**Chlamydia pneumoniae** infection in adolescents with type 1 diabetes mellitus

Antonietta Rizzo,1 Rossella Paolillo,1 Dario Iafusco,2 Francesco Prisco2 and Caterina Romano Carratelli1

1Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, Faculty of Medicine and Surgery, Second University of Naples, Naples, Italy
2Department of Pediatrics, Second University, Naples, Italy

Chlamydia pneumoniae, an intracellular bacterium, is associated with respiratory diseases, reinfectivity and chronic diseases such as cardiovascular disease, hypertension and stroke. The risk of infection is higher and infections are a serious clinical problem in patients with type 1 (insulin-dependent) diabetes mellitus (T1DM). Although diabetes mellitus and hyperglycaemia are considered possible risk factors for various types of aetiological agents, the epidemiological evidence concerning C. pneumoniae infection is scanty. The aim of the present study was to evaluate the impact of glycosylated haemoglobin (HbA1c) levels, an indicator of a hyperglycaemic state, on C. pneumoniae infection and disease chronicity; in addition we compared the duration of diabetes with the occurrence of C. pneumoniae infection. C. pneumoniae blood real time PCR and serology (IgG, IgA and IgM) assays by an ELISA method were performed. C. pneumoniae DNA was detected in 46.5 % [95 % confidence interval (CI)=35.1–57.9 %] of the patients with T1DM; this prevalence is higher (P<0.05) than in non-diabetic paediatric controls, 10.5 % (95 % CI=3.6–17.4 %). IgG/IgA C. pneumoniae antibody positivity was significantly (P≤0.05) more common in patients in poor metabolic control (HbA1c >9 %) versus patients in good metabolic control (HbA1c <7 %), suggesting that the metabolic control of the disease is compromised in the patients with T1DM. In conclusion, adolescents with T1DM were more likely to show signs of infection with C. pneumoniae compared with healthy adolescents and the results suggest an increased risk of progressing from an acute C. pneumoniae infection to a chronic form.

**INTRODUCTION**

Infective agents may predispose to, or trigger, some chronic non-communicable diseases, and may be infective triggers for some types of diabetes (Hadley, 2006; McNally et al., 2006). Type 1 diabetes mellitus (T1DM) is considered to be a chronic immune-mediated disease with a subclinical prodrome of variable duration. It is characterized by selective loss of insulin-producing β-cells in the pancreatic islets in genetically susceptible subjects (Knip et al., 2005). Environmental factors have been implicated in the pathogenesis of T1DM, both as triggers and potential factors of β-cell destruction (Dahlquist, 1995; Akerblom & Knip, 1998; Akerblom et al., 2002), although the contribution of any individual exogenous factor has yet to be defined. The risk of infection is higher and infections are serious clinical problems in patients with diabetes mellitus (Pozzilli & Leslie, 1994; Joshi et al., 1999). The mechanisms of this increased susceptibility to various infections are not fully elucidated and no biological explanation for this increased susceptibility has been given (Toplak et al., 1991). It is speculated that such diseases probably reflect and connect to long-term effects of a change in lifestyle and thereby a different exposure to certain bacteria that have been inherently associated with human societies (Daneman, 2006). A very important group of bacteria among these organisms is chlamydia, which triggers regulatory immune cell populations (Daneman, 2006; Halme et al., 2000).

Chlamydia pneumoniae, an intracellular bacterium, is associated with respiratory diseases, reinfectivity and chronic diseases such as cardiovascular disease (Yamaguchi et al., 2004; Kaul et al., 2000), hypertension (Cook et al., 1998), stroke (Wimmer et al., 1996; Fagerberg et al., 1999) and an atherogenic lipid profile (Murray et al., 1999). Previous studies have suggested that C. pneumoniae residing in circulating monocytes offers a means of distribution from the primary colonization region into other organs to initiate and participate in the maintenance of a local immunological response and inflammation (Carratelli et al.,...
C. pneumoniae can infect a wide range of human cells and survive and multiply within macrophages: elementary bodies and DNA have been observed in endothelial cells, smooth muscle cells and macrophages within atheromatous arteries (Kuo et al., 1995; Muhlestein et al., 1996). C. pneumoniae establishes a latent infection in some cell types, with life-long persistence of the bacteria in the infected body, participating in the formation of atherosclerotic plaques and potentiating the inflammatory reaction at the site of infection (Roubalová et al., 2007). It can establish persistent infection in macrophages, associated with an alteration of their function, and can modulate the host immune response (Roubalová et al., 2007; Bulut et al., 2002); these immune cell populations are probably the deterrent to some autoimmune diseases such as T1DM (insulin-dependent) (Sechi et al., 2008).

Viable C. pneumoniae are readily detectable in peripheral blood mononuclear cells (PBMC) obtained from healthy donors, indicating that the persistent presence of viable bacteria in the bloodstream might be a risk factor for the development of atherosclerosis (Yamaguchi et al., 2004; Wong et al., 1999). Furthermore, several investigations suggest that macrophages participate in the pathogenesis of diabetic nephropathy (Kanauchi et al., 2000) and it is possible to hypothesize that chronic C. pneumoniae infection within macrophages may contribute to its progression. Most studies have demonstrated that the prevalence of C. pneumoniae infection is higher in patients with diabetes mellitus than in those without, at least in the presence of poor glycaemic control (Rayfield et al., 1982). Glycosylated haemoglobin (HbA1c), an indicator of a hyperglycaemic state, reflects long-term glycaemic control and is a more stable measurement than fasting plasma glucose (Rohlffing et al., 2000).

The objectives of this study were to document the impact of HbA1c levels on C. pneumoniae infection and disease chronicity, and we compared the duration of diabetes with the occurrence of C. pneumoniae infection in a cohort of adolescents with T1DM.

**METHODS**

**Study population.** We studied 73 adolescent patients with T1DM, 39 males and 34 females, with a median age of 14.0 years (interquartile range (IQR)=12.0-15.0), recruited from the Paediatric Diabetes Clinic of the Second University of Naples, Italy. Information on past medical history, including symptoms and duration of diabetes, and demographic data, was obtained for all patients on a standardized questionnaire. We considered only subjects with three or more HbA1c measurements in the previous 2 years and we excluded those diagnosed with diabetes for less than 1 year. Excluded were subjects with diseases of the heart, kidneys or with endocrine organ alteration. Total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and triglyceride levels were taken from medical records or determined if not carried out within the previous 6 months. A random population sample of subjects and siblings of the patients were the control group, which was made up of 76 subjects with normal glucose levels and normal HbA1c, 35 males and 41 females, with a median age of 14.0 years (IQR=12.8-15.0).

The cases and controls were recruited prospectively during the study period. Patients and controls who had received antibiotics prior to blood sampling were excluded from the study. The characteristics of the study population are shown in Table 1.

Approval for the study was obtained from the local Research Ethics Committees, and informed consent was obtained from all participants.

**Glycaemic control.** The glycaemic control was evaluated with the HbA1c levels in peripheral blood. HbA1c was measured in all 149 enrolled adolescents by the point-of-care immunoassay using the DCA Vantage Analyser (Siemens Healthcare Diagnostics). We classified all of the subjects into five groups, according to the American Diabetes Association guidelines (American Diabetes Association Standards of medical care in diabetes, 2008): an HbA1c level of 4–5.9 % is considered normal; an HbA1c level of 6–6.9 % is the recommended glycaemic control; 7–7.9 %; 8–8.9 %; and 9–10 % is poor glycaemic control. As we aimed to assess the association between glycaemic control and the prevalence of C. pneumoniae disease, we excluded those diagnosed with diabetes for less than 1 year, as HbA1c is frequently normal in the early post-diagnosis period; we considered only subjects with three or more HbA1c measurements in the previous 2 years.

**Collection of samples.** Venous blood samples were collected after an overnight fast. Participants received their usual evening insulin glargine dose the evening before or were continued on their insulin pump at the usual basal rate. The laboratory technician was blinded to the names and clinical condition of the patients and controls. For each participant, samples for serology were stored at −20 °C until determination.

PBMC specimens were obtained from all subjects for PCR. DNA was extracted from the PBMC as described by Fukano (2004), eluted in a final volume of 200 μl, aliquoted and stored at −20 °C.

**Real-time PCR (RT-PCR) for C. pneumoniae.** The C. pneumoniae-specific sequences of the PCR primers and the probe were selected from a 53 kDa protein of C. pneumoniae with Sequence Detection Systems 1.6.3 and synthesized (Applied Biosystems) (Fukano, 2004). The PCR product generated was 116 bp and the sequences of the primers and the TaqMan probe were as follows: forward primer 5′-AACCAAGGTAGCAAACAAATAC-3′; reverse primer 5′-CCAGATTTCGACACCCGCTT-3′ and TaqMan probe 5′-CAGCTGTCAGACAAGCGATCACCGC-3′ (Miayshita et al., 2007). A search was performed with the BLAST program to check the specificity of the primers and the probe. The PCR was performed in 96-well MicroAmp optical plates (Applied Biosystems) with a reaction mixture consisting of 12.5 μl TaqMan Universal Master mix plus dUTP and uracyl N-glycosylase (AmpErase UNG; Applied Biosystems). The primers and the probe were in a total reaction volume of 25 μl for both the purified C. pneumoniae DNA series and the clinical specimens. Amplification and detection of the PCR products were performed with an ABI Prism 7700 sequence detection instrument (Applied Biosystems), as suggested by the manufacturer, using all the default program settings. Briefly, the cycling conditions were as follows: after 2 min at 50 °C and 10 min at 95 °C, the samples were submitted to 50 cycles, each consisting of a step at 95 °C for 15 s, followed by a step at 60 °C for 1 min. The Sequence Detection Systems 1.6.3 was used for analysis after RT-PCR. The sensitivity of the test, determined by performing titration of chlamydial cultures to analyse the lowest level of detection of the target gene, was approximately 95 % (Dowell et al., 2001).
The positive PCR result was accepted if two or more tests were positive. If a positive reaction could not be repeated, the sample was considered negative.

**Serology.** Sera from patients and control subjects were tested for the presence of specific IgG, IgA, and IgM antibodies against the *C. pneumoniae* antigen, made up of elementary bodies, using an ELISA kit, which is a species-specific test (Eurospital Spa). In this test, IgG, IgA, and IgM *C. pneumoniae* antibodies from the samples bind to the *C. pneumoniae* antigen attached to the polystyrene surface of the Microstrip wells. The results were calculated according to the manufacturer’s instructions. In this test, a primary *C. pneumoniae* infection is defined by a predominant IgM response. A positive IgA antibody level is considered a sign of chronic infection, as two IgG antibodies or *C. pneumoniae* DNA were considered negative. If a positive reaction could not be repeated, the sample was considered negative.

**Statistical analysis.** Presence or absence of IgG (≥128), IgA (≥64), IgG/IgA association, IgM (≥20) and chlamydial DNA was considered as an independent nominal variable and the distribution of these components in patients and controls was examined using the Fisher’s exact test.

The *C. pneumoniae* DNA distribution between patients with different durations of diabetes (more or less than 5 years) and healthy controls was evaluated using Fisher’s exact test. Bonferroni’s correction for multiple comparisons was applied.

As described above, values of HbA1c concentration were divided into five groups and considered categorical independent variables. We compared the presence of anti-*C. pneumoniae* antibodies or *C. pneumoniae* DNA between the different groups by using the χ² test. Bonferroni’s corrections were calculated because of multiple comparisons. For the rank correlation we used the Spearman rank correlation test.

A P-value <0.05 was considered statistically significant. Statistical analyses were performed with the MedCalc software.

**RESULTS**

**Summary of study subjects**

A total of 149 adolescents were enrolled at the hospital during the study period; the study subjects comprised 73 patients and 76 controls. The clinical features of the study population are summarized in Table 1. No significant differences between the groups were detected for age or gender.

**RT-PCR results**

For the 149 samples examined, *C. pneumoniae* DNA, detected by RT-PCR, was isolated from the PBMC of 42 individuals (28.18%), i.e. from 34 patients and eight healthy controls (Table 1).

The prevalence of *C. pneumoniae* DNA in the PBMC of the patient group differs significantly (P<0.05) from the healthy controls (46.6%, CI=35.1–57.9%, and 10.5%, CI=3.6–17.4%, respectively); the differences observed between patients with a diabetes duration of less than 5 years (19.1%) and those with a duration of more than 5 years (27.4%) was statistically non-significant.

**Serology**

In this study the diagnostic criteria to verify a recent *C. pneumoniae* infection were RT-PCR positivity for *C. pneumoniae* and specific IgM ≥20; a single IgG titre (≥128) is unreliable if used as the sole criterion for the serodiagnosis of acute *C. pneumoniae* infection, as two IgG antibody end point titres are necessary for the diagnosis. The diagnostic criteria for reinfection or chronic *C. pneumoniae* infection were RT-PCR positivity for *C. pneumoniae*, specific IgG antibody levels ≥128 and/or elevated IgA titres (≥64) in the absence of an IgM titre; a positive IgA titre in the presence of a positive IgM titre indicated a primary infection.

Of the 73 patients, 45 (61.6%) had pre-existing IgG antibodies compared with nine (11.8%) individuals in the control group, 40 (54.8%) had a positive IgA antibody titre compared with two (2.6%) in the control group, 12 (16.4%) had a positive IgM antibody titre for *C. pneumoniae*, while all controls had a negative IgM antibody titre (see Table 2 for statistical significance).

Combined seropositivity for IgG/IgA was found in 40 patients (54.8%) and one healthy individual (1.3%) in the
Table 2. Association between serological status and C. pneumoniae DNA and chronic chlamydial infection

<table>
<thead>
<tr>
<th>No. of individuals (%)</th>
<th>IgG ≥128</th>
<th>IgA ≥64</th>
<th>IgM ≥20</th>
<th>DNA</th>
<th>IgG/IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>73</td>
<td>45</td>
<td>40</td>
<td>12</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>(61.6)*</td>
<td>(54.8)*</td>
<td>(16.4)*</td>
<td>(46.6)*</td>
<td>(54.8)*</td>
</tr>
<tr>
<td>Controls</td>
<td>76</td>
<td>9 (11.8)</td>
<td>2 (2.6)</td>
<td>0 (0)</td>
<td>8 (10.5)</td>
</tr>
</tbody>
</table>

*P<0.05 Patients versus controls (Bonferroni’s correction applied).

Diabetic group and control group, respectively (P<0.05) (Table 2).

Seropositivity for IgA, expression of a chronic infection, was significantly higher in the adolescents with T1DM compared with the healthy controls (54.8 and 2.6%, respectively, P<0.05).

Blood glucose control

In patients with diabetes, the elevated prevalence of chronic C. pneumoniae infection is closely related to metabolic control.

In the healthy controls (HbA1c ≤5.9%), the prevalence of infection was 10.5%. For diabetic patients in good glycaemic control (HbA1c 6–6.9%), it was 15.4%. In those with intermediate metabolic control with HbA1c 7–7.9%, the prevalence was 28.6% and in those with intermediate metabolic control with HbA1c 8–8.9%, the prevalence was 60% (P<0.05 versus patients with good glycaemic control and P<0.001 versus control). In patients with poor glycaemic control, the prevalence rose to 61.9% (P<0.05 versus patients with good glycaemic control and P<0.001 versus control) (Fig. 1a). The patients with intermediate and poor glycaemic control presented a statistically significant increase versus the controls and those with good metabolic control (Fig. 1a).

In addition, there was a significant positive correlation between the HbA1c level and the IgG/IgA antibody levels (P<0.05, with the Spearman rank correlation test), suggesting a relationship between the degree of glucose control and a chronic state. The total percentage of IgG/IgA C. pneumoniae-antibody-positive patients was significantly higher in the HbA1c groups of 8–8.9% (68%, versus patients with good glycaemic control P<0.05 and versus control P<0.001) and 9–10% (76.2%, versus patients with good glycaemic control P<0.005 and versus control P<0.001) (Fig. 1b).

DISCUSSION

Common infectious diseases like those caused by C. pneumoniae may be closely related to chronic non-communicable diseases. An association between T1DM and C. pneumoniae infection has long been cited (Müller et al., 2003; Toplak et al., 1996) and several studies have shown an increased risk of C. pneumoniae infection in individuals with type 1 and type 2 diabetes (Roubalová et al., 2007; Lutsey et al., 2009).

In the present study, we assessed by RT-PCR the occurrence of C. pneumoniae infection in PBMCs in T1DM patients. We detected the presence of C. pneumoniae DNA in 46.6% of adolescents with T1DM; this presence was higher than that in non-diabetic paediatric controls (10.5%). In agreement with previous data, we found a correlation between the prevalence of chlamydia infection and poor metabolic control, as determined by a high HbA1c level (Toplak et al., 1996). We measured the frequency of chronic C. pneumoniae infection in the serum of T1DM patients. IgA C. pneumoniae antibody positivity was significantly more frequent in patients in poor metabolic control (HbA1c >9) versus patients in good metabolic control, suggesting that adolescents with diabetes may be at increased risk of progressing from acute C. pneumoniae infection to a chronic form. Although the number of cases and controls in our study is rather small,
an association between hyperglycaemia and the infection and its chronicity seems to emerge.

At present, we do not know whether a high HbA1c level increases the risk of C. pneumoniae disease progression or whether hyperglycaemia occurs as a consequence of C. pneumoniae infection. In fact, the role of C. pneumoniae infection in diabetic patients is still not clear (Iliescu et al., 2000; Müller et al., 2003) and uncertainty remains around whether C. pneumoniae is a risk factor for T1DM; however, it is clear that C. pneumoniae infection, like other infections, complicates diabetes management (Young et al., 2009). Moreover, the development of diabetes may be a factor promoting C. pneumoniae systemic dissemination, and the development of atherosclerotic plaques in diabetic patients may be accelerated by the presence of C. pneumoniae in the blood (Yamaguchi et al., 2006; Mahony et al., 2000; Jha et al., 2009). The study by West et al. (2009) does not preclude the possibility of the presence of persistent C. pneumoniae in the lung or in atherosclerotic plaques. It has been hypothesized that PBMCs only serve as transport vehicles from the lungs to the arterial wall, where a chronic infection can take place (Müller et al., 2003). Animal models have shown that hyperglycaemia promotes C. pneumoniae dissemination from the lung to the peripheral blood and causes higher bacterial loads (Yamaguchi et al., 2006); a mouse model carried out by Wang et al. (2009) indicated that in obese C57BL/6 mice, C. pneumoniae infection induced significantly increased insulin resistance that persisted long after bacterial clearance.

The development of diabetes is likely to cause a compromised host-permitting infection by immune suppression (Feigin & Shearer, 1975; Johnson, 2000; Orrett, 2000) and this evasion from the host defence system may promote long-term C. pneumoniae survival and growth in the lung or peripheral blood cells. The increased presence of C. pneumoniae in adolescents with diabetes may be secondary to defects in the host defence and immune cell functions, increasing their risk of progressing from an acute C. pneumoniae infection to a chronic form.

Current knowledge on the involvement of C. pneumoniae in chronic inflammatory diseases, exemplified in coronary artery disease, reveals that C. pneumoniae-infected macrophages disseminate to secondary organs and exacerbate pathological processes (Campbell & Kuo, 2004). It is possible that diabetes makes C. pneumoniae management more difficult and that chronic stimulation of the inflammatory system by C. pneumoniae may affect diabetes management and outcome.

The mechanisms through which the micro-organism may cause a predisposition to diabetes may be related to the capacity of C. pneumoniae to use diverse mechanisms to enter the host cells, most likely multiple mechanisms for its attachment and internalization, which may differ depending on the host cell type (Kaukoranta-Tolvanen et al., 1992). Disturbances in the function of white blood cells seem to be common in diabetes and thus would result in higher susceptibility to infection (Toplak et al., 1996). Previous studies focusing on the chlamydial glycan, a high mannose oligosaccharide that promotes attachment and internalization of chlamydiae, demonstrated that ligands of the mannose-6-phosphate/insulin-like growth factor 2 (IGF2) receptor affect the ability of C. pneumoniae to infect endothelial cells (Chen & Stephens, 1997; Chen et al., 2004). IGF2, a monocyte-derived soluble factor, was shown to enhance infection of endothelial cells by C. pneumoniae. An experimental study also demonstrated an IGF2 increase in diabetic patients (Yi et al., 2001), suggesting the possible mechanisms of involvement of an IGF2 receptor in C. pneumoniae infection (Lin et al., 2000, 2001).

In conclusion, our findings in a cohort of adolescents with T1DM suggest that an elevated HbA1c level is not only a risk factor for C. pneumoniae infection onset but also a possible cofactor increasing the risk of disease chronicity. Although the mechanism by which hyperglycaemia per se is involved in infectious diseases remains obscure, early identification of hyperglycaemia and appropriate behavioural and therapeutic intervention may be beneficial for the prevention of C. pneumoniae infection, especially when the number of diabetic individuals is increasing rapidly. There is a need for rational and standardized screening, and monitoring of C. pneumoniae infection in adolescents with diabetes, and we recommend that prospective controlled studies be conducted to avoid the chronic complications related to metabolic control.

REFERENCES


