Chapter VII

The role of *Chlamydia* in primary sclerosing cholangitis

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ABSTRACT

Background/Aims: The etiology of primary sclerosing cholangitis (PSC) is unknown. We recently found evidence of elevated seroprevalence of *Chlamydia* antibodies. This finding was retested against three control groups. Moreover, serological subtyping in *Chlamydia trachomatis* and *Chlamydia pneumoniae* was carried out. In addition, *Chlamydia* specific heat shock proteins were assessed. Finally, the presence of *Chlamydia* in liver tissue of PSC patients was looked for.

Methods: Anti-lipo polysaccharide (LPS) antibodies were determined by ELISA in 41 well-established PSC patients and three race matched control groups (an inflammatory bowel disease (IBD) group, n=35; a non-IBD patients group, n=39; a healthy blood donor (BD) group, n=40). Subtyping in *C. trachomatis* and *C. pneumoniae* serotypes by specific anti-major outer membrane protein (MOMP) assays was done in the four groups. Immunohistochemical staining using specific markers for *Chlamydia* was done on liver biopsies of 14 PSC patients. Furthermore, *Chlamydia*-heat shock protein60 (HSP60) antibodies were determined in the PSC- and BD-groups.

Results: Seroprevalence of anti-*Chlamydia*-anti-LPS antibodies was markedly elevated in the PSC patients compared to the IBD-, the non-IBD-, and the BD-groups. The outcomes in the *C. trachomatis* and *C. pneumoniae* anti-MOMP assays did not correlate with the anti-LPS positive PSC sera. HSP60 antibodies were significantly elevated in the PSC-group compared to the BD-group. The presence of *Chlamydia* in liver tissue could not be demonstrated.

Conclusions: Our findings suggest an association between PSC and (previous) infection with *Chlamydia*. This supports the hypothesis of an aberrant immune response directed towards certain constituents of the bile duct, possibly incited via mimicry by a *Chlamydia* infection.

Key words: primary sclerosing cholangitis; *Chlamydia*; pathogenesis; autoimmune disease; serology; immunohistochemistry.
INTRODUCTION

The aetiology of primary sclerosing cholangitis (PSC) is unknown. It is generally believed to be a primary immune mediated disorder. This notion comes from the close association of PSC with inflammatory bowel disease. Only few reports have been published concerning the possible role of microbiological agents in the aetiology of PSC. Portal bacteremia in ulcerative colitis has been suggested, but never demonstrated (1-4). As far as viruses are concerned, only reovirus type 3 and cytomegalovirus have been studied in PSC. Both agents were ruled out as a likely cause of PSC (5, 6).

We recently performed a large serological survey comprising 22 different common viruses, as well as Chlamydia species and Mycoplasma pneumoniae. A clear association between PSC and Chlamydia spp. was found (Chapter VI).

The aim of the present study is to further substantiate this association. We therefore performed a case-control study comparing the serological results in the PSC cohort to three different control groups. Furthermore, serological subtyping into C. trachomatis and C. pneumoniae was performed in an attempt to identify the prevailing subspecies. Immunohistochemistry on liver biopsies from PSC patients was done in an attempt to demonstrate the organisms in portal tracts. Lastly, the putative role of Chlamydia-specific heat shock protein 60 was studied.

MATERIALS AND METHODS

Patients

Cases consisted of 41 consecutive PSC patients, accrued from our GI outpatient department. The diagnosis of PSC was established by ERCP (n=39), liver biopsy (n=2), or both (n=16). All cases were Caucasian, their median age was 46 years (range 21-76), 25 of them were male. Median duration of overt disease at the time of serum sampling was 4 years (range 0-17). Concurrent inflammatory bowel disease (IBD) was present in 30 patients. One patient used azathioprine and one patient was on low-dose prednisolone, both for control of colitis.

Two race matched control groups were constructed, collected from the same GI outpatient clinic as the case-group. One IBD control group (IBD) consisted of 35 Caucasian patients with endoscopically established inflammatory bowel disease, without liver test abnormalities, nor a history suggestive of liver disease. A second non-IBD control group (non-IBD) consisted of 39 randomly selected Caucasian patients with general GI complaints, who did not have a history suggestive of IBD,
nor of liver disease. All patients in this group had normal liver tests. A third control group was assembled from 40 healthy blood donors (BD), in order to estimate the prevalence of *Chlamydia* antibodies in the general population. Age and gender characteristics of the various groups are shown in table 1.

**Table 1.**

<table>
<thead>
<tr>
<th></th>
<th>Cases PSC n=41</th>
<th>Controls IBD n=35</th>
<th>Controls Non-IBD n=39</th>
<th>Controls BD n=40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>46 (21-76)</td>
<td>35 (21-65)</td>
<td>40 (21-76)</td>
<td>36 (21-61)</td>
</tr>
<tr>
<td>Gender m / f (%)</td>
<td>25 / 16</td>
<td>13 / 22</td>
<td>11 / 28</td>
<td>33 / 7</td>
</tr>
<tr>
<td></td>
<td>(61/39)</td>
<td>(37/63) (*)</td>
<td>(28/72) (*)</td>
<td>(82/18) (*)</td>
</tr>
</tbody>
</table>

PSC = primary sclerosing cholangitis  
IBD = inflammatory bowel disease  
non-IBD = general GI outpatients  
BD = healthy blood donors

*) = gender distribution significantly different from PSC group by Chi-square testing

**Serologic testing**

The presence of IgG-, IgM-, and IgA- antibodies against *Chlamydia* spp. was determined using a broad *Chlamydia*-specific anti-lipopolysaccharide (LPS) assay (Medac, Hamburg, Germany). For characterization into serotypes the following assays were used: *Chlamydia trachomatis* (IgG and IgA): Merlin anti-major outer membrane protein (MOMP) ELISA, (Labsystems Oy, Helsinki, Finland), *Chlamydia pneumoniae* (IgG and IgA): Elegance anti-MOMP ELISA, (Bioclone, Marrickville, Australia). For all three assays cut-off values were used as specified by the manufacturers. Anti *Chlamydia*-specific heat shock protein (HSP) titers were determined in the PSC- and BD- groups by ELISA after coating with the *Chlamydia* HSP60 Cpn60 (StressGen Biotechnologies Co., Victoria, BC, Canada).
**Immunohistochemistry**

In vivo presence of *Chlamydiae* species was assessed by immunofluorescence staining of 10 liver biopsies from the PSC group and 4 other PSC patients, all biopsied in 1994, using 14 gauge Quick-Core biopsy needles (William Cook, Bjaerverskov, Denmark). Paraffin sections were incubated with 1:25 diluted 16.3B6 and 1:400 diluted RR402, both generously provided by the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. 16.3B6 is a broad anti *Chlamydia-LPS* antibody and RR402 is a specific *Chlamydia pneumoniae* membrane protein antibody. As second and third step goat-anti-mouse biotin and streptavidine horse radish peroxidase (HRP) were applied, respectively. Visualisation was made possible by diaminobenzidine staining. *Chlamydia pneumoniae* infected and non-infected Hep2 cells served as positive and negative controls, respectively. For detection of *Chlamydia trachomatis*, deep frozen sections from 14 PSC patients, who were all part of the PSC serum group, were used. After fixation in acetone, the specimens were incubated with a *C. trachomatis* monoclonal antibody (Siva Microtrak, Behring Diagnostics Inc., Cupertino, CA, USA), in a 1:10 dilution at room temperature for 1 hr. Labelling was done with rabbit-anti-mouse IgG, and conjugation with HRP. Peroxidase activity was made visible with amino-ethylcarbazole H₂O₂. *C. trachomatis* infected and non-infected McCoy cell suspensions served as positive and negative controls, respectively. Six deep frozen surgical liver biopsies from patients with liver metastases from colorectal cancer, primary cholangiocarcinoma, or hepatocellular carcinoma served as additional controls.

**Statistical analysis**

To investigate the association of PSC with the presence of serum antibodies to *Chlamydia species*, differences in proportion of positive sera in the three assays between cases and controls were determined. Odds ratios (OR) were calculated with exact 95% confidence intervals (95% CI) according to Mehta *et al.* (7). To assess whether a positive anti-LPS test result represented in fact seropositivity for either *C. trachomatis* or *C. pneumoniae* antibodies, correlations between the three assays were estimated by Phi-coefficients. The putative role of *Chlamydia*-specific HSP was assessed by comparing the anti-HSP titers of the PSC group with the BD group with the use of Mann-Whitney U testing. The proportion of liver tissue specimens in the PSC group that showed positive staining with the *C. trachomatis* monoclonal antibody was compared with the control
group using Chi-square testing.
For statistical analysis the SPSS 8.0 package (SPSS Inc., Chicago, IL, USA) and EpilInfo version 5 (Centers for Disease Control, Atlanta, GA, USA) were used.

RESULTS

Comparison with control groups

The results of the Medac anti-LPS *Chlamydia* ELISA assay in the PSC group were compared with the outcomes for the three control groups and odds ratios (OR) were calculated (see figure 1 and table 2). No significant differences were observed regarding the proportions of patients positive for IgG between the groups. In contrast, the proportion of IgM positive samples in the PSC group (29%) was much higher compared to the control groups (IBD: 6%, non-IBD: 18%, and BD: 0%, respectively). This difference was statistically significant for the comparison of the PSC group versus the IBD- (OR = 6.8) and the donor (OR = not applicable) groups. When the IgA results between the PSC group and the control groups were compared, the odds of a positive titer in the PSC group were much higher compared to the IBD- (OR = 7.9), the non-IBD- (OR = 6.6), and the donor groups (OR = 19.5).

Table 2.
Odds ratios and confidence intervals for the presence of IgG-, IgM-, and IgA- antibodies in the anti-LPS assay for the PSC cohort versus the control groups

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>PSC vs. IBD</td>
<td>1.8 (0.6-5.6)</td>
<td>6.8 (1.3-66.3) *</td>
<td>7.9 (2.3-25.8) *</td>
</tr>
<tr>
<td>PSC vs. non-IBD</td>
<td>1.2 (0.4-3.7)</td>
<td>1.9 (0.6-6.4)</td>
<td>6.6 (2.2-20.7) *</td>
</tr>
<tr>
<td>PSC vs. BD</td>
<td>1.5 (0.5-4.5)</td>
<td>n.a. (3.4 - ∞) *</td>
<td>19.5 (5.6-70.2) *</td>
</tr>
</tbody>
</table>

OR (95% CI) = odds ratio with 95% confidence interval
n.a. = not applicable; OR cannot be calculated because no positive results were encountered in the BD group
*) = difference significant
Fig 1. Proportion of positive sera in the three assays per immunoglobulin subclass. * = difference statistically significant from PSC group.
Characterization into serotypes

The Merlin anti-MOMP IgG for *Chlamydia trachomatis* was found positive in 9/40 PSC patients (one sample missing). The IgA showed positive titers in 15/40 PSC patients. The degree of correlation with the anti-LPS outcomes was very small and non-significant (Phi = 0.164, p = 0.29 and Phi = -0.01, p = 0.95 for IgG and IgA respectively). The Elegance ELISA, which is specific for *Chlamydia pneumoniae*, showed for the IgG assay 31/41 positive titers, and for the IgA 30/41 positive test results, proportions which were almost identical to the Medac test findings. However, the correlations with the Medac assay results were only weak as estimated by Phi-coefficients (Phi = 0.21, p = 0.186 for IgG, and Phi = -0.30, p = 0.056 for IgA, respectively). The results of the two anti-MOMP ELISAs are depicted in figure 1. When calculating the odds ratios between the PSC group and the various control groups, the significant differences observed in the anti-LPS test were not retained in the *C. trachomatis* or the *C. pneumoniae* assay. The odds ratios with 95% confidence intervals are listed in table 3.

Table 3.

<table>
<thead>
<tr>
<th></th>
<th><em>C. trachomatis</em></th>
<th></th>
<th><em>C. pneumoniae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgA</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>PSC vs. IBD</td>
<td>0.6 (0.2-1.9)</td>
<td>1.9 (0.6-6.2)</td>
<td>0.77 (0.2-2.6)</td>
</tr>
<tr>
<td>PSC vs. non-IBD</td>
<td>0.5 (0.2-1.5)</td>
<td>0.8 (0.3-2.2)</td>
<td>0.6 (0.2-2.0)</td>
</tr>
<tr>
<td>PSC vs. BD</td>
<td>1.9 (0.5-8.0)</td>
<td>3.9 (1.1-15.3) *)</td>
<td>0.6 (0.2-1.9)</td>
</tr>
</tbody>
</table>

*) = difference significant
Heat shock protein antibodies

Anti *Chlamydia*-specific heat shock protein titers in the PSC group were significantly higher compared to the outcomes in the BD group, (median 320 OD versus 190 OD, Mann-Whitney U: p < 0.001). This is shown in figure 2.

![Box plots of anti-HSP titers in the PSC- and BD-groups](image)

Fig 2. Box plots of anti-HSP titers in the PSC- and BD-groups

OD = optical density units
3 cases missing

Immunohistochemistry

The anti-LPS immunohistochemical staining was dubious in one out of 14 PSC patients, but otherwise negative. The *Chlamydia pneumoniae* specific staining yielded several positive cells in the portal tracts in only one of 14 patients, who was not in the PSC serum group. The *C. trachomatis* specific antibody staining yielded no apparent positive staining portal tracts cells. Interestingly, slight to moderate diffuse cytoplasmic staining of the bile duct epithelium, mainly at the apical side, was observed in 8/14 PSC specimens. An example is shown in figure 3. This phenomenon was seen in one of the six control liver specimens only, which came from a female with cholangiocarcinoma. However, the difference in proportion of positive samples between the PSC liver tissue specimens and the controls did not reach a statistically significant level (p = 0.12).
DISCUSSION

The results of this study confirmed our earlier observation of increased seroprevalence of anti-LPS Chlamydial antibodies in a PSC cohort, when compared with three matched control groups. This applies in particular to IgA antibodies. The Medac anti-LPS assay detects antibodies against an epitope on the LPS molecule that is shared by all subtypes of the genus Chlamydiales. To find out if the anti-LPS positive sera in fact represented seropositivity for either C. trachomatis or C. pneumoniae, specific assays for these subtypes were run on all sera. With regard to C. pneumoniae, a high proportion of positive sera was observed in the PSC group, as
well as in the three control groups. However, the correlations between the outcomes for the anti-LPS assay and both anti-MOMP ELISAs for the individual patients were only weak and not statistically significant.

There are several possible explanations for the observed discrepancies.

1) Lower specificity of the anti-LPS assay compared to the anti-MOMP ELISAs. If this were the case, the outcomes for the former test might represent seropositivity for other *Chlamydia* spp.. Some years ago, a novel *Chlamydia* species named *C. pecorum*, was described (8, 9). Moreover, very recently, Ossewaarde and Meijer found molecular evidence for the existence of yet additional members of the order *Chlamydiales* (10).

2) Alternatively, positive anti-MOMP IgA titers may largely represent past infection, whereas IgA reactivity in the anti-LPS assay may represent persisting infection. Yet, presence of *Chlamydiae* spp. by immunohistochemical staining in liver tissue specimens was not apparent.

The low number of positive IgM sera compared to IgG and IgA outcomes and the lack of positive immunohistochemical staining in the liver biopsies, both argue against persisting *Chlamydia* infection. We hypothesized that a relatively trivial micro-organism such as *Chlamydia* may give rise to autoantibodies that cross-react with certain constituents of the bile duct system in genetically susceptible hosts. Moreover, PSC is a chronic disease with an insidious onset and it may well be that an inciting noxious event precedes the onset of overt disease by many years. Autoantibodies could result from a variety of mechanisms including (1) incorporation of host antigens into viral or bacterial structures or pathogen-induced alteration of host antigens, (2) pathogen-induced alterations in immunoregulatory systems, (3) cross-reactivity (molecular mimicry), and (4) induction of anti-idiotypic antibodies which stimulate host cell receptors (11). For these pathogenetic pathways the responsible micro-organism need not be present anymore (hit and run phenomenon). Recently, in BALB/c mice it was shown that *Chlamydia* can induce heart disease through antigenic mimicry of a heart muscle-specific protein (12). In this respect, our finding of slight to moderate staining of bile duct epithelium at the brush border side in 8 out of 14 liver biopsies from PSC patients after incubation with the *C. trachomatis* immunohistochemical antibody, may be of interest.

Another interesting feature of *Chlamydia* is the ability to elicit anti heat shock proteins which are involved in tubal damage (13, 14). In our study there was a striking difference in the prevalence and height of the titers of anti-HSP60 between the PSC-group and the BD-group.

In conclusion, our findings suggest an association between PSC and previous
infection with *Chlamydia* spp.. This supports the hypothesis of the development of an auto immune response to certain constituents of the bile ducts in genetically susceptible individuals as the pathophysiologic mechanism that leads to PSC, in which *Chlamydiae* may play a role as the inciting noxious agent. Further research should focus on more sensitive techniques such as in situ hybridization and PCR to determine traces of the presence of *Chlamydia* spp. in portal tracts, on subtyping the involved *Chlamydia*, and on the possible role of anti-*Chlamydial*-HSP antibodies in mediating chronic inflammation.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


REFERENCES


