

## Continuous B-Cell Epitopes in *Chlamydia trachomatis* Heat Shock Protein 60

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**B-cell peptide epitopes in chlamydial heat shock protein 60 (hsp60) were elucidated with antisera from 13 rabbits immunized with *Chlamydia trachomatis* serovars B, C, and L2 and antisera from eight women with *C. trachomatis*-associated ectopic pregnancies. Thirteen major epitopes were identified with the human sera, 10 of which were also observed with rabbit antisera. Seven of the 13 epitopes recognized by human antisera exhibited cross-reactive antibody binding to homologous peptide sequences in human hsp60. Self-reactive B-cell immunity to hsp60 may contribute to chlamydial disease pathogenesis.**

Immune response to *Chlamydia trachomatis* heat shock protein 60 (hsp60) may determine chlamydial disease pathology (7). Native and recombinant chlamydial hsp60 elicits delayed mononuclear cell inflammation when applied to the conjunctivae of immunologically primed experimental animals (7, 8). Women with fallopian tube obstructions due to *C. trachomatis* infections have antibody responses to chlamydial hsp60 disproportionate to those to other chlamydial antigens. This is shown when such women are compared with other individuals with noncomplicated chlamydial infections (2, 3, 10). Thus, an emerging concept regarding chlamydial disease immunopathogenesis is that a subset of *C. trachomatis*-infected individuals develops B- and T-cell responses to hsp60 and that these responses result in the tissue-damaging sequelae of chlamydial infection. Chlamydial hsp60 is a good candidate antigen for engendering immunopathology, in part because of the great heterogeneity in individual immunoresponsiveness. In mice, antibody responses to chlamydial and other microbial hsp60s are genetically determined and, in part, are linked to *H-2* genes (1, 14). The mechanisms by which immune responses to hsp60 prove deleterious is unknown, but since hsp60s are highly conserved evolutionarily, cross-reactive immune responses between the chlamydial and human homologs may be important (11). We therefore characterized antibody responses to specific hsp60 peptides with sera engendered in humans by natural infections and in rabbits by immunization with whole *C. trachomatis* elementary bodies (EBs).

Antisera were raised against viable *C. trachomatis* serovar B, C, and L2 EBs in six, five, and two female New Zealand White rabbits, respectively, as described previously (13, 15). The clone of recombinant hsp60 fusion protein of *C. trachomatis* used in this study was a generous gift from R. S. Stephens (University of California, San Francisco). *C. trachomatis* hsp60 is expressed as a fusion protein consisting of glutathione *S*-transferase and the complete sequence of chlamydial hsp60 (4). The production and purification of glutathione *S*-transferase fusion proteins have been described previously (4, 14). Serum samples from eight women with ectopic pregnancies who were seropositive for *C. trachomatis* antibody were evaluated. The clinical and histopathologic features of the chlamydial infections in these women have been previously reported (3). They represent a

subset of 19 women in a previous study in each of whom sufficient serum was available to carry out the assays described below.

The amino acid sequence of *C. trachomatis* hsp60 as deduced from the DNA sequence by Cerrone et al. (4) was used to direct the synthesis of 533 overlapping 12-mer peptides covering the entire sequence and overlapping by all but a single residue with a commercially available kit (Cambridge Research Biochemicals, Cambridge, U.K.) (5). Antibodies in sera from immunized rabbits and those in sera from infected women were assayed for binding to peptides by peptide-enzyme-linked immunosorbent assay (ELISA) as described previously (12, 13, 15). The eight human serum samples were also titrated for antibody to chlamydial recombinant hsp60 by an ELISA.

Figure 1 shows the peptide epitope patterns in hsp60 recognized by the 13 rabbits immunized with viable serovar B, C, and L2 EBs, respectively. A pattern of common

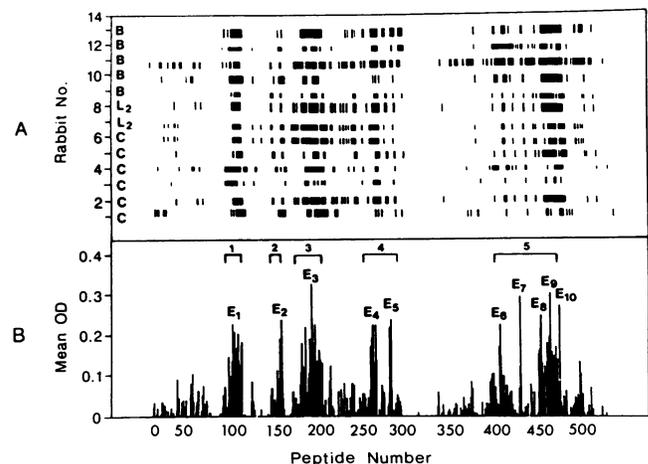


FIG. 1. Antigenicity of 533 overlapping 12-mer peptides encompassing the entire hsp60 sequence of *C. trachomatis*. (A) Antisera were raised in 13 rabbits immunized with EBs of *C. trachomatis* serovars B (four rabbits), L2 (two rabbits), and C (six rabbits) as indicated. Bar codes signify reactive peptides. (B) Composite binding patterns averaged among the individual antiserum samples and expressed as mean OD units are shown. Sera were tested at a dilution of 1:250. The bracketed regions 1 to 5 indicate the five immunogenic regions.

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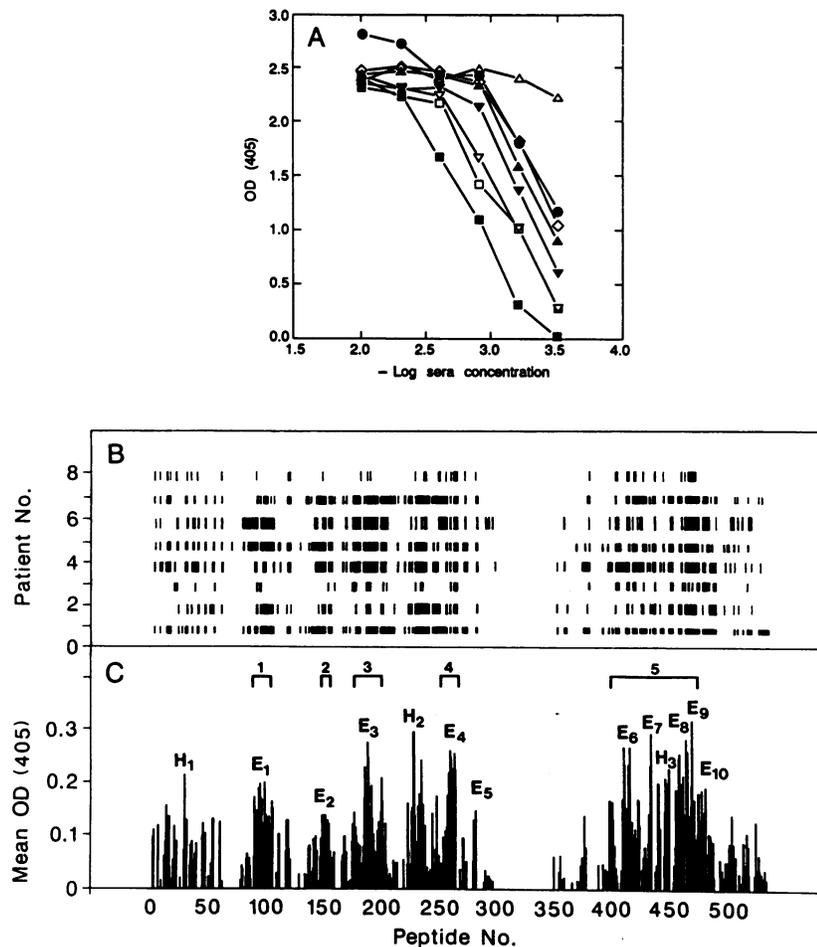


FIG. 2. Serum samples from eight women with *C. trachomatis*-associated ectopic pregnancies were assayed for antibody titers to purified recombinant *C. trachomatis* hsp60 fusion protein in an ELISA (A) and for peptide binding (B and C). Pepsan results for the eight human serum samples (tested at a dilution of 1:400) were displayed in the format described in the legend to Fig. 1. H<sub>1</sub>, H<sub>2</sub>, and H<sub>3</sub> represent three new epitopes identified only with human sera. E<sub>1</sub> through E<sub>10</sub> represent the identical peptide epitopes as identified with rabbit antisera.

epitopes among all rabbits was consistently observed, although individual heterogeneity was also apparent. No marked difference in epitope patterns was apparent among the three different serovars for rabbits immunized with EBs, consistent with the known conservation of hsp60 among all members of the genus *Chlamydia* (9). Although epitopes are distributed throughout the entire sequence, the region from amino acid (aa) 283 to 409 was consistently immunorecessive. When both binding titers and reactive frequencies of all 13 rabbit serum samples were considered, five distinct immunogenic regions (aa 94 to 111, 147 to 163, 188 to 205, 258 to 294, and 410 to 491) were resolved within the linear sequence (Fig. 1B). Region 5, located toward the C terminus, is the most complex and is composed of multiple epitopic peaks. The other four regions have single predominant or, at most, two epitopic peaks. Ten epitopes characterized by high frequencies of response (greater than or equal to seven rabbits) and high titers of binding ( $\geq 0.2$  optical density [OD] units) are located within the five regions. One rabbit served as a control and was immunized with HeLa cell debris. Its serum was tested by the pepsan assay, and no peptide bound antibody with an OD of  $\geq 0.1$  (data not shown).

Serum samples from eight women with *C. trachomatis*-

associated ectopic pregnancies were also evaluated for the epitope specificity of the hsp60 antibodies by the pepsan assay. All women had antibodies with high titers to recombinant chlamydial hsp60, as determined by ELISA (Fig. 2A). Epitope scanning revealed a pattern of immunogenic regions similar to that observed with antisera of rabbits immunized with chlamydial EBs. In addition to the 10 epitopic peaks identified with rabbit antisera, three new epitopes (H<sub>1</sub>, H<sub>2</sub>, and H<sub>3</sub> in Fig. 2B) were detected with the human sera. The major new immunogenic region is aa 226 to 249, labelled epitope H<sub>2</sub>. Two minor epitopes are located at aa 29 to 41, labelled H<sub>1</sub>, and at aa 446 to 461, labelled H<sub>3</sub>. Two human serum samples not reactive with recombinant hsp60 in the ELISA were also tested by the pepsan assay. No peptides were bound with an OD of  $\geq 0.1$  by either serum sample (data not shown).

We compared the chlamydial hsp60 amino acid sequences for the 13 major epitopes recognized by sera from humans with the homologous sequences in the human mitochondrial chaperonin 60 protein (6) (Table 1). Both sets of 13 epitopes for chlamydial and human hsp60 vary in their sequences. We used the homologous region in the human sequences for synthesis of 12-mer peptides of each epitope. Pin-bound peptides were tested by the pepsan assay with the eight

TABLE 1. Comparison of amino acid core sequences of *C. trachomatis* hsp60 peptide epitopes with that of human mitochondrial chaperonin 60 protein

Epitope <sup>a</sup>	Peptide sequence <sup>b</sup>
H <sub>1</sub> .....	<sup>26</sup> VTLGPKGRHVVI <sup>27</sup> **M*****T*I*
E <sub>1</sub> .....	<sup>94</sup> VLAELIYTEGLR <sup>92</sup> ***RS*AK**FE
E <sub>2</sub> .....	<sup>151</sup> SANNDAEIGNLI <sup>149</sup> ***G*K****I*
E <sub>3</sub> .....	<sup>188</sup> DVVDGMNFNRGY <sup>186</sup> EIIE**K*D***
H <sub>2</sub> .....	<sup>228</sup> SGIKDFLPVLQQ <sup>226</sup> *S*QSIV*A*EI
E <sub>4</sub> .....	<sup>260</sup> ATLVGNRIRGGF <sup>258</sup> S***L**LKV*L
E <sub>5</sub> .....	<sup>282</sup> GDRRKAMFEDIA <sup>280</sup> **N**NQ*K*M*
E <sub>6</sub> .....	<sup>411</sup> ILPGGGTALIRC <sup>410</sup> *VL***C**L**
E <sub>7</sub> .....	<sup>438</sup> NEDEQIGARIVL <sup>432</sup> ***QK**IE*IK
H <sub>3</sub> .....	<sup>480</sup> SAPLKQIAANAG <sup>447</sup> KI*AMI**K***
E <sub>8</sub> .....	<sup>489</sup> NAGKEGAIIFQQ <sup>486</sup> ***V**SL*VEK
E <sub>9</sub> .....	<sup>464</sup> GAIIFQQVMSRS <sup>461</sup> *SL*VEKI*QS*
E <sub>10</sub> .....	<sup>470</sup> QVMSRSANEGYD <sup>467</sup> KI*QS*SEV***

<sup>a</sup> E<sub>1</sub> to E<sub>10</sub> signify epitopes defined with both rabbit and human antisera, and H<sub>1</sub>, H<sub>2</sub>, and H<sub>3</sub> represent epitopes seen only with human antisera.

<sup>b</sup> Top sequences are from *C. trachomatis* GroEL hsp60 (4), and bottom homologous sequences are from human mitochondrial chaperonin 60 protein (6).

human serum samples, and immunologic cross-reactivity was determined by comparing relative antibody binding to peptides of chlamydial hsp60 with binding to homologous peptides of human hsp60 (Fig. 3). The data show that six epitopes (E<sub>1</sub>, E<sub>2</sub>, E<sub>5</sub>, H<sub>3</sub>, E<sub>8</sub>, and E<sub>9</sub>) are relatively specific to the chlamydial sequence, with the human antisera binding to the chlamydial peptide sequence at a significantly ( $P < 0.05$ ) higher OD than it does to the human peptide sequence. In particular, epitope E<sub>8</sub> is absolutely specific to the chlamydial sequence. The remaining seven epitopes (H<sub>1</sub>, E<sub>3</sub>, H<sub>2</sub>, E<sub>4</sub>, E<sub>6</sub>, E<sub>7</sub>, and E<sub>10</sub>) were cross-reactive between the chlamydial and human hsp60 sequences.

These results expand on those previously reported by Cerrone et al. (4). These researchers used deletion fragments of the glutathione S-transferase hsp60 fusion protein and found that the B-cell epitopes as recognized by antisera from five women with *C. trachomatis*-associated pelvic inflammatory disease or ectopic pregnancies were restricted to fragments at the carboxy terminus between aa 274 to 402 or aa 405 to 544. Using the pepscan assay, we found that antigenic sites are distributed throughout the entire sequence, although the COOH half of the protein expresses predominantly chlamydia-specific epitopes.

The goal of this study was to determine whether the antibodies that are induced to chlamydial hsp60 are cross-reactive between human and chlamydial hsp60. Direct sequence comparison of epitopes from chlamydial hsp60 with homologous regions in human mitochondrial hsp60 shows that antibodies to the major epitopes are directed to regions that vary in sequence. However, when peptides homologous to the human sequence are used in the pepscan assay, antibodies that bind to the human sequence are found in sera from women with *C. trachomatis*-associated fallopian tube

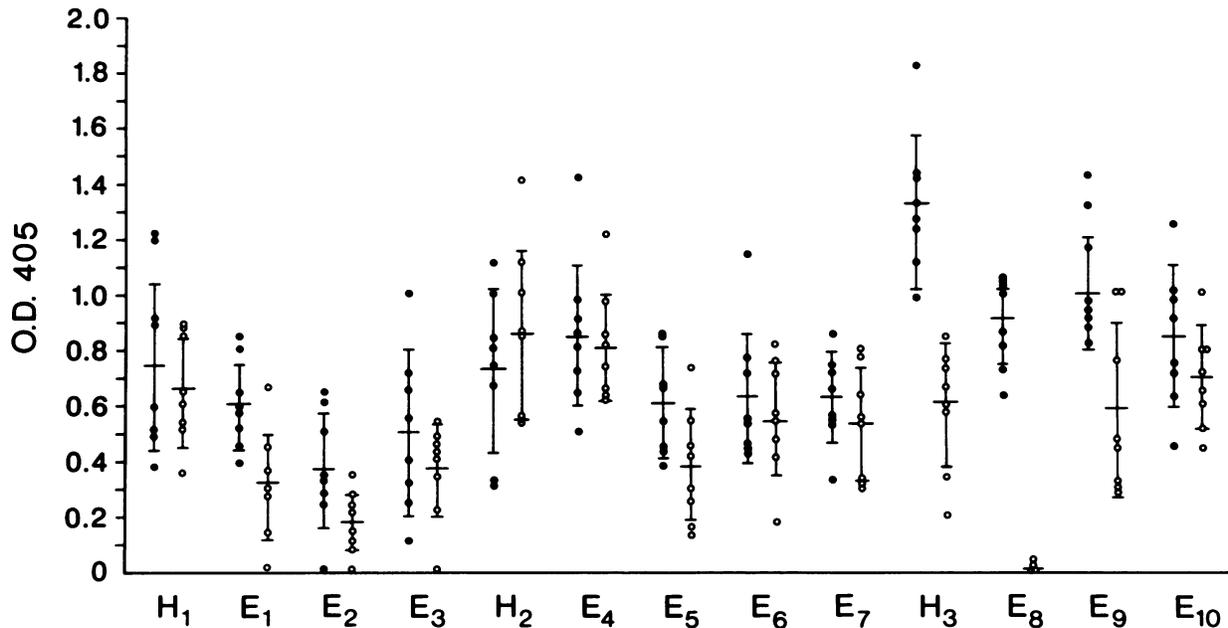


FIG. 3. Thirteen major peptide epitopes from chlamydial hsp60 were used to detect homologous sequences in human mitochondrial hsp60 (6). 12-mer peptides were synthesized in the pepscan format and tested with the eight serum samples from women with *C. trachomatis*-associated ectopic pregnancies (each sample was tested at a dilution of 1:200). Closed circles represent binding to the *C. trachomatis* sequences, and open circles represent binding to the homologous human sequences. Antibody binding was significantly ( $P < 0.05$ ) greater to the chlamydial peptide than to the human peptide for E<sub>1</sub>, E<sub>2</sub>, E<sub>5</sub>, H<sub>3</sub>, E<sub>8</sub>, and E<sub>9</sub>. No human peptide bound antibody better than the chlamydial peptide, and H<sub>1</sub>, E<sub>3</sub>, H<sub>2</sub>, E<sub>4</sub>, E<sub>6</sub>, E<sub>7</sub>, and E<sub>10</sub> were cross-reactive.

damage. Differences between chlamydial and human hsp60 in their amino acid sequences did not accurately predict which epitopes were specific or cross-reactive. Antigenic cross-reactivity may depend on specificity or epitope-paratope immunochemistry, as has been seen previously in studies of the antigenic structure of the chlamydial major outer membrane protein (13).

We hypothesize that cross-reactive antibodies are induced by chlamydial hsp60 during infection. The role played by such antibodies in disease pathogenesis remains to be clarified. The present observations are consistent with the notion that autoimmune responses to human hsp60 as mediated by serum antibodies or by T cells may contribute to chlamydial disease pathology. Further elucidation of T-cell epitopes, major histocompatibility complex-binding peptides, and peptide-specific cytokine secretion by T-cell subsets is needed in order to fully evaluate the immunologic properties of chlamydial hsp60 and its relationship to disease pathogenesis.

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