Chlamydia trachomatis-associated tubal factor subfertility: immunogenetic aspects and serological screening

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Chlamydia (C.) trachomatis female genital tract infections usually remain asymptomatic and untreated. Therefore, an adequate immune response, rather than antibiotic treatment, is essential to clear the pathogen. Most women will effectively clear C. trachomatis infections, but some will have persistent C. trachomatis infections, which may ascend to the upper genital tract and increase the risk of tubal factor subfertility. Pattern recognition receptors (PRRs) of the toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) families recognize C. trachomatis and initiate the immune response. Host immune factors are determinants of the course of C. trachomatis infections. Genetic variations in TLR and NOD genes may affect receptor function, leading to inadequate recognition of C. trachomatis, an inadequate immune response, and consequently an increased risk of persistence and late sequelae. For the risk assessment of tubal pathology in subfertile women, C. trachomatis immunoglobulin (Ig) G antibody testing (CAT) in serum is widely used. A positive CAT is indicative of a previous infection but not of a persistent infection. Measuring serological markers of persistence, of which C-reactive protein (CRP) seems promising, in CAT-positive women may identify a subgroup of subfertile women with persistent C. trachomatis infections and the highest risk of tubal pathology.

Key words: Chlamydia trachomatis/immunogenetics/serological markers/tubal factor subfertility

Introduction

A large inter-patient variability exists in the course and outcome of a C. trachomatis infection. Some women clear the infection adequately without developing tissue damage, whereas others get a persistent infection, which increases the risk of tubal damage and tubal factor subfertility. The course and outcome of infectious diseases are generally determined by virulence factors of the pathogen, environmental factors and host immune factors. Regarding C. trachomatis female genital tract infections, pattern recognition receptors (PRRs) of the innate immune system are suggested to be involved in clearance of the infection. Genetic variations in PPRs may contribute to persistence, thereby increasing the risk of tubal pathology.

A better understanding of the role of persistent C. trachomatis infections in tubal factor subfertility may be useful in optimizing the fertility work-up by incorporating screening tests for persistent C. trachomatis infections, aiming to accurately estimate the risk of persistence and identify those women who are at highest risk of tubal pathology.

This review will address the following items:
(i) The normal immune response to infections.
(ii) The course of a C. trachomatis infection: recognition of the pathogen.
(iii) Screening for C. trachomatis-associated tubal factor subfertility.
(iv) Summary and future perspectives.

The normal immune response to infections

Innate immune system

The innate immune system is a general, non-specific system, which is the first line of defence against pathogens that are unknown to the host. Key elements of the innate immune system are macrophages, neutrophils, dendritic cells and natural killer (NK) cells. Several studies have suggested that besides the above-mentioned immune cells, epithelial cells play an important role in the early immune response to infections (Rasmussen et al., 1997; Quayle, 2002; Stephens, 2003).

Both epithelial cells and circulating cells of the innate immune system possess cell-surface-bound or intracellular PPRs. The two most important families of PRRs are the toll-like receptor (TLR) family and the nucleotide-binding oligomerization domain (NOD) proteins. PRRs recognize and bind pathogen-associated molecular
patterns (PAMPs), which are components on and in foreign organisms. Binding of a PRR to its PAMP initiates several intracellular reactions, including a signal transduction cascade with nuclear factor (NF)-κB as the end product. NF-κB is able to bind to specific DNA sequences in the nucleus, thereby enhancing the production of pro-inflammatory cytokines. Some PRRs, such as cluster of differentiation 14 (CD14), (partly) exist in a soluble extracellular form and act as a co-receptor. Initiation of the innate immune response then occurs by binding of an extracellular PAMP–PRR complex to a transmembrane PRR. Because different PRRs recognize different PAMPs, the PRR system provides a complex and flexible initiation of the innate immune response. Figure 1 shows the initiation of the innate immune response by PAMP–PRR complexes.

When a pathogen enters the body, epithelial cells are the first line of defence. The epithelial PRRs bind to the pathogen, and the epithelial cells start to secrete chemokines (which attract circulating cells of the innate immune system to the site of infection) and other pro-inflammatory cytokines. When the circulating cells of the innate immune system arrive at the site of infection, their PRRs bind to the pathogen. Subsequently, macrophages, neutrophils and dendritic cells ingest the pathogen by phagocytosis and destroy it within the cell. NK cells directly destroy the pathogen by cytolysis. Macrophages and dendritic cells are able to express pathogen components (antigens) bound to major histocompatibility complex (MHC) proteins (also known as human leukocyte antigens) on their surface and to serve as antigen-presenting cells (APCs), which can activate the acquired immune system. Circulating cells of the innate immune system also produce pro-inflammatory cytokines.

**Acquired immune system**

The acquired (or adaptive) immune system is a specific system, which develops after the first contact with a pathogen. It builds up a memory against the pathogen, which is responsible for a quick immune response following re-infection. The acquired immune system consists of a humoral arm (with B lymphocytes, mainly targeting extracellular pathogens) and a cell-mediated arm (with T lymphocytes, mainly targeting intracellular pathogens), which closely interact.

In the humoral arm, B lymphocytes are activated by APCs (cells of the innate immune system or T lymphocytes). Activated B lymphocytes develop into plasma cells and produce antibodies [(immunoglobulins (Igs)], which neutralize the antigen or directly destroy the pathogen. An antibody–antigen complex can also activate the complement system. Furthermore, B lymphocytes can serve as APCs for T lymphocytes.

In the cell-mediated arm, T lymphocytes are activated by APCs (cells of the innate immune system or B lymphocytes). Most T lymphocytes are T helper (Th) cells. Th cells produce pro-inflammatory cytokines. The Th1 subclass produces interleukin (IL)-12 and interferon γ, which support the cell-mediated system. The Th2 subclass produces IL-4, IL-5, IL-6 and IL-10, which support the humoral system. The relative contributions of the two respective subclasses of Th cells determine whether the cell-mediated or the humoral arm is predominant. Cytotoxic T cells (or killer cells) directly attack and destroy a pathogen and produce pro-inflammatory cytokines. Suppressor T cells provide a negative feedback mechanism to protect the host against an excessive immune response (i.e. hyperinflammation).

**Complement system**

The complement system consists of a group of over 20 proteins. Most of them are circulating in an inactive form (precursors). Once the complement system is activated, a cascade of reactions leads to active end products, which enhance the immune response or destroy the pathogen. Activation of the complement system can be induced by an antibody–antigen complex (classical pathway) or by membrane components of the pathogen (alternate pathway).

**The course of a C. trachomatis infection: recognition of the pathogen**

**Clearance of a C. trachomatis infection**

In most women, a normal immune response to a C. trachomatis infection will occur, resulting in an adequate clearance (Golden et al., 2000; Joyner et al., 2002; Morré et al., 2002; Molano et al., 2005). The host is exposed to the pathogen during a short period, leading to no or minimal tissue damage. A key element of a normal immune response to a C. trachomatis infection is an adequate recognition of the pathogen by PRRs on and in epithelial cells in the genital tract and initiation of the immune response. The role of PRRs of the TLR and NOD families in C. trachomatis recognition and an early initiation of the immune response will be discussed in this review and is summarized in Table I. The role of pro- and anti-inflammatory cytokines in the immune response will not be covered by this review.

**TLRs**

TLRs are cell-surface-bound or intracellular PRRs. So far, 11 different TLRs have been identified. The PAMPs of all TLRs, except TLR10, are known (Akira and Takeda, 2004). Binding of a TLR 720
to its PAMP initiates the immune response by triggering the NF-kB signal transduction cascade. It is plausible that TLRs play a role in the host defence mechanism against *C. trachomatis* genital tract infections, because some TLRs are able to recognize *C. trachomatis* PAMPs and are expressed in epithelial cells in the human genital tract.

TLR2 is the PRR for the *C. trachomatis* component peptidoglycan (Schwandner et al., 1999; Yoshimura et al., 1999), and TLR4 is the PRR for the *C. trachomatis* components lipopolysaccharide (LPS) and heat shock protein (hsp) (Poltorak et al., 1998; Ohashi et al., 2000). TLR2 and TLR4 are expressed in the human female genital tract (Pioli et al., 2004; Fazeli et al., 2005) and in the human uterine epithelial cell line ECC-1 (Schaefer et al., 2004). TLR2 is also expressed in a cloned murine tubal epithelial cell line (Derbigny et al., 2005). Differential expression along the human genital tract has been observed for TLR2, mainly expressed in the tubes and endometrium and weakly expressed or even absent in the ectocervix (Pioli et al., 2004; Fazeli et al., 2005). These differences in expression may be related to the different functions of the different parts of the genital tract: protection against sexually transmitted pathogens without disturbing the functional vaginal commensal flora and toleration of semen and embryonic implantation.

TLR9 recognizes bacterial DNA (Hemmi et al., 2000). So far, the expression of TLR9 has not been studied in the human female genital tract, although TLR9 expression has been found in the human uterine epithelial cell line ECC-1 (Schaefer et al., 2004). Its precise role in *C. trachomatis* female genital tract infections remains to be established.

TLR1, TLR3, TLR5 and TLR6 are also present in the human female genital tract (Pioli et al., 2004; Fazeli et al., 2005), but they do not recognize *C. trachomatis* PAMPs. This suggests that these TLRs may play a role in the host defence against non-*C. trachomatis* and/or polymicrobial genital tract infections.

Animal studies are able to provide information on the role of PRRs in *C. trachomatis* infections that cannot be obtained by human studies, although results of animal studies may not be freely translated to the human *in vivo* situation. Knockout (KO) mouse technology offers the opportunity to remove entire genes of interest from the genome, to compare the course and outcome of infectious diseases between KO mice and wild-type (WT) mice, which possess the gene of interest. Darville et al. (2003) have designed a KO mouse model to study the role of TLR2 and TLR4 in the course and outcome of a *C. muridarum* infection, which is the mouse variant of *C. trachomatis*. WT mice with normal TLR2 and TLR4 genes served as controls. The *in vitro* cytokine production of macrophages was down-regulated, but not totally inhibited, in macrophages derived from TLR2 KO mice, whereas it was up-regulated in macrophages derived from TLR4 KO mice. The *in vivo* resolution of a *C. muridarum* infection was equally efficient in KO and WT mice, indicating that the remaining and/or compensatory immune mechanisms seem to lead to sufficient clearance. Remarkably, TLR2 KO mice developed less tubal pathology in comparison with WT mice, despite a down-regulated cytokine production. These findings suggest that TLR2 genetic variations provide a balanced immune response leading to efficient clearance, rather than hypo- or hyperinflammation, and serve as protection against tissue damage (Darville et al., 2003).

**NOD proteins**

NOD proteins are intracellular PRRs. The family of NOD proteins contains at least 25 proteins, including NOD1 and NOD2 (Inohara and Núñez, 2003). NOD1 and NOD2 are also referred to as caspase recruitment domain 4 (CARD4) and CARD15, respectively. NODs are able to recognize intracytoplasmatic bacterial PAMPs, such as LPS and peptidoglycan (Inohara et al., 2001; Girardin et al., 2003). Binding of a NOD to its PAMP activates the NF-κB signal transduction cascade, which initiates the immune response.

Because *C. trachomatis* is an intracellular pathogen containing LPS and peptidoglycan, a role of intracellular NODs in the recognition of *C. trachomatis* has been suggested. This is supported by findings of three recent studies (Derbigny et al., 2005; Opitz et al., 2006; Ohashi et al., 2000; Girardin et al., 2003).
NOD1 and NOD2 are expressed in a cloned murine fallopian tube C. trachomatis (Opitz et al., 2006). Welter-Stahl et al. (2006) have found that C. trachomatis produces at least the rudimentary proteoglycan motif recognized by NOD1. The third study has proved that NOD1 and NOD2 are expressed in a cloned murine fallopian tube epithelial cell line (Derbiigny et al., 2005).

Clinical aspects of clearance

Up to 70–80% of the C. trachomatis infections in women are asymptomatic, and therefore unrecognized and untreated (Rahm et al., 1988). A normal immune response (rather than antibiotic treatment) is essential to clear the pathogen and to protect women from ascendance of the infection to the upper genital tract and/or transmission to a sexual partner. Studies on the natural course of untreated C. trachomatis lower genital tract infections in women show spontaneous clearance rates of 30% in the first few weeks to months, ~50% in 1 year, 80% in 2 years and 94% in 4 years (Golden et al., 2000; Joyner et al., 2002; Morré et al., 2002; Molano et al., 2005).

Although these studies indicate that most infected women seem to have an adequate local immune response, a subset of infected women will have a long-lasting C. trachomatis infection and thereby an increased risk of late sequelae. The above-mentioned studies on spontaneous clearance of C. trachomatis lower genital tract infections may even underestimate the percentage of women at risk of complications, because clearance from the lower genital tract does not necessarily mean that the infection has not already ascended to the upper genital tract. Given a worldwide prevalence of 50 million new C. trachomatis infections in women each year, a clinically significant group of infected women may be at risk of late sequelae (World Health Organization, 2001).

Persistence of a C. trachomatis infection

In some women, a C. trachomatis infection will not be cleared adequately, which may result in a persistent infection. There is no generally accepted definition of persistence. From a clinical point of view, persistence involves exposure of the host to the pathogen during a longer period, increasing the risk of ascendance to the upper genital tract, endosalpingeal tissue damage and tubal factor subfertility. However, no consensus exists on the length of this period. From a scientific point of view, persistence is assumed to be characterized by a chronic low-grade immune response and/or the presence of aberrant C. trachomatis particles. In this review, we use both the clinical and scientific description of persistence.

The course of a C. trachomatis infection (i.e. whether the infection will be cleared or persist) may be determined by virulence factors of the pathogen, environmental factors or host immune factors.

Virulence factors of the pathogen

Several studies have evaluated whether different serovars are associated with differences in clinical course of C. trachomatis infections, i.e. symptomatic versus asymptomatic infection, lower versus upper genital tract infection and clearance versus persistence (Ito et al., 1990; Persson and Osser, 1993; Dean et al., 2000; Morré et al., 2000; Geisler et al., 2003; Molano et al., 2005).

Serovars D, E and F account for most C. trachomatis infections (Persson and Osser, 1993; Morré et al., 2000; Geisler et al., 2003; Molano et al., 2005). Two studies reported a significant relationship between serovars and symptoms: serovar F and the less-common serovar K were associated with a symptomatic course (Morré et al., 2000; Geisler et al., 2003), whereas serovar Ia was found in asymptomatic women only (Morré et al., 2000). However, both studies could not confirm each other’s findings (Morré et al., 2000; Geisler et al., 2003), and Persson and Osser (1993) could not find any relationship between serovars and symptoms.

In asymptomatic untreated patients, spontaneous clearance from the cervix occurred more often in women infected with the common serovars F and G, whereas persistent C. trachomatis infections were observed more frequently among serovars D and E and the less-common serovars B, H, I, J and K (Molano et al., 2005). Remarkably, despite antibiotic treatment, serovars H, I and J were able to persist for 2 or 3 years in the lower genital tract of women (Dean et al., 2000). In a mouse model, the duration of lower genital tract infection was longest with serovars D and E, and ascendance to the upper genital tract occurred more often in mice infected with serovar D as compared with that in mice infected with serovar H (Ito et al., 1990).

Studies on the association between different C. trachomatis serovars and clinical course and outcome of the disease are relevant not only in the fertility field but also in the oncology field. It has already been shown that C. trachomatis cervical infections are associated with cervical cancer by increasing the risk of persistence of the high-risk types of the oncogenic human papillomavirus (Samoff et al., 2005). Serovar studies have revealed that exposure to certain single C. trachomatis serovars (G, I and D) or to multiple C. trachomatis serovars is associated with the development of cervical squamous cell carcinoma (Anttila et al., 2001).

In brief, studies on the association between virulence of the most common serovars and the course of C. trachomatis infections did not yield consistent and clinically applicable results. A hypothesis which is currently under investigation is that genetic variations in the plasticity zone (i.e. a virulence region in the bacterial genome) may account for intra-serovar or strain differences in the course and outcome of C. trachomatis infections (Read et al., 2000; Read et al., 2003; Carlson et al., 2004).

Environmental factors

The risk of tubal pathology following pelvic inflammatory disease (PID) is dependent on the number of episodes and the severity of the disease (Weström, 1980; Weström et al., 1992) (Table II). In a large follow-up study in women with laparoscopically verified pelvic inflammatory disease (PID) in relation to the risk of tubal factor subfertility (adapted from Weström et al., 1992).

<table>
<thead>
<tr>
<th>Number of episodes of PID</th>
<th>Severity of PID</th>
<th>n</th>
<th>%</th>
<th>Risk of tubal factor subfertility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>Mild</td>
<td>312</td>
<td>25</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>450</td>
<td>36</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>229</td>
<td>18</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>All grades</td>
<td>991</td>
<td>80</td>
<td>8.0</td>
</tr>
<tr>
<td>Two</td>
<td>Mild</td>
<td>185</td>
<td>15</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>65</td>
<td>5</td>
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<td>Total</td>
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</table>
PID diagnosed between 1960 and 1984, the risk of tubal factor subfertility was about 10% after one episode of PID, 20% after two episodes and 40% after three episodes (Table II). C. trachomatis accounted for ~40% of all PIDs in this study, although it should be noted that routine C. trachomatis testing was introduced in their clinic only in 1977 and was therefore not applied in all PID cases (Weström et al., 1992). The incidence of tubal factor subfertility increased significantly with the severity of PID at laparoscopy and was 0.6% after one mild episode, 6% after one moderately severe episode and 21% after one severe episode of PID (Weström et al., 1992) (Table II).

A large follow-up study (over 13,000 participants) has evaluated the risk of subfertility following a positive C. trachomatis test on samples obtained from the cervix and/or urethra (Andersen et al., 2005). Birth rates and time to birth were comparable between women tested positive and negative (Andersen et al., 2005). It should be noted that nearly all positive cases received antibiotic treatment. Therefore, the risk of subfertility following untreated C. trachomatis lower genital tract infections is assumed to be higher.

Although precise data are not available, it is suggested that the presence of multiple micro-organisms in the genital tract increases the risk of tubal pathology. In large community-based and school-based screening programmes in the UK and in the USA, 4–12% of all C. trachomatis-infected women had a co-infection, such as Neisseria (N.) gonorrhoeae (Harindra et al., 2002; Nsuumi et al., 2004). Studies in women attending clinics for sexually transmitted diseases have shown a 13–28% rate of co-infections in 2004. Studies in women attending clinics for sexually transmitted infections (Jeremias et al., 1999; Smirnova et al., 2003; Franchimont et al., 2004; Peeters et al., 2004; Frosham, 2005). Regarding C. trachomatis ocular infections, a 40% genetic predisposition was noted in a Gambian twin study, supporting the relevance of genetics in C. trachomatis infections (Bailey et al., 1998).

As discussed previously, a normal immune response to a C. trachomatis infection is based on an adequate recognition of the pathogen by, amongst others, TLRs and NODs on epithelial cells in the genital tract. In the next paragraphs, the role of genetic variations in genes encoding TLRs and NODs as potential risk factors for persistent C. trachomatis infections is discussed (see also Table I).

Variations in TLR genes. It is likely that TLR2, TLR4 and TLR9 play a role in the recognition of C. trachomatis in the genital tract, because they are able to recognize C. trachomatis PAMPs and because they are expressed in the human female genital tract. It is assumed that genetic variations in TLR genes may result in aberrant receptor density on or in cells or in dysfunctional receptors, leading to an inadequate recognition of C. trachomatis and an increased risk of persistence. However, only a few human studies have tested this hypothesis.

Regarding TLR4, it is known that only homozygous carriage of the TLR4 +896 A>G (also referred to as Asp299Gly) and Thr399Ile SNPs affects the LPS receptor function, whereas heterozygous carriage has no effect on the LPS receptor function (Erridge et al., 2003). Because almost all carriers of the common TLR4 +896 A>G SNP are heterozygous, no significant association between this SNP and C. trachomatis-associated tubal pathology has been found in a cohort of 71 subfertile women (Morré et al., 2003). In a larger cohort, the same results were found for TLR4 +896 A>G, as well as for TLR9 –1237 T>C and TLR9 +2848 G>A, although a trend was observed towards a higher risk of tubal pathology among carriers of these SNPs (den Hartog et al., submitted for publication). Also for the –260 C>T variation in the CD14 gene, the LPS-sensing co-receptor of TLR4, no involvement in the development of C. trachomatis-associated tubal pathology was found (Ouburg et al., 2005). So far, the studies mentioned are the only human studies on the role of TLR genetic variations and susceptibility to C. trachomatis genital tract infections. This limited number of human studies may be because of difficulties in collecting adequate sample sizes, because patients who have undergone a C. trachomatis infection and have had evaluation of the tubal function and carry a single or multiple genetic variations are exceedingly rare. Multicentre trials might resolve this drawback. Although functional SNPs in the TLR2 gene have been described in relation to infection and inflammation (Lorenz et al., 2000; Sutherland et al., 2005; Yim et al., 2006), no studies have been performed yet for C. trachomatis infections.

Further studies are needed to investigate the precise role of TLRs in C. trachomatis genital tract infections, in particular to determine whether TLR genetic variations act in a damaging way, as generally assumed, or in a protective way, as suggested by Darville et al. (2003) in their KO mouse model.

Variations in NOD genes. The precise role of NOD proteins in the intracellular recognition of C. trachomatis in the genital tract has not been established, although several studies suggest that NODs are involved in the immune response to C. trachomatis.

### C. trachomatis-associated tubal factor subfertility

Host immune factors

Introduction to immunogenetics. Because at this time neither virulence factors of the pathogen nor environmental factors do seem to play a major role in the difference of the clinical course of C. trachomatis infections, host immune factors are considered more important determinants of the inter-patient variability in the course and outcome.

Immunogenetic studies evaluate the role of genetic variations in immunologically important host genes in the course and outcome of infectious diseases. Among these genetic variations are single-nucleotide polymorphisms (SNPs), in which one nucleotide has been substituted, inserted or deleted, and variations in the number of repetitive DNA sequences (variable number of tandem repeats). Carrying a genetic variation may have direct or indirect biological consequences. Potential direct biological consequences of carrying a genetic variation are translation of an aberrant protein or up- or down-regulation of the translation of a normal protein. If a genetic variation is not functional, i.e. it does not change the function of the gene studied, it may have indirect biological consequences when it is inherited together with another, sometimes unidentified, functional gene nearby (linkage).

During the past years, immunogenetic studies have provided more insight into the inter-patient variability of the course and outcome of infectious diseases. Several studies have found an association between carriage of genetic variations and infectious diseases, such as hepatitis, inflammatory bowel diseases, meningococcal infections and Ureaplasma urealyticum lower genital tract infections (Jeremias et al., 1999; Smirnova et al., 2003; Franchimont et al., 2004; Peeters et al., 2004; Frodsham, 2005). Regarding C. trachomatis ocular infections, a 40% genetic predisposition was noted in a Gambian twin study, supporting the relevance of genetics in C. trachomatis infections (Bailey et al., 1998).

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genital tract infections (Inohara et al., 2001; Girardin et al., 2003; Derbigny et al., 2005; Opitz et al., 2005; Welter-Stahl et al., 2006). If this association could be confirmed, NOD genetic variations may be risk factors of inadequate recognition and persistence in C. trachomatis infections.

Several genetic variations in the NOD2 genes have been associated with the susceptibility to inflammatory bowel disease (Hugot et al., 2001; Ogura et al., 2001; Hampe et al., 2002; Murillo et al., 2002; McGovern et al., 2005). Carrying a NOD2 genetic variation seems to result in hyporesponsiveness to enteric bacteria, increasing the risk of chronic bowel inflammation. Hugot et al. (2001) have also identified a so-called gene-dosage effect: the higher the number of genetic variations in a patient, the higher the risk of Crohn’s disease. As compared with patients without NOD2 variations, the relative risk of Crohn’s disease was three in heterozygous carriers of a single variation, 38 in homozygous carriers of a single variation and 44 in heterozygous carriers of two variations (Hugot et al., 2001).

NOD1 is also a ubiquitous cytosolic receptor for peptidoglycan from Gram-negative bacteria, and recent studies have suggested that C. trachomatis and C. muridarum do, in fact, produce at least the rudimentary proteoglycan motif recognized by NOD1. Nonetheless, NOD1 deficiency has no effect on the duration of infection, the intensity of cytokine secretion or the extent of pathology in vaginally infected mice, compared with WT controls (Welter-Stahl et al., 2006). Thus, Chlamydia may not produce sufficient peptidoglycan to stimulate NOD1-dependent pathways efficiently in infected animals, or other receptors of the innate immune system may compensate for the absence of NOD1 during Chlamydia infection in vivo as has been shown by Netea et al. (2005).

The studies mentioned encourage investigation of whether NODs play a role as PRRs for C. trachomatis and, if so, whether genetic variations increase the risk of an aberrant immune response and persistence.

Conclusive remarks on immunogenetics. Carriage of a single variation in a single host gene does not necessarily lead to late sequelae of infectious diseases, especially in the case of a polygenic multivariate infection such as C. trachomatis. The immune system is a complex and flexible system, and compensatory routes will, to a certain extent, provide alternative pathways to trigger the immune response. For instance, blockage of the NOD1 pathway can be partially overcome by functional TLRs (Netea et al., 2005), and not only PRRs but also the complement system are involved in pathogen recognition. Furthermore, heterozygous carriage of some genetic variations may not have a large effect on the function of the gene (Erridge et al., 2003). It is also hypothesized that the risk of late sequelae increases with the number of genetic variations, as found for NOD variations in Crohn’s disease (Hugot et al., 2001) and TLR variations in meningococcal infections (Smirnova et al., 2003). We have studied whether carrying multiple genetic variations in four PRR genes plays a role in the development of C. trachomatis-associated tubal pathology. The results showed a higher risk of tubal pathology in carriers of at least two genetic variations (73%) as compared with carriers of less than two variations (33%) (den Hartog et al., submitted for publication). Although this cohort was too small to obtain significant differences and larger cohorts are needed to retest this hypothesis, it is tempting to suggest that carrying multiple genetic variations, rather than a single genetic variation, is a determinant of the risk of late sequelae such as tubal pathology following a C. trachomatis infection (den Hartog et al., submitted for publication).

In general, the main goal of immunogenetic studies is to provide more insight into the immunopathogenesis of infectious diseases. Regarding C. trachomatis female genital tract infections, the precise role of PRRs and their genetic variations remains to be elucidated. As long as this is not being clarified, there is no place for clinical application of immunogenetic analyses in screening for tubal pathology.

Clinical aspects of persistence

If a cervical C. trachomatis infection is not cleared adequately, the infection may ascend from the lower to the upper genital tract and/or may be transmitted to a sexual partner. Ascendance to the endometrium, tubes and pelvis may result in a (silent) PID and an increased risk of tubal factor subfertility. Histological evidence of endometritis has been found in 30–40% of women with cervicitis (Paavonen et al., 1985a; Wiesenfeld et al., 2002) and in 70% of women with suspected PID (Paavonen et al., 1985b). The microorganism itself has been isolated from the endometrium in one-third of women with a C. trachomatis cervicitis and/or urethritis (Jones et al., 1986). Salpingitis has been demonstrated in 10% of women with endometritis (Cates and Wasserheit, 1991). Tubal pathology accounts for 20–25% of the cases of subfertility in developed countries (Collins et al., 1995; Collins and Van Steirteghem, 2004) and up to 80% in developing countries (Collet et al., 1988).

The pathogenesis of C. trachomatis-associated tubal pathology is not yet fully understood. Two mechanisms are assumed to be responsible for the development of tubal damage following a persistent C. trachomatis infection. The first and probably most important mechanism is by a persistent infection causing a chronic low-grade immune response, which attacks and destroys the host cells (LaVerda et al., 1999). Secondly, C. trachomatis itself can damage the host tubal epithelial cells when its replication cycle has been completed and elementary bodies are released by cytolysis of the host cell. The latter mechanism does not appear to play a major role in persistent infections, because persistence is characterized by reduced replication of the dormant pathogen (AbdellRahman and Belland, 2005; Mpiga and Ravaarinoro, 2006). These aberrant Chlamydia particles have been identified in the genital tract, whereas previously these aberrant forms were only visualized in cell culture under special conditions (Bragina et al., 2001). More studies are needed to elucidate the precise immunopathogenesis of C. trachomatis infections. The hypothesis that persistence and low-grade inflammation are associated with tubal pathology is presently the subject of investigation.

Screening for C. trachomatis-associated tubal factor subfertility

Because most C. trachomatis infections remain asymptomatic, a patients’ history will usually not be helpful in assessing the risk of a previous C. trachomatis infection (Rahm et al., 1988; Logan et al., 2003). Several test methods to assess this risk are available.

The reference standard for diagnosing tubal pathology in subfertile women is laparoscopy with tubal testing, by which tubal patency and the presence of peri-adnexal adhesions can be assessed. However, a laparoscopy has several disadvantages. First,
it is an invasive and expensive procedure (in the Netherlands, about 1000 euros) (Fiddelers et al., 2005), requiring general anaesthesia. Operating facilities may not be easily available in every clinic. Furthermore, it holds a 1.5% risk of surgical complications (e.g. bleeding and infection) (Chapron et al., 1998). Owing to these disadvantages, laparoscopy with tubal testing is unsuitable to be applied as a screening procedure in sub fertile women on a large scale. It would be preferable to estimate the risk of tubal pathology before laparoscopy, to select only high-risk patients for this procedure. Two frequently used screening methods to assess the risk of tubal pathology are hysterosalpingography (HSG) and serological testing.

HSG

Today, several methods are used to evaluate tubal patency in sub fertile women, e.g. HSG, hysterosalpingo (contrast) sonography and transvaginal hydrolaparoscopy, of which HSG is most widely used and has been evaluated most extensively. Compared to laparoscopy with tubal testing, HSG is less expensive (in the Netherlands, about 150 euros) (Fiddelers et al., 2005) but also less accurate in diagnosing tubal pathology. HSG has a sensitivity of 58% and a specificity of 77% for diagnosing tubouterine abnormalities (defined as at least unilateral tubal obstruction and/or hydrosalpinx and/or peri- adnexal adhesions) as compared with laparoscopy (Dabekausen et al., 1994). A meta-analysis has been performed to determine the accuracy of HSG in diagnosing tubal patency and adhesions separately (Swart et al., 1995), as compared with laparoscopy with tubal testing, HSG has a sensitivity of 65% and a specificity of 85% for diagnosing tubal patency, whereas HSG is unreliable for diagnosing peri-adnexal adhesions (Swart et al., 1995). The low sensitivity of HSG (tubal pathology at laparoscopy despite normal HSG findings) may be because of peri-adnexal adhesions not visualized during the procedure itself or at the abdominal X-ray after 24 h or of incorrect interpretation of the HSG results. The specificity of HSG is higher, but still ~20% of women without tubal pathology at laparoscopy have abnormal HSG findings. These false-positive HSG findings may be because of tubal spasms, dissimilar tubal filling pressure, too high viscosity of the contrast medium used or technical failure (Dabekausen et al., 1994). Another disadvantage of HSG is the risk of infection, which is up to 10% in patients with tubal pathology (Forsey et al., 1990). Furthermore, HSG is considered a painful test by patients.

Because HSG has a limited predictive value for tubal disease and holds a risk of febrile morbidity, it is questioned whether HSG is the best screening test in high-risk patients. Owing to the disadvantages of both laparoscopy and HSG, clinicians have tried to find an inexpensive and non-invasive test, which could accurately discern high-risk from low-risk patients for tubal factor sub fertility. Ideally, on the basis of the results of such a screening test, one would subject high-risk patients to diagnostic testing (i.e. laparoscopy) and delay additional invasive and expensive testing in low-risk patients. For this purpose, serological screening tests have been developed.

Chlamydia antibody testing

Since the association between C. trachomatis IgG antibodies in serum and tubal pathology has been noted (Punnonen et al., 1979), serum Chlamydia IgG antibody testing (CAT) has been introduced as a screening test for tubal pathology in the fertility work-up. Following C. trachomatis infections, which mainly affect adolescents, a decade or more may pass until women present with sub fertility. Serum IgG antibodies are known to remain detectable for many years (Gijsen et al., 2002), even after antibiotic treatment (Puolakkainen et al., 1986; Chaim et al., 1992; Flura et al., 1993; Henry-Suchet et al., 1994). Therefore, CAT is considered a useful tool in sub fertile women to reflect a previous C. trachomatis infection which has mostly occurred more than a decade ago. The costs of CAT are low (in the Netherlands, about ten euros) (Fiddelers et al., 2005) and the patients’ discomfort is negligible.

The negative predictive value (NPV) of CAT in sub fertile women is 85–90% (Mouton et al., 2002; Veenemans and Van der Linden, 2002; Akande et al., 2003; Land et al., 2003; Logan et al., 2003), although NPVs around 75% have been reported (Eggert-Kruse et al., 1997; Tiitinen et al., in press). Because of the high NPV, the presence of tubal pathology in patients with a negative CAT is unlikely.

The positive predictive value (PPV) of CAT in sub fertile women is lower than the NPV and ranges from 30 to 65% (Eggert-Kruse et al., 1997; Mouton et al., 2002; Veenemans and Van der Linden, 2002; Akande et al., 2003; Land et al., 2003; Logan et al., 2003). The results reported on the diagnostic accuracy of CAT are heterogenous because of differences in CAT tests, threshold levels for a positive test, reference standard and definition of tubal pathology used (Land et al., 1998; Land et al., 2003) (Table III). However, the main limitation of CAT is the number of false-positive results, i.e. positive CAT in the absence of tubal pathology, as reflected by the low PPV. A major concern of this high false-positive rate is that laparoscopies will be performed in women without tubal pathology. Unintended cross-reactivity with highly prevalent C. pneumoniae IgG antibodies has been suggested to account for false-positive test results in some CAT tests (Gijsen et al., 2001; Land et al., 2003). Probably an even more important cause of false-positive CAT results is that a positive CAT is a marker of a previous C. trachomatis infection but does not reflect the course of the infection and neither the extent of tubal damage. Therefore, CAT is not useful in discriminating between clearance and persistence of a C. trachomatis infection, whereas persistence is an important risk factor for tubal pathology. To screen for persistent C. trachomatis infections, the value of serological markers of persistence in identifying sub fertile women at highest risk of tubal disease has been evaluated over the last few years. Part of the findings is summarized in Table IV.

Serological markers of persistence

High-sensitivity C-reactive protein

The acute phase protein C-reactive protein (CRP) is a general serological marker of inflammation. CRP levels are >10 mg/l in acute infections and <1 mg/l in the absence of an infection. CRP levels between 1 and 10 mg/l (so-called elevated levels within the normal range) are assumed to reflect a low-grade inflammation (Pearson et al., 2003) and can be detected using a high-sensitivity (hs) CRP test. The role of elevated hs-CRP levels as markers of an ongoing low-grade inflammation has been evaluated in studies on the relationship between persistent C. pneumoniae infections and cardiovascular diseases. These studies have shown that the known
association between *C. pneumoniae* and cardiovascular diseases is even stronger in the presence of slightly elevated hs-CRP levels (Roivainen et al., 2000; Gattone et al., 2001; Johnston et al., 2001).

The value of hs-CRP, in addition to CAT, in predicting the risk of tubal factor subfertility has recently been studied (den Hartog et al., 2005) (Table IV). This study showed that combining CAT, which has a PPV of 62%, an NPV of 90% and an odds ratio (OR) of 13.9, with hs-CRP significantly increases the predictive value for tubal pathology (PPV 86%, NPV 86%, and OR 39.7). CAT/hs-CRP seems a clinically important set of serological screening tests, which increases the low PPV of CAT without lowering the

NPV (den Hartog et al., 2005). These results must be confirmed in larger studies.

**IgG to Chlamydia hsp 60**

*Chlamydia* hsp 60 (chsp60) is a *Chlamydia* genus-specific protein, serving as a strong antigenic target for the immune system (Morrison et al., 1989; Kaufmann, 1990). Antibodies to chsp60 have been suggested as markers of chronic inflammation (Kaufmann, 1990) and may therefore be good predictors for the risk of tubal pathology. Studies have shown a strong association between anti-chsp60 antibodies and tubal factor subfertility. Anti-chsp60 antibodies are significantly more prevalent in subfertile women with tubal...
C. trachomatis-associated tubal factor subfertility

Summary

C. trachomatis genital tract infections in women usually remain asymptomatic. Therefore, a normal immune response, rather than antibiotic treatment, is essential for clearing the pathogen. In most women with C. trachomatis infections, the immune response will be adequate and the pathogen will be cleared effectively. However, in some women, C. trachomatis infections will persist and ascend to the upper genital tract, increasing the risk of late sequelae such as tubal factor subfertility. PRRs of the TLR and NOD families play a substantial role in recognizing C. trachomatis and initiating the immune response. Virulence factors of the pathogen and environmental factors are suggested to be determinants of the risk of tubal pathology, but the role of host immune factors is considered of more importance. Genetic variations in TLR and NOD genes are presumed to affect receptor function, leading to an inadequate recognition of C. trachomatis, an inadequate immune response and subsequently an increased risk of persistence and late sequelae. However, the precise role of the PRR genetic variations in C. trachomatis female genital tract infections and tubal pathology remains to be elucidated.

To assess the risk of tubal pathology in subfertile women in an inexpensive and non-invasive way, CAT (measuring C. trachomatis IgG antibodies in serum) has been introduced in the fertility work-up and is nowadays commonly used in the Netherlands. The predictive value of CAT for tubal pathology is limited, because the presence of C. trachomatis IgG antibodies reflects a previous infection, but not a persistent infection. Therefore, CAT is not suitable to identify subfertile women with persistent C. trachomatis infections, who have the highest risk of tubal pathology. Serological markers of persistent C. trachomatis infections, such as hs-CRP, may help identify these women. The first study on the value of CAT/hs-CRP in predicting tubal factor subfertility has shown that the PPV of CAT (62%) increases to 86% if both CAT and hs-CRP are positive. Although several studies have reported encouraging findings, further evaluation is needed to decide whether markers of persistence deserve a place in the fertility work-up.

Future perspectives

Further immunogenetic studies may provide more insight into the immunopathogenesis of C. trachomatis female genital tract infections in general and into the role of PRRs and their genetic variations in particular. Owing to the expected low prevalence of subfertile women who have contracted a C. trachomatis infection and have had evaluation of tubal function and carry a single or multiple genetic variations, immunogenetic analyses are not expected to become clinically relevant as screening methods for tubal factor subfertility.

It is more likely that serum markers of persistence of the micro-organism will be added to CAT in the fertility work-up, because a non-invasive method to obtain samples of the upper genital tract for the detection of persistent C. trachomatis using nucleic acid amplification techniques does not exist. The combination of CAT and hs-CRP (reflecting a previous C. trachomatis infection and persistence of the micro-organism, respectively) appears to be a promising valuable set of serological tests to identify women at highest risk of tubal pathology. The role of chsp60 IgG as an additional marker of chronic inflammation is the promising topic of several studies. From a theoretical point of view, the ultimate goal of the fertility investigation would be to have a screening method available with 100% accuracy in ruling in and ruling out C. trachomatis-associated tubal pathology. This ultimate screening method is not available.

So far, many studies have measured serum antibodies, which are products of the humoral immune response, to estimate the risk of a previous C. trachomatis infection. However, clearance of intracellular pathogens such as C. trachomatis is known to depend also on the Th1 response of the cell-mediated arm (Bailey et al., 1995; Holland et al., 1996; Hawkins et al., 2002). A recent study has evaluated the role of measuring the cell-mediated immune response in predicting the risk of tubal factor subfertility and has shown that an in vitro lymphocyte response to C. trachomatis was
significantly more often detected in women with tubal factor subfertility as compared with subfertile controls (Tiitinen et al. , 2006). In predicting tubal pathology, adding markers of the cell-mediated immune system to antibody testing improved the value of measuring markers of the humoral response alone (Tiitinen et al. , 2006). If further studies can confirm these findings and test methods to evaluate the cell-mediated immune response become commercially available, measuring the cell-mediated immune response may be implemented in the fertility work-up as a screening method for tubal factor subfertility.

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