Identification of Human T Cell Targets Recognized during *Chlamydia trachomatis* Genital Infection

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The specificity of the human T cell response to *Chlamydia trachomatis* was investigated by stimulating lymphocytes from 16 case patients with urogenital infection by use of a size-fractionated serovar D lysate. Considerable heterogeneity was found among case patients, and multiple protein fractions were recognized in each specimen. Mass spectrometry analysis of the 30–42-kDa T cell–stimulating region identified 10 *C. trachomatis* proteins. Of these, CT583, CT603, and CT610 were identified as strong antigens that induced significantly higher levels of IFN-γ secretion in PBMCs from case patients, compared with PBMCs from control donors. All 3 proteins were recognized in specimens from case patients infected with serovars D–F, the most prevalent serovars. McDonald-Kreitman and Tajima’s D tests involving clinical isolates from the same samples showed evidence for frequency-dependent selection on *ct583*. We predict that CT583 is a target of acquired protective immune responses in humans.

Genital infection with *Chlamydia trachomatis* is the most common sexually transmitted bacterial infection worldwide, with >90 million cases annually [1]. The infection can result in chronic changes in the genital tract, causing infertility, abdominal pain, and ectopic pregnancy [2, 3]. *C. trachomatis* is also the causative agent of trachoma, a chronic infection of the conjunctiva that is characterized by extensive scarring and blindness. There are at least 15 serovars of *C. trachomatis*; serovars A–C mainly produce trachoma, whereas serovars D–K are the agents of sexually transmitted genital infection [4]. Although the genetic variability among trachoma-associated serovars is not well described, several studies have demonstrated a high degree of sequence variability in sexually transmitted *C. trachomatis* serovars [5–8]. A major factor in the generation of genetic variability among bacterial pathogens is likely the evasion of host immune responses. Rare bacterial variants encoding antigenic types that are infrequently recognized by host immune responses will initially have a selective advantage, which is often frequency dependent [9]. The identification of genes encoding bacterial antigens that are subject to such selection may therefore help find antigens that are targets of protective immune responses [10].

Although it is generally accepted that T cells of the murine immune system play a major role in the control of bacterial multiplication [11–13], the cells involved in the protective immune response in humans remain unclear [14, 15]. An immunoepidemiological study with a cohort of female sex workers from Nairobi showed that individuals infected with HIV were more susceptible to subsequent genital infection with *C. trachomatis* than were individuals not infected with HIV, suggesting that CD4+ T cells play a role in protection against *C. trachomatis* infection [16]. This hypothesis is supported by recent data from another study involving female sex workers in Kenya, which showed that a high level of IFN-γ secretion by PBMCs stimulated with *C. trachomatis* heat shock protein 60 (HSP60) was the strongest predictor of protection against reinfection [17]. Despite the putative importance of the T cell response for protection against...
C. trachomatis genital infection, relatively few antigens recognized by human T cells have been characterized so far. These antigens include the major outer membrane protein (MOMP) [18, 19], HSP60 [20, 21], the histone-like protein Hc1 [20], OMP2 [22, 23], and CT521 [24]. MOMP elicits specific T cell responses against epitopes in both variable and conserved regions during C. trachomatis genital infection [25, 26], and it has been proposed that host immune responses against MOMP may select for mutants that escape immunological recognition [9, 27, 28].

In this study, 96 paired blood and urine samples were collected from case patients with a diagnosed C. trachomatis genital infection and used to identify antigens frequently recognized by T cells.

SUBJECTS, MATERIALS, AND METHODS

Patients and samples. Ninety-six C. trachomatis−infected case patients and 24 control donors were enrolled in this study; details about the study subjects are described elsewhere [24]. In brief, 96 case patients attending an outpatient sexually transmitted diseases clinic at Bispebjerg Hospital (Copenhagen, Denmark) were included after they provided informed consent. The genotype of the C. trachomatis isolates from 79 case patients was determined by direct sequencing of the full-length omp1 genes in accordance with the methods of Ortiz et al. [26]. Serovar E, identified in 27 (29%) of 94 case patients, was the most prevalent serovar. This was followed by serovar D (in 20 [21%]) and serovar F (in 17 [18%]), whereas serovars G, H, I, J, and K were recovered relatively infrequently (in 2–6 [2%–6%]). Amplification by the omp1 PCR protocol failed in 12% of the samples. Samples from 57 of 96 case patients that responded positively to a whole C. trachomatis serovar D lysate were selected for further investigation. PBMCs from 16 of these 57 case patients (8 women and 8 men) were stimulated with protein fractions to identify frequently recognized fractions. To study the recognition of the recombinant proteins, 2 groups of cases patients, one with 10 patients (5 women and 5 men) and one with 40 patients (20 women and 20 men), were randomly selected. Twenty-four blood donors with no history of testing positive for C. trachomatis−infected were selected as control donors. The study was approved by the Local Ethical Committee for Copenhagen (01-008/03).

Fractionation of C. trachomatis serovar D lysate. The C. trachomatis serovar D strain (UW-3/Cx) was propagated in HeLa 229 cells (ATCC) and fractionated into 30 narrow–molecular weight fractions, using a multielution technique described elsewhere [24].

Protein identification by mass spectrometry. Protein fractions from the Multi-Eluter were transferred to VivaSpin 500 spin columns with a cutoff of 3 kDa (VivaScience AG) and centrifuged to a volume of 25 μL. The samples were washed and centrifuged twice with 200 μL of 50 mmol/L NH4HCO3 (pH 7.8) and finally diluted to 50 μL with the same buffer and 0.05 μg trypsin (sequencing grade, modified; Promega). After digestion overnight at 37°C, 5-μL samples were purified on Eppendorf GELoader tips packed with Poros R2/50 reversed-phase column material (Applied Biosystems) [29] and eluted directly onto the matrix-assisted laser desorption/ionization (MALDI) target plate, using 0.8 μL of α-cyano-4-hydroxycinnamic acid (10 mg/mL) in 70% acetonitrile and 0.1% trifluoroacetic acid. Mass spectra were either acquired on a PerSeptive Voyager Elite MALDI MS instrument (Applied Biosystems) in the reflector mode by means of delayed extraction or on a 4700 MALDI MS Proteomics Analyzer (Applied Biosystems) in the MS/MS mode. Proteins were identified using the Mascot search engine (Matrix Science).

Expression and purification of recombinant C. trachomatis genes. The full-length genes for the 10 C. trachomatis proteins (CT067, CT538, CT561, CT582, CT583, CT603, CT610, CT678, CT679, and CT681) were amplified from C. trachomatis serovar D [30] and expressed in the pDESTTM17 expression vector (Invitrogen). Plasmids containing ct538 and ct678 did not produce a recombinant protein. Recombinant proteins were initially purified by metal chelate affinity chromatography, essentially as described elsewhere [31, 32], followed by electroelution from SDS-PAGE gel, precipitation with 80%–95% acetone (HPLC grade, Aldrich) to remove SDS, and washing with 95% ethanol. Recombinant proteins were then resuspended in 50 mmol/L TRIS (pH 7.5), 150 mmol/L NaCl, and 40% glycerol and stored at −20°C.

T cell assay and cytokine analysis. PBMCs were separated and cultivated as described previously [24]. Antigens were added in the following concentrations: C. trachomatis serovar D fractions, 2 μg/mL; and all recombinant proteins, 5 μg/mL. Phytohemagglutinin (2 μg/mL) was used as a positive control, and cell cultures without antigen were included as negative controls. The cells were incubated at 37°C in humidified air (5% CO2 and 95% air). The supernatants were harvested on day 5 for quantification of IFN-γ. The amount of IFN-γ supernatant was determined by standard sandwich ELISA, as described previously [24]. The detection limit of the assay was 20 pg/mL, and the amount of IFN-γ released into unstimulated wells was always below the level of detection.

Amplification of ct583, ct603, ct610, and omp1. DNA fragments containing the genes and flanking regions of ct603, ct610, and ct583, and omp1 were amplified from urine samples and laboratory strains by nested PCR, using the specific primers 5'-ATGCTGCTAATCGCTTATTCT and 5'-TCTAATAGTTTTCAAGCTTAT (for ct603), 5'-ATGTTGCTAAGCTTAT (for ct583), and 5'-AGTATCTGAGAAATG (for omp1), 5'-GCTTCTATCCTTGGACACAAAC (for ct583), and 5'-GGTTGCGCGTTTTCTTTC (for omp1) in a first-round PCR, and chlamyd-
ial DNA was extracted as the template as described elsewhere [28]. The respective products were used as template in second-round PCR, using the primers 5’-ATGAAAACAATCGCTGTTAATGTTCATC and 5’-CACAGATTTCGTTAATTCTTCAAGTGA (for ct583), 5’-ATGAAATTTACTGTTGCAGTGGTTTGG and 5’-GATGTTGAGAGAGGAAAATTCTGAAGAA (for ct603), 5’-CGCACAGAGAATTCTCGACATACTATC and 5’-ATGAAAATAACTCCGATCAAAACACG (for ct610), and 5’-CTAGGACGCAGTGCCGCC and RVS1163 [28] (for omp1).

A 20-cycle amplification process, consisting of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, was performed using Phusion polymerase (Finnzymes). Nucleotide sequencing was performed directly on the PCR products by MWG-Biotech AG, using specific sequencing primers.

Alignment and analysis. Nucleotide sequences were aligned by the Clustal V program [33], using the orthologous genes from the MoPn strain as an outgroup. The McDonald-Kreitman (MK) test [34] was performed using the DnaSP program [35] to determine whether the neutral model of molecular evolution could explain the observed data. Tajima’s D test was used to predict whether selection was occurring, on the basis of the difference between the number of segregating sites and the average number of nucleotide substitutions [36]. Positive values of Tajima’s D that are significantly different from 0 support the hypothesis that frequency-dependent selection is maintaining polymorphisms in the population, and negative values indicate directional selection. This test was performed using the program DnaSP and a sliding window method (window size, 100 nucleotides; step, 25 nucleotides). The 95% CIs for D, assuming a β distribution, were −1807 to 2026 for ct603, and −1806 to 2026 for ct610. All values of D that fell outside these intervals were considered to be significant, resulting in the rejection of the hypothesis that mutations were neutral [36].

Statistical methods. The Mann-Whitney rank sum test was used to analyze differences in immune responses between groups. Comparisons of the prevalence of positive T cell responses between groups were performed using Fisher’s exact test. A P value of <.05 was considered statistically significant.

RESULTS

T cell reactivity against native C. trachomatis antigens. To characterize the T cell stimulatory activity, a C. trachomatis lysate was separated by SDS-PAGE into 30 narrow fractions [24], and the individual protein fractions were examined for their ability to induce IFN-γ secretion in PBMCs from 16 case patients (8 women and 8 men) with representative C. trachomatis infection (figure 1). The T cell responses against the individual fractions were expressed as the prevalence of positive responses, defined as an IFN-γ level of >168 pg/mL (the cutoff level was based on the mean value for control donors [±2 SDs]). On the basis of these criteria, the most frequently recognized fractions (i.e., those recognized in >60% of the patients) were in the 5–12-kDa, 14–20-kDa, 30–42-kDa, and 55–70-kDa molecular-weight regions (figure 1). To identify individual proteins that could be responsible for the T cell stimulatory activity, fractions in the 30–42-kDa region were digested with trypsin and subjected to peptide mass mapping by MALDI-MS. The resulting peptide mass maps were matched against those in the NCBI nonredundant database, and 10 proteins (CT067, CT538,
observed for 20 control donors (figure 2B). All 3 proteins induced significantly higher levels of IFN-γ in case patients, compared with control donors (P < .05). To investigate whether the observed T cell reactivity is serovar dependent, the genotype of *C. trachomatis* isolates from the same 50 case patients described above was determined by direct sequencing of full-length omp1 genes. Table 1 summarizes the prevalence of positive T cell responses, according to the genotype of the infecting *C. trachomatis* isolate, in 36 samples for which the genotype could be determined. All 3 proteins were recognized in case patients infected with the 3 most frequently observed serovars (i.e., serovars D, E, and F). Although CT603 and CT610 were equally well recognized in case patients with serovar G, H, J, or K infection, CT583 appeared to be less frequently recognized in this group (P = .04, by Fisher’s exact test).

**Sequence diversity in T cell antigens.** To investigate the selective pressures operating on the T cell antigens identified here, we amplified the *ct583, ct603, and ct610* genes in isolates from urine specimens of the same case patients described above, and the nucleotide sequences were determined by direct sequencing of the PCR products. The nucleotide diversity (π) was low for *ct583* (π = 0.0025), *ct603* (π = 0.0045), and *ct610* (π = 0.0022), compared with that for the *omp1* gene (π = 0.13996), suggesting that the genes for these T cell antigens are relatively conserved in *C. trachomatis*. Each set of *C. trachomatis* sequences was then aligned with the orthologous gene from *Chlamydia muridarum*, and the data were subjected to the MK test [34]. The MK test determines whether the ratio of replacement to synonymous polymorphisms within species is equal to the ratio of replacement to synonymous substitutions between species, as predicted by the neutral theory of molecular evolution [34]. Because the MK test is generally considered insensitive to population subdivisions [37], gene sequences from all serovars were pooled to detect signatures of selection. Findings of the MK test were significant for *ct583* (P = .0016), pointing to an excess of replacement polymorphisms (table 2). To further investigate these findings, we performed a sliding-window analysis of Tajima’s D values across the *ct583, ct603, and ct610* genes.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Serovars D–F (n = 28)</th>
<th>Serovars G–K (n = 8)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT583</td>
<td>16</td>
<td>1</td>
<td>.04</td>
</tr>
<tr>
<td>CT603</td>
<td>11</td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td>CT610</td>
<td>17</td>
<td>4</td>
<td>.70</td>
</tr>
</tbody>
</table>

**NOTE.** Cutoff levels of antigen-specific IFN-γ (defined as the upper 95% confidence limit of the geometric mean response in control patients) for CT583, CT603, CT610 were 265 pg/mL, 400 pg/mL, and 1340 pg/mL, respectively. 

* By Fisher’s exact test.
Table 2. Findings of the McDonald-Kreitman test to determine the type of selection acting on *Chlamydia trachomatis* genes *ct583*, *ct603*, and *ct610*.

<table>
<thead>
<tr>
<th>Gene (no. of specimens), substitution type</th>
<th>Fixed differences, no.</th>
<th>Polymorphic changes, no.</th>
<th>(P^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ct583</em> (n = 31)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synonymous</td>
<td>101</td>
<td>1</td>
<td>.016</td>
</tr>
<tr>
<td>Nonsynonymous</td>
<td>33</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>ct603</em> (n = 25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synonymous</td>
<td>83</td>
<td>5</td>
<td>1.000</td>
</tr>
<tr>
<td>Nonsynonymous</td>
<td>24</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>ct610</em> (n = 34)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synonymous</td>
<td>91</td>
<td>1</td>
<td>.135</td>
</tr>
<tr>
<td>Nonsynonymous</td>
<td>26</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Orthologous genes from the *Chlamydia muridarum* MoPn strain were used as the outgroup.

* By Fisher’s exact test, to test the null hypothesis of no effect of selection.

.ct610* sequences. Consistent with findings of the MK test, this analysis identified a region in *ct583* (nucleotides 276–375) that exhibited a significant positive departure from 0 (\(D = 2.12\); \(P < .05\)), indicating that frequency-dependent selection was operating on this locus (data not shown).

**DISCUSSION**

By analyzing a large number of paired blood and urine samples from a group of patients with *C. trachomatis* genital infection, the present study aimed to identify antigens, particularly those with signatures of natural selection, frequently recognized by T cells. Stimulation of PBMCs from 16 case patients infected with *Chlamydia* organisms, representing several serovars, with a serovar D fraction panel led to the identification of 4 T cell–stimulating regions (mass, 5–12, 14–20, 30–42, and 55–70 kDa). Elsewhere, we focused on the 16–20 kDa region and identified CT521 as an antigen frequently recognized by T cells [24]. Here, we focused on the 30–42 kDa region and identified 3 T cell–stimulatory antigens, CT583, CT603, and CT610 (figure 2A and 2B). A human CD8 epitope has previously been identified in CT610 [38], whereas CT583 and CT603 are novel T cell–stimulatory antigens. All 3 antigens are frequently recognized by PBMCs from patients with *C. trachomatis* genital infection, suggesting that they are strong targets of natural T cell responses to *Chlamydia* organisms.

It is well-known that not all antigens recognized by T cells are protective. Given the assumption that the immune system exerts a strong selection pressure on protective antigens, it has been proposed that molecular population genetic analyses that identify antigens that are subject to frequency-dependent selection can help to predict targets of protective immunity [10]. Accordingly, molecular population genetic and immunological analyses have been used to identify principal targets of human immunity to malaria [39, 40]. The selective pressure on *ct583*, *ct603*, and *ct610* in clinical isolates was therefore examined by the MK test, which determines whether the pattern of nucleotide substitutions at synonymous and nonsynonymous sites deviates from that predicted by the neutral model of molecular evolution [34]. The MK test revealed an excess number of replacement polymorphisms in *ct583*. This result was supported by findings of Tajima’s D test, which showed a positive departure from neutrality in the 5’-region (nucleotides 276–375). Frequency-dependent selection may be a plausible explanation for this phenomenon. Mutants that arise in the population and have the potential to avoid detection by the immune system (referred to as escape variants) would have a survival advantage, leading to their increased frequency in the population. In agreement with this hypothesis, we observed that the prevalence of positive responses to recombinant CT583 (serovar D) was significantly higher among patients with serovar D, E, or F genital infection, compared with patients with serovar G, H, J, or K infection (\(P = .04\)) (table 1). This observation may suggest that CT583-specific T cell responses are serovar restricted; however, it should be mentioned that the differences in T cell responses observed between the different groups of donors may also be associated with differences in the history of exposure to *C. trachomatis* or with polymorphisms in genes encoding HLA molecules. The present findings that CT583 is subject to diversifying selection and that it displays a certain serovar-restricted T cell response may therefore suggest that this antigen plays a role in host-pathogen interactions. It has previously been speculated that the high prevalence of serovars D, E, and F worldwide may be associated with epitopes that enhance infectivity and transmission [41], and it is possible that CT583 plays a role in this respect. CT583 was annotated in the TIGR *Chlamydia* genome database as a hypothetical protein of unknown function, and native CT583 has not yet been demonstrated in any of the proteome studies conducted so far [42, 43]. However, microarray data have shown that the gene is transcribed ~16 h after attachment to the epithelial cells [44], supporting the present finding that CT583 becomes exposed to the host immune system during the natural course of *C. trachomatis* infection.

In contrast, we did not detect evidence of diversifying selection for the T cell–recognized antigens CT603 and CT610. Both antigens may be involved in the control of apoptosis induced by *Chlamydia* organisms. A recent study has suggested that CT610 (referred to as “*Chlamydia* protein associating with death domains”) may induce apoptosis through the caspase-dependent pathway [45], and CT603 (a thiol-specific antioxidant peroxidase) may be involved in antiapoptotic activity, as it may form part of the thioredoxin system, which plays an important role in protecting against oxygen stress by reducing peroxides to harmless products [46]. The notion that CT603 and CT610 play a role in the survival of *Chlamydia* organisms during the intracellular
phase of infection is further supported by the observation that their production up-regulated 2–3-fold when C. trachomatis–infected cells are treated with IFN-γ [47].

In conclusion, the present study has identified 3 antigens that are targets of cell-mediated immune responses during C. trachomatis genital infection. One of these antigens, CT583, may be subject to diversifying selection, which, we hypothesize, is produced by effective responses mounted by the host immune system against epitopes within the central part of the protein. Future studies in the mouse model will determine the protective value of these antigens.

Acknowledgments
We thank Vita Skov, Lisbeth Schack Abrahamsen, Annette Hansen, and Inger Christiansen for excellent technical assistance. Jes Dietrich is acknowledged for critical reading of the manuscript.

References

Human C. trachomatis T Cell Antigens • JID 2007:196 (15 November) • 1551