the power of analysis to detect possible subtle smoking effects.

Subjects and methods

Subjects

The study subjects were recruited from the population-based Finnish Twin Cohort. This cohort comprises > 16000 same-gender twin pairs born before 1958 and alive in 1975. The determination of zygosity and the assessment of the representativeness of subject selection have been described in detail elsewhere [19]. A questionnaire including items on smoking habits and concomitant chronic diseases was sent to the 86 twin pairs, monozygote (MZ) or of undefined zygosity (UZ), who in a previous study in 1990 [20] had reported the highest discordance for smoking assessed as pack-years. Blood samples were obtained from 129 twins (13 male and 33 female pairs, and 18 male and 19 female single twins), yielding a final participation rate of 75%. The proportion of MZ twins was 59%. The number of smoking males remained relatively small, because several recent studies have been carried out targeting the male twin pairs discordant for smoking in the Twin Cohort [21, 22]. Written informed consent was obtained from all the study subjects, and the study protocol was approved by the local ethical committee.

The subjects who reported regular smoking daily or nearly daily were defined to be current smokers, the ones who had been regular smokers in the past were ex-smokers, and the ones who had never been regular smokers were non-smokers. Heavy smoking was defined as ≥ 10 cigarettes (or corresponding amount of cigars or pipe tobacco) daily. One occasional smoker (female) was categorised as a non-smoker. The mean ages of the males according to the smoking status – current, ex- and non-smokers – were 49, 50 and 49 years, respectively, while the mean ages of the females were 46 years for current and non-smokers and 45 years for ex-smokers (Table 1). The proportion of current heavy smokers was similar for both genders (64% for men, 68% for women). In 21% of the currently smoking men and in 16% of the respective women the cumulative smoking exposure was 30 pack-years or more.

Serum and heparinised blood samples were collected upon enrolment. A second serum sample was obtained c. 3 months later from the subjects in whom the first sample had shown an elevated IgA level (≥ 40) to verify the stability of the elevated IgA level suggestive of persistent C. pneumoniae infection [1, 6]. Heparinised blood samples were analysed within 24 h, and serum samples were frozen at −20°C for later serial analysis.

Serum antibody measurements

C. pneumoniae-specific serum antibodies were measured by the micro-immunofluorescence (micro-IF) test as described earlier [23]. Elementary bodies of C. pneumoniae, strain Kajaani 6, were used as an antigen, and the samples were titrated from 1 in 8 (IgG) or 1 in 10 (IgA) dilution to the end point. Fluorescein isothiocyanate-conjugated anti-human IgG (Kallestad, Austin, TX, USA), or anti-human IgA (Sigma), each at a 1 in 40 dilution, was used as the conjugate. Before analysing the IgA antibody level, the samples were treated with Gull'sorbs reagent (Gull Laboratories, Salt Lake City, UT, USA) to remove the IgG antibodies, which may interfere with the IgA measurement [24]. All serum samples were also tested for C. trachomatis and C. psittaci IgG antibodies with elementary bodies in a pool of three immunotypes (B, E and D) of C. trachomatis (Washington Research Foundation, Seattle, WA, USA), and a mixture of two C. psittaci strains (OA and 6BC; Slovak Academy of Sciences, Bratislava, Slovakia) as antigens. Antibody titres were read by one of the investigators with an Axiosophot microscope (Zeiss, Oberkochen, Germany) at a magnification of ×400. All paired sera were tested in parallel. The positivity criterion for an elevated IgA antibody level was defined as a titre of ≥ 40, and that for an elevated IgG as a titre of ≥ 128.

Measurement of lymphoproliferation response

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood by Lymphoprep (Nyegaard, Oslo, Norway) gradient centrifugation, washed three times with Hank's balanced salts solution and suspended in RPMI-1640 medium containing human AB serum 10% and supplemented with streptomycin. The PBMCs (5 × 10^6 cells/well) were culture-stimulated in triplicate on round-bottomed 96-well plates with the optimal concentration of antigen in a total volume of 200 μl and incubated in a humidified atmosphere of CO2 at 37°C for 6 days as described previously [25].

C. pneumoniae and C. trachomatis elementary body antigens were purified for the LP assay according to...
Surcel and colleagues [25] and used in a total protein concentration of 1 µg/ml. Purified protein derivative (PPD, Statens Serum Institute, Copenhagen, Denmark) was used as a control antigen at a final concentration of 10 µg/ml and Pokeweed mitogen (Gibco, Paisley, UK) was used as a control mitogen at a final concentration of 12.5 µg/ml in each experiment. Antigen-stimulated LP responses were expressed in terms of a stimulation index (SI): the ratio of the mean LP response of triplicate cultures in the presence of the antigen to the mean LP response in its absence.

**Differentiation of mononuclear cell subtypes**

The mononuclear cell subsets were stained with fluorescein (FITC)-conjugated monoclonal antibodies anti-CD4, CD8, CD21 (B cells) and CD11b (monocytes) (Caltag Lab., San Francisco, CA, USA) and analysed by Facs analysis (Becton Dickinson, San Jose, CA, USA).

**Statistical methods**

The data were analysed biphasically. In the first phase the data were pooled and analysed as a general population stratified by gender and smoking status by the Mann-Whitney U-test and χ² test, and in the second phase an analysis of the twin pairs discordant for smoking was performed by the Wilcoxon signed rank test. The data were expressed as means and SEM. Analysis of covariance (ANCOVA) was performed to test the significance of the potential confounding factors, gender and smoking status, for the IgA and IgG antibody levels with age as a covariate. A p value of < 0.05 was considered significant.

**Results**

**Pooled data analysis**

In the pooled data including all subjects, no significant differences were found when current or ex-smokers were compared to non-smokers, either in the humoral immune response – in the geometric mean titres (GMTs) of IgG and IgA antibody levels – or in the cellular function, although a tendency towards a depressed LP response both to the specific antigen and the mitogen among current male smokers was observed (Tables 1 and 2). Nor were significant smoking-associated differences found in mononuclear cell numbers (CD8+ T cells, B cells or monocytes), with the exception of CD4+ T cells, which showed a significant increase in the mean number among female smokers compared to female non-smokers (40.4 versus 34.0, respectively, p = 0.03).

When the genders were compared in each smoking category, marked differences in the specific antibody levels emerged: among non-smokers, the GMTs of IgA and IgG antibodies were significantly higher (p = 0.01 and p = 0.006, respectively) in males than in females. Similarly, among current smokers, a significant difference in the GMT of IgG antibodies was found between the genders, males having higher values than females (p = 0.005) (Table 1). In contrast, no significant gender differences could be demonstrated either in the CMI response or in the mononuclear cell numbers, although in the cellular function a depressed LP response to C. pneumoniae and to the mitogen was found among currently smoking males compared to currently smoking females (Table 2). Moreover, in the analysis of covariance, gender proved to be the only significant factor (p = 0.0001 for IgG antibody level) (Table 3).

**Analysis of twin pairs**

The paired data analysis including only complete twin pairs revealed a smoking-associated effect on the C. pneumoniae-specific immune response: significantly higher GMTs of IgA antibodies were found in ever-smoking (i.e., currently or formerly smoking) male twins than in their non-smoking co-twins (p = 0.03). Furthermore, the cellular function showed a reciprocal trend; LP responses in currently or formerly smoking male twins were clearly depressed as compared to their non-smoking twin brothers, although the difference in this small number of twin pairs did not reach significance (Table 4).

The only significant difference in mononuclear cell numbers between twin pairs was again found for CD4+ T cells among females, currently or formerly smoking women having a higher mean number than their non-smoking co-twins (39.4 versus 34.8, respectively, p = 0.03).

No diagnostic titre changes indicative of acute infection were observed in any of the paired serum samples from males, whereas one female non-smoker showed a diagnostic seroconversion to C. pneumoniae in the IgG and IgA titres, indicating recent infection.

**Discussion**

The results derived from the pair analysis demonstrated that the GMT of IgA antibodies was significantly higher in currently or formerly smoking men than in their non-smoking twin brothers. Because of the limited number of male pairs, current and ex-smokers were analysed together. We consider this unlikely to cause any disturbing effect on the results, because it has been shown previously in a large population study that the relative risk for C. pneumoniae seropositivity (a high seropositive status) was similarly increased for currently (p = 0.03) and formerly (p = 0.01) smoking men as compared to non-smokers [26].

In accordance with these results, a relationship between smoking and elevated IgA antibody levels
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Short-lived IgA antibodies with a half-life of < 1 week [28] have been suggested to be a marker of persistent or chronic infection [1, 6] whereas more long-lived IgG antibodies are considered to indicate past infection and exposure [27]. The significant difference in the GMTs of the specific IgA antibodies of male twin pairs between men who had ever smoked and non-smokers suggests that smoking may increase the risk of males to become chronically or recurrently infected with C. pneumoniae. This elevated IgA level in smoking male twins was stable, because no seroconversions were found in any of the paired sera from males. Seroconversions were also rare among females, as only one diagnostic titre change was detected. Antibodies to C. trachomatis or C. psittaci were found only occasionally and independently of the antibodies to C. pneumoniae (data not shown), which indicates the specificity of the micro-IF test. Only an even, complete fluorescence pattern of elementary bodies was accepted as a positive finding.

The tendency to inverse relationship between humoral and CMI responses is not an unexpected finding; the Th1 and Th2 cell subsets have been shown to have cross-inhibitory properties that largely explain the general mutual exclusion of cell-mediated and antibody responses [29]. Elevated specific IgA antibody levels and depressed antigen-specific cellular function in currently or formerly smoking male twins as compared to their non-smoking co-twins, suggest a predominance of the Th2 immune type response in these men. As the secretion of IgA antibodies by human B cells has been shown to be induced by IL-10, a typical product of Th2 cells [30], and as smoking is known to favour the Th2-type response among smokers [10, 14], it is possible that the biased cytokine balance in many male smokers increases the susceptibility of these men to persistent infection.

Overall, men displayed higher specific antibody levels than women irrespective of smoking status. The gender difference found in the C. pneumoniae-specific humoral response has been reported previously [26, 27, 31], but no explanation for this phenomenon has been found so far. As regards C. trachomatis, the antibody profile in the general population by age and gender is entirely different [32].

It is possible that the smoking males who show evidence of prolonged exposure to C. pneumoniae are at an increased risk for developing irreversible tissue scarring in the lungs which, in turn, might play a role in the development of chronic airway obstruction [6]. As the pairs in the present study have a completely similar (MZ) or partly or completely similar (UZ) genetic background and share the same family history, and as the smoking and non-smoking co-twins have been reported to be similar with respect to personality

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**Table 1.** Subject characteristics, seropositivity and geometric mean titres (GMT) of IgG and IgA antibody levels according to gender and current smoking status (pooled data including all subjects)

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Gender</th>
<th>Asthma</th>
<th>COPD</th>
<th>COPD + HD</th>
<th>HD</th>
<th>COPD</th>
<th>COPD + HD</th>
<th>HD</th>
<th>COPD</th>
<th>COPD + HD</th>
<th>HD</th>
<th>COPD</th>
<th>COPD + HD</th>
<th>HD</th>
<th>COPD</th>
<th>COPD + HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current smoker</td>
<td>M</td>
<td>14</td>
<td>49</td>
<td>4.66</td>
<td>1.23</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/14</td>
<td>35.7</td>
<td>20.00</td>
<td>2/14</td>
<td>14.86</td>
<td>0</td>
<td>1/14</td>
<td>14.86</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>25</td>
<td>128</td>
<td>4.16</td>
<td>1.98</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2/25</td>
<td>69.5</td>
<td>23.75</td>
<td>1/25</td>
<td>93.2</td>
<td>0</td>
<td>1/25</td>
<td>93.2</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>M</td>
<td>14</td>
<td>52</td>
<td>4.64</td>
<td>1.25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/14</td>
<td>25.0</td>
<td>14.86</td>
<td>1/14</td>
<td>14.86</td>
<td>0</td>
<td>1/14</td>
<td>14.86</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>15</td>
<td>60</td>
<td>4.63</td>
<td>1.24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/15</td>
<td>25.0</td>
<td>14.86</td>
<td>1/15</td>
<td>14.86</td>
<td>0</td>
<td>1/15</td>
<td>14.86</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>M</td>
<td>16</td>
<td>62</td>
<td>4.64</td>
<td>1.25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/16</td>
<td>25.0</td>
<td>14.86</td>
<td>1/16</td>
<td>14.86</td>
<td>0</td>
<td>1/16</td>
<td>14.86</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>45</td>
<td>170</td>
<td>4.63</td>
<td>1.24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/45</td>
<td>25.0</td>
<td>14.86</td>
<td>1/45</td>
<td>14.86</td>
<td>0</td>
<td>1/45</td>
<td>14.86</td>
</tr>
</tbody>
</table>

n, Number of subjects meeting the criterion; N, number tested. HD, defined as coronary heart disease, acute myocardial infarction or both. (but not between smoking and IgG antibody levels)
Table 2. Cell-mediated immune (CMI) response according to gender and current smoking status

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Gender</th>
<th>N</th>
<th>n</th>
<th>-Cpn</th>
<th>-Ctr</th>
<th>-PPD</th>
<th>-PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current smoker</td>
<td>M</td>
<td>14</td>
<td>10</td>
<td>10.65 (4.0)</td>
<td>7.32 (3.1)</td>
<td>87.42 (29.6)</td>
<td>122.08 (42.5)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>25</td>
<td>17</td>
<td>16.07 (5.4)</td>
<td>9.69 (2.6)</td>
<td>110.21 (25.5)</td>
<td>197.84 (32.7)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>M</td>
<td>14</td>
<td>10</td>
<td>24.41 (9.7)</td>
<td>11.79 (4.4)</td>
<td>130.84 (46.3)</td>
<td>164.21 (44.8)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>15</td>
<td>10</td>
<td>10.28 (4.1)</td>
<td>7.31 (2.0)</td>
<td>53.31 (18.0)</td>
<td>138.79 (58.2)</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>M</td>
<td>16</td>
<td>11</td>
<td>27.30 (8.8)</td>
<td>17.06 (8.7)</td>
<td>138.25 (37.7)</td>
<td>153.23 (53.7)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>44</td>
<td>39</td>
<td>14.31 (4.3)</td>
<td>8.64 (3.1)</td>
<td>68.84 (13.5)</td>
<td>201.76 (25.8)</td>
</tr>
</tbody>
</table>

Values are expressed as means and SEM (pooled data). n, number of subjects with heparinised blood sample; N, number of subjects in the group; SI-Cpn, stimulation index for C. pneumoniae; SI-Ctr, stimulation index for C. trachomatis; SI-PPD, stimulation index for purified protein derivative; SI-PWM, stimulation index for Pokeweed mitogen.

Table 3. Significance of gender and current smoking status for geometric mean titres (GMT) of IgA and IgG antibody levels derived from the analysis of covariance

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GMT IgA p-value</th>
<th>GMT IgG p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (covariate)</td>
<td>0.228</td>
<td>0.166</td>
</tr>
<tr>
<td>Gender</td>
<td>0.066</td>
<td>0.0001</td>
</tr>
<tr>
<td>Smoking status</td>
<td>0.770</td>
<td>0.933</td>
</tr>
</tbody>
</table>

Table 4. C. pneumoniae specific humoral and cell-mediated immune (CMI) response in twin pairs discordant for smoking

<table>
<thead>
<tr>
<th>Gender</th>
<th>Smoking status</th>
<th>Number of pairs</th>
<th>IgA GMT</th>
<th>IgG GMT</th>
<th>Number of pairs</th>
<th>-Cpn</th>
<th>-Ctr</th>
<th>-PPD</th>
<th>-PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>ever-smokers</td>
<td>13</td>
<td>42.19</td>
<td>150.20</td>
<td>6</td>
<td>13.38</td>
<td>4.08</td>
<td>144.95</td>
<td>156.09</td>
</tr>
<tr>
<td></td>
<td>non-smokers</td>
<td></td>
<td>20.00</td>
<td>176.20</td>
<td></td>
<td>34.45</td>
<td>13.09</td>
<td>156.23</td>
<td>165.17</td>
</tr>
<tr>
<td>Female</td>
<td>ever-smokers</td>
<td>33</td>
<td>12.60</td>
<td>52.97</td>
<td>20</td>
<td>12.90</td>
<td>8.28</td>
<td>99.58</td>
<td>178.92</td>
</tr>
<tr>
<td></td>
<td>non-smokers</td>
<td></td>
<td>12.86</td>
<td>47.02</td>
<td></td>
<td>13.89</td>
<td>6.73</td>
<td>82.55</td>
<td>195.38</td>
</tr>
</tbody>
</table>

Values of stimulation indices (SI) expressed as means (SEM). Ever-smoker, current or ex-smoker; IgA GMT, geometric mean titre of IgA antibodies; IgG GMT, geometric mean titre of IgG antibodies; SI, see legend to Table 2.

Table 4. C. pneumoniae specific humoral and cell-mediated immune (CMI) response in twin pairs discordant for smoking

Factors, life changes, occupational class, marital status and educational level [33], it is unlikely that factors other than smoking could explain the significant difference in the specific IgA levels between the twin pairs. The comparison of twin pairs is considered to increase the power of analysis for detecting effects of smoking, and indeed, revealed differences in this study. Whether passive smoking has any effect on immune responses to C. pneumoniae remains to be studied.

C. pneumoniae may produce chronic, silent infection in the bronchi or alveoli analogous to subclinical C. trachomatis infection in the fallopian tube. A large body of data has confirmed the causative role of C. trachomatis infection in pelvic inflammatory disease and its sequelae, tubal obstruction and infertility, over a course of years [32]. Whether similar mechanisms exist in chronic C. pneumoniae lung infection is not yet known. As most C. pneumoniae infections are mild or asymptomatic it is evident that many infections will never be detected. The vast majority of information about immunity to C. pneumoniae has been derived from serological analyses; little emphasis, if any, has been put on cell-mediated immunity, in spite of the intracellular nature of the agent. To our knowledge this is the first attempt to assess the humoral and CMI responses to C. pneumoniae in a case-control study. The demonstration of an enhanced humoral response and the tendency to a depressed CMI response in smoking men may add to understanding about the mechanism involved in the establishment of persistent chlamydial infection.

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