Chlamydia trachomatis from Individuals in a Sexually Transmitted Disease Core Group Exhibit Frequent Sequence Variation in the Major Outer Membrane Protein (omp1) Gene

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Abstract

60 cervical Chlamydia trachomatis infections identified by antigen detection from 51 prostitute women in Nairobi, Kenya were evaluated for sequence polymorphism in the major outer membrane protein (omp1) gene. DNA from clinical specimens was amplified by the polymerase chain reaction and cycle sequenced through variable domains (VD) 1, 2, and 4. 37 (63%) samples had variant VD sequences, 19 (32%) samples had prototype VD sequences, and 4 (6%) samples contained omp1 sequences from two or more C. trachomatis strains. Among the 37 variant strains, 18 had two or fewer nucleotide substitutions in one or two VDs and represented point mutational drift variants. 19 strains had a larger number of nucleotide changes and displayed mosaic omp1 sequences that may have been generated by omp1 VD recombination. We conclude that the prevalence of C. trachomatis omp1 DNA polymorphism is substantial among prostitute women in Nairobi, Kenya and that this is the likely result of immune selection pressure. (J. Clin. Invest. 1994. 94:458-463.) Key words: Chlamydia trachomatis • omp1 • antigenic variation

Introduction

The major outer membrane protein $(MOMP)^1$ gene (omp1) of *Chlamydia trachomatis* is a single copy gene with multiple alleles. Each omp1 allele specifies a specific serologic variant or serovar of the organism (1). Sequence analysis shows that omp1 has four variable regions or domains (VD1 to VD4) interspersed with five constant regions (1, 2). Antibody responses to the *C. trachomatis* MOMP are primarily directed to the VDs (3). Furthermore, monoclonal antibodies to an epitope

J. Clin. Invest.

sequence within a VD can neutralize the organism in cell culture and can distinguish between VD sequences which vary by only one or two amino acids (4, 5).

Limited epidemiologic data and phylogenetic analysis suggest that omp1 polymorphism is relatively stable and the tempo for genetic change is slow (4, 6, 7). However, MOMP-specific monoclonal antibody typing of C. trachomatis isolated from cases of trachoma and sexually transmitted chlamydial infections has identified a surprisingly large number of examples of atypical immunoreactivity (8-11). Detailed sequencing studies of the omp1 gene by Dean et al. (8) identified that atypical patterns of monoclonal antibody reactivity patterns mapped entirely or predominantly to amino acid sequence changes in the MOMP VDs. Lampe et al. (11) molecularly studied three variants of serovar D, two of serovar I, and one of serovar L₂ and suggested that sequence variation arises by point mutation or, possibly, by recombination of MOMP VDs. Recently, we reported that 38% of 49 clinical samples of C. trachomatis identified in Winnipeg from individuals with sexually acquired chlamydial infections also had sequence polymorphism in the omp1 gene (12). In aggregate, these observations suggest that the omp1 gene may exhibit a far greater degree of polymorphism than is currently appreciated.

Polymorphism in antigen-specifying genes for sexually transmitted pathogens may be most apparent among isolates originating from a sexually transmitted disease (STD) core group. This hypothesis derives from the assumption that immune responses that occur among STD core group members who are repeatedly exposed to an STD pathogen will produce a significant barrier to persistence of the pathogen in the core group (13). Thus, strain diversification of a major antigen specifying gene (omp1 in the case of *C. trachomatis*) will be necessary to allow ecologic success for the pathogen in the face of strain specific immunity in the host population (14).

To test this hypothesis, 60 C. trachomatis infections identified by antigen detection among women who work as prostitutes in the Pumwani district of Nairobi, Kenya were evaluated for sequence variation in the omp1 VDs. The results show that C. trachomatis omp1 sequence variation is extraordinarily prevalent among core group members, presumably in response to immune selection.

Methods

Study population

In April 1991, a cohort of prostitute women working in the Pumwani district of Nairobi, Kenya was established to study the immunoepidemi-

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^{1.} Abbreviations used in this paper: MOMP, major outer membrane protein, omp1, outer membrane protein gene, VD, variable domain.

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Table I. Summary of Sequence Analysis of VD1, VD2, and VD4 of Chlamydia trachomatis omp 1 for 60 Strains Isolated among Pumwani Prostitute Cohort between April 1991 and April 1992

	Serovar	Number	Percentage in each group
			%
Prototype	Е	15	32
	G	4	
Minor variants	С	3	
	К	7	20
	L ₁	1	30
	L_2	7	
Major variants	I-like/H-like	4	
-	L ₁ -like/L ₂ -like	11	22
	L ₂ -like/L ₁ -like	2	32
	L ₃ -like/H-like	2	
Mixed		4	6

ology of sexually transmitted *C. trachomatis* infection. At enrollment demographic, sexual, reproductive, and medical histories were obtained in a standard format interview and a complete physical examination including vaginal speculum and bimanual examination was performed. Sera for HIV serology, CD4 T cell enumeration, and endocervical swabs for *Neisseria gonorrhoeae* culture and *C. trachomatis* antigen detection were obtained at enrollment. Women were scheduled for routine follow-up at two weekly intervals. At each visit, a limited interview regarding condom use, number of sexual partners and STD symptoms was conducted and a general and genital examination including vaginal speculum and bimanual was performed. At each visit, endocervical swabs for gonococcal and chlamydial infection were obtained. Chlamydia infected women were treated with doxycycline 100 mg orally twice daily for 7 d and a test-of-cure performed 4 d after completion of treatment.

Laboratory tests

Chlamydia antigen detection. Cervical swabs were collected and evaluated for chlamydial antigen using a commercially available enzyme immunoassay (Clearview; Unipath, Nepean, Ontario, Canada). Specimens were processed according to the manufacturer's instructions.

C. trachomatis omp1 gene characterization. Samples found to contain chlamydial antigen were stored at -20° C and shipped to Winnipeg where DNA was extracted with 0.1 M Tris-HCl, pH 8.0, 0.10 M NaCl, 0.005 M EDTA, 1% sodium dodecyl sulfate, and 50 μ g/ml proteinase K as previously described (12). DNA was further extracted with an equal volume of phenol and chloroform and precipitated with sodium acetate and isopropanol to remove nonspecific inhibitors of the polymerase chain reaction (PCR).

Omp1 gene amplification and variable domain DNA sequencing. An 879-bp product of the omp1 gene was amplified by PCR using previously described flanking and nested primers (12). Amplification with PCR involved the use of 2 U of Thermus aquaticus (Taq) polymerase and annealing temperatures of 55 or 60°C and an extending temperature of 72°C for 2 to 3 min. The DNA sequence of VD1, VD2, and VD4 was determined by the double stranded DNA dideoxynucleotide chain termination method using 5' end-labeled [³²P]ATP oligonucleotide primers as described (12). Fig. 1 shows the position of the primers with respect to the omp1 gene VD organization and the legend describes the nucleotide sequence of each primer. Sequences for VD1, 2, and 4 among samples identified in the current study were compared with the VD sequences from the 15 prototype *C. trachomatis* serovars as reported by Yuan et al. (15).

Clonal analysis of omp1. Omp1 was re-amplified from the secondary PCR reaction using 2 μ l of the PCR product as template and re-designed primers. Oligonucleotide primers were re-synthesized based on primer 3 and primer 4 and incorporating a 5' KpnI restriction site and six additional unrelated bases. The sequence for modified primer 3 is: CCGTTAGGTACC-TGACTTTGTTTTCGACCGTGTTTT; and the sequence for modified primer 4 is: CTGCAAGGTACCTTTTCTAGATTT-CATCTTGTTCAATTG. The KpnI site is underlined. The resulting omp1 PCR product was subjected to phenol:chloroform extraction followed by ethanol precipitation and dissolved in TE buffer. Proteinase K was added to a final concentration of 200 μ g/ml, incubated for 30 min at 37°C and SDS was added to a final concentration of 0.5% and incubated at 68°C for 10 min. The reaction mixture was extracted once with phenol:chloroform and once with chloroform before ethanol precipitation. The DNA was digested with KpnI and subjected to electrophoresis. The correct sized band was excised from the gel, DNA was electroeluted and purified with phenol/chloroform extraction before ligation to dephosphorylated pUC19 restricted with KpnI. A fraction of the ligation reaction was used to transform competent DH5 a E. coli. White colonies were picked and grown up. The plasmid was purified and the insert fragment was amplified using M13 reverse sequencing primer (AGCGGATAA-CAATTTCACACAGGA) and M13 universal sequencing primer (CGCCAGGGTTTTCCCCAGTCACGAC). The PCR product was visualized on an ethidium bromide stained 1% agarose gel. The cycle sequencing for omp1 VD1, VD2, and VD4 (using the purified plasmid that contained the correct sized insert) was done under the same condition as described above.

C. trachomatis Omp1 primers

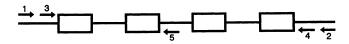


Figure 1. The relationship of the five primers used in the present study to the three variable domains (VDs), VD1, VD2, and VD4 which were sequenced and in relationship to the overall sequence organization of the Chlamydia trachomatis omp1 gene is shown in the figure. The nucleotide sequence of the primers and their number position in the omp1 DNA sequence is as follows: Primer 1 (sense) GCC GCT TTG AGT TCT GCT TCC TC (34 → 56); Primer 2 (antisense) ATT TAC GTG AGC AGC TCT CTC AT (1176 \rightarrow 1154); Primer 3 (sense) T GAC TTT GTT TTC GAC CGT GTT TT (198 → 221); Primer 4 (antisense) TTT TCT AGA TTT CAT CTT GTT CAA T/CTG (1077 \rightarrow 1051); Primer 5 (antisense) ACA TTC CCA C/GAA/G AGC TGC (621 \rightarrow 604). Primers 1 and 2 were used to initially amplify, and primers 3 and 4 were used for reamplification. Primer 3 was used to sequence VD1, primer 5 VD2, and primer 4 VD4. These sequence data are available from EMBL/GenBank/DDBJ under accession number M14738.

Prototype	1	64 600		CCT	ACA	GCT	ACT	ACA			VD	I GCA		CCA	TCC						83 GAG											
		Å	K	P	Ť	Ä	Ť	T	G	m	A	A	A	P	S	T	TGT	T	GLA	AGA 2	E											
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		139									v	110										160										
Prototype	L1	GAT	AAT	GAA	AAT	CAA	AGC	ACG	GTC	***		GAT	GCT	GTA	CCA	AAT	ATG	AGC	TTT	GAT	CAA											
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Prototype	ĸ	ACA	***	AÇA	CAA	TAT	TCT	AAG	ΠI	AAT	ACA	GCG	AAT	CTT	GTT	CCT	AAC	ACT	GCT	TTG	GAT	CGA	GCT									
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Figure 2. Nucleotide and amino acid sequence comparison of prototype and seven clinical isolates that varied at one or two sites in one or two of three sequenced variable domains (VDs).

Results

Study population. 259 women have been enrolled and have completed a mean length of follow-up of 13.6 ± 7.9 mo with 10.6 ± 7.8 visits. 164 (63%) women were HIV seropositive at enrollment with a mean CD4 T cell count of 437 ± 273 . 231 positive chlamydial isolates were identified from 2575 specimens. 113 women had 231 chlamydial infections with 56 women having a single positive chlamydia test and 57 women having two or more positive tests.

VD sequence analysis of the C. trachomatis omp1 gene. The first 60 C. trachomatis infections occurred among 51 women and these samples were used in the current study. DNA was extracted from antigen-positive specimens and was characterized by DNA sequencing of omp1 VD1, 2, and 4. Table I summarizes the data. 11 distinct genotypes were identified and 10 of the 11 genotypes were identified on two or more occasions from two or more different individuals. Four genotypes (serovar E, L₁-like/L₂-like, K, and L₂) accounted for 67% of the isolates. 19 (32%) isolates had prototype VD sequences and 37 (62%) isolates had variant VD sequences. Four (6%) samples contained omp1 DNA sequences from multiple serovars and presumably represent mixed infection with two or more C. trachomatis strains.

Among the 37 isolates with variant sequences, 18 (30%)

had two or fewer nucleotide substitutions in one or two VDs and were classified as minor variants. Fig. 2 shows the DNA and inferred amino acid sequences in the relevant VDs for each type of minor variant.

19 (32%) isolates had substantial sequence change and were classified as major omp1 variants. Each major omp1 variant was isolated on more than one occasion from more than one person. Figs. 3, 4, 5, and 6 illustrate the DNA and inferred amino acid sequence for VD1, 2, and 4 for each of the major variants. These strains appear to represent omp1 mosaics mixing VDs from different serovars.

Among the major and minor omp1 variant genotypes, 21 nucleotide substitutions occurred in 18 codons. 19 nucleotide substitutions were in nonsynonymous codons. 17 nucleotide substitutions were transitional types in which purine transition predominated (7 A \rightarrow G, 6 G \rightarrow A, 2 C \rightarrow T, and 2 T \rightarrow C).

Clonal analysis of omp1. To investigate whether omp1 mosaic sequences resulted from PCR artifact, two types of experiments were performed. Omp1 DNA from the second cycle amplification was reamplified with modified primers 3 and 4 containing KpnI restriction sites. Omp1 from each of the four major variant types was reamplified, ligated in pUC19 and cloned into *E. coli.* Re-sequencing of VD1, 2, and 4 from recombinant pUC19 showed that each major variant was a single molecular species with mosaic VD sequences. This excludes primer prefer-

		64									VD	1									83										
Prototype	L1	GCC	AAG	CCT	ACA	GCT	ACT	ACA	GGC	AAT	GCT	GCA	GCT	CCA	TCC	ACT	TGT	ACA	GCA	AGA	GAG										
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Prototype	L2					۸	G											•••		•••											
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Nairobi	L1											٠ï.									•••										
		•	•	•	•	•	•	•	•	•	•	۷	•	•	•	•	•	•	•	•	•										
		139									v	D11										160									
Prototype		CAT	-			-	460	406	GTC			GAT	GCT		GTA	CCA	AAT	ATG	AGC	TTT	GAT	CAA	TCT								
Prototype		5	~	E	~~~·	~~~~	5	T	v	ĸ	ĸ	D		-	v	P	N	M	S	F	D	Q	S								
Prototype	12			٦,	- ۲		601	` ^	Ťт	τĈ.	с, т	AG.	AAG	CTT																	
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Nairobi	11	•																													
Adirobi	•••											•	•	-						L											
																															317
		288														VD	10										~ ~ ~		~ ~ ~		
Prototype	EL1.	TTA	GCT	ACA	GCT	ATC	: 111	GAT	ACT	ACC	ACG	CTT	AAC	CCA	ACT	ATT	GCT	GGA	GCT	GGC	GAG	GIG	~~~	GCI	AAC	GUA	646	661	UNG		
		L		Т		I	F	D	Т	T	T	L	N	P	- T	1	•	G	•	G	E	v	ĸ	•	N	•	E .	6	e.	Ľ	u
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Figure 3. Nucleotide and amino acid sequence comparison of prototype and a clinical isolate of mosaic L_1 -like/ L_2 -like sequences in variable domains (VDs) 1, 2, and 4.

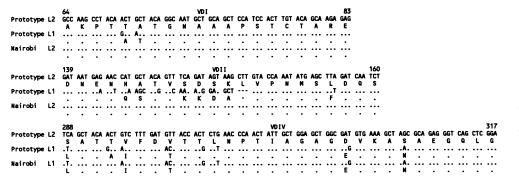


Figure 4. Nucleotide and amino acid sequence comparison of prototype and a clinical isolate of mosaic L_2 -like/ L_1 -like sequences in variable domains (VDs) 1, 2, and 4.

ence in the presence of multiple omp1 alleles as a potential explanation for omp1 mosaic sequences.

Next, experiments were performed in an attempt to generate in vitro mosaic omp1 molecules during PCR amplification. Omp1 DNA from prototype serovar H and I were coamplified and directly sequenced. These DNAs were chosen because H/ I mosaic was observed in the current study. Direct sequencing showed that only a mixed sequencing pattern was detectable. To evaluate at a clonal level the experiment was repeated, this time using the modified primers 3 and 4. After reamplification, omp1 was ligated into pUC19 and cloned into *E. coli*. Sequencing of 10 randomly selected clones showed that six contained serovar H VD1, 2, and 4 sequences, four contained serovar I VD1, 2, and 4 sequences, and none was mosaic. We conclude that serovar H and I mosaics are not readily generated under these PCR conditions.

Discussion

STD pathogens are endemic in human populations through persistence in small groups of individuals who are highly sexually active and connected in common sexual networks (13). These groups are termed core groups and individuals within such groups are high frequency transmitters of STD pathogens. They not only frequently transmit STD pathogens to others, but are also frequently exposed to new infections themselves. Significant immune barriers which arise within the core group because of frequent exposure to the STD agent must be overcome if STD pathogens are to achieve ecologic success in this type of epidemiologic circumstance. In general, STD pathogens seem to have either of two strategies when dealing with host immune responses. Some STD pathogens appear to limit antigenicity by infecting immunologically privileged sites (neurons in the case of herpes simplex type II) or by having few surface proteins

on the organism (rare outer membrane proteins in the case of Treponema pallidum) (16). The second major mechanism observed with other STD pathogens is to vary surface antigens as best defined for opa or pilin proteins in the case of N. gonorrhoeae. The genetic basis for antigenic variation can involve either gene rearrangement as in the case of gonococcal opa and pilin antigens or allelic polymorphism as in the case of single copy genes which vary among the population of the pathogen (9). The omp1 gene of C. trachomatis is the major antigen specifying gene of this organism and its product, MOMP is a target for neutralizing and protective antibodies (4). C. trachomatis antigenic variation is due to allelic polymorphism of the single copy ompl gene (1). Current data have been interpreted to suggest that the tempo for genetic change at the omp1 locus is slow and that omp1 DNA sequences remain largely intact for specific serovars isolated at distant geographic sites and over time scales measuring several decades (4). Under these conditions one might predict that C. trachomatis would have difficulty in persisting in STD core groups and thus in human populations because of the occurrence of herd immunity within the core group to a limited number of serovars of the organism.

However, conclusions regarding the tempo for genetic change in chlamydiae have been based on few isolates and none involved multiple isolates from an STD core group. The present study is based on a relatively large number of *C. trachomatis* infections among individuals in an STD core group and suggests a much more dynamic picture of omp1 polymorphism.

Overall, 11 distinct genotypes were observed. Genetic diversity at a locus (h) can be estimated by the following formula: $h = 1 - \Sigma (x_i)^2 [n/(n-1)]$ where x_i equals the frequency of the i th allele and *n* equals the number of isolates (17). For omp1 genotypes detected in the present study, h = 0.81. Thus, the omp1 locus is indeed highly polymorphic.

Among the 56 single strain infections observed in this study,

		64									VD	ı											83									
Prototype	L3		GAG	CCT	ACT	ACC	AGC	GAT	ACA	GCG	GGC	TTA	TCA	AAC	GAT	CCA	ACA	ACA	AAT	GTT	GCT	CGT	CCA									
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Figure 5. Nucleotide and amino acid sequence comparison of prototype and a clinical isolate of mosaic L₃-like/H-like sequences in variable domains (VDs) 1, 2, and 4.

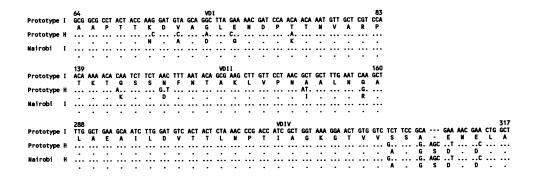


Figure 6. Nucleotide and amino acid sequence comparison of prototype and a clinical isolate of mosaic I/H sequences in variable domains (VDs) 1, 2, and 4.

37 (66%) had variant sequences in VD1, VD2, or VD4 regions of the omp1 gene. When compared with prototype VD sequences from the 15 standard *C. trachomatis* serovars, 19 (51%) of the 37 variants appeared to involve a major antigenic shift, and 18 (49%) appeared to be minor antigenic drift variants.

The genetic mechanisms for diversification at the omp1 locus are unknown. Stephens et al. (1) suggested that accumulated clustered base substitution for closely related serovars and insertion and deletions for distantly related serovars accounts for omp1 diversification. Detailed analysis of the nucleotide changes in the chlamydial omp1 VD sequences observed in this study suggests that mutational drift is not random since 17 of 21 nucleotide changes were transition-type and 19 of 21 resulted in nonsynonymous codons. The former observation may reflect the specificity of a mutator mechanism and the latter suggests the presence of phenotypic selection. With other procaryotes, genetic analysis has shown that mutations affecting proofreading, postreplication repair of copying errors, or in PolI raise the frequency of specific base substitutions and thus, enzymes acting at any of these sites may be involved in the origin of omp1 mutations (18).

Analysis of the 21 nucleotide substitutions observed in the present study revealed that 17 involved transition mutation. Since Taq polymerase can also produce base substitutions that are also predominantly of transitional type, it may be that the origin of some of these mutations is due to Taq polymerase (19–21). However, the following observations make this suggestion unlikely: identical mutations were detected in epidemiologically distinct infections on multiple occasions; repeated cycle sequencing of omp1 variants yielded identical sequences; most (19/21) nucleotide changes were in nonsynonymous codons suggesting the occurrence of phenotypic selection; and most transition base substitutions involved purines (13/17) and not pyrimidines as is favored by Taq polymerase (20).

Lampe et al. (11) suggested recombination may be a genetic mechanism for omp1 diversification. This suggestion was based upon detailed study of *C. trachomatis* strains that had serovar I sequences in VD1, 2, and 3, and serovar H sequence in VD4. Several of the major variants identified in the current study are also compatible with a recombinational mechanism including four isolates identical to the one studied by Lampe et al. (11). Logically, recombination should depend on co-infection of the host with multiple *C. trachomatis* serovars and interestingly, 6% of the identified infections in the present study involved multiple strains of *C. trachomatis*. Before concluding that the omp1 mosaic sequences observed in the present study represent omp1 recombinants, it is important to exclude PCR artifact. In

particular, the occurrence of multiple strain infection with two or more omp1 alleles raises the possibility for at least two artifactual mechanisms for the generation of mosaicism during PCR amplification. Firstly, primer preference with differential amplification of omp1 VDs during cycle sequencing may be able to generate apparent mosaic sequences. This was excluded by cloning omp1 from the four major variants and resequencing VD1, 2, and 4. The results showed that each cloned variant omp1 had identical mosaic sequences as initially identified in the clinical samples. Secondly, a different type of PCR artifact can give rise to mosaic molecules when related alleles are coamplified and incomplete PCR products are generated. Such incomplete products may hybridize to the alternate allelic template and be extended during subsequent PCR cycles, thus yielding mosaic molecules (21, 22). To explore this possibility, prototype serovar I and serovar H omp1 were coamplified, subsequently cloned and 10 clones were sequenced. Six were serovar H, four were serovar I, and none was mosaic. Thus, under the experimental conditions used in the present study, mosaic omp1 was not readily generated in the presence of two closely related omp1 alleles.

The data from this study suggest that point mutation and perhaps recombination are involved in determining the repertoire for omp1 polymorphism. The large number of polymorphic omp1 isolates suggests that the tempo for genetic change in omp1 is much greater than previously thought. We conclude that sequence variability in omp1 may contribute to the ecologic success of *C. trachomatis*. Determination of the genetic mechanism for omp1 diversification will be of considerable interest.

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