

Antigenic Analysis of the Chlamydial 75-Kilodalton Protein

GUANGMING ZHONG AND ROBERT C. BRUNHAM*

*Department of Medical Microbiology, University of Manitoba, Winnipeg,
Manitoba, Canada R3E 0W3*

Received 19 July 1991/Accepted 30 December 1991

Both B- and T-cell immunogenicity of a chlamydial 75-kDa protein was analyzed by using 131 partially overlapped decapeptide homologs of the 75-kDa protein from *Chlamydia trachomatis* serovar L2. Six rabbit antiserum specimens raised with serovars B, C, and L2 were used to assay the antibody reactivities of the decapeptides. Seventy-five of the 131 decapeptides were recognized by at least one antiserum specimen, and two peptides were found to be immunodominant and surface accessible on native organisms. The same set of decapeptides were cleaved from the pins and tested for their T-cell-stimulating activity in an in vitro proliferation assay. A single decapeptide was able to stimulate proliferation of chlamydial antigen-primed lymph node T cells from BALB/c mice.

Recent studies have substantially advanced understanding of the antigenic properties of *Chlamydia trachomatis*. This knowledge is of importance in elucidating the immunopathogenesis of chlamydial disease and in the development of immunoprophylaxis. A chlamydial protein with a mass of approximately 75 kDa has been found to commonly induce immune responses in naturally infected humans, and recent genetic investigations indicate that the 75-kDa protein is highly conserved among different serovars and is related to the heat shock protein 70 (hsp70) family (1, 4, 8). Monospecific rabbit antisera raised against the cloned 75-kDa recombinant protein were found to bind to native chlamydial organisms and neutralize the infectivity of two distinct serovars in cell culture (4). Because of these properties we evaluated the antigenic properties of the 75-kDa protein by using synthetic peptides.

C. trachomatis serovars B (TW5/OT), C (TW3/OT), and L2 (434/BU) were propagated in HeLa 229 cells, and the elementary bodies (EBs) were purified as described previously (10, 11). Purified EBs were used to raise rabbit antisera or used as antigen to adsorb antisera.

The amino acid sequence of the serovar L2 75-kDa protein as deduced from the DNA sequence (1) was used to synthesize 131 decapeptides with five overlapping residues (7). A tripeptide (D-P-G) containing a formic acid cleavage site (D-P bond) was included at the C terminus of each decapeptide linking the peptide to polyethylene rod (12). After use for B-cell epitope mapping, peptide pins were cleaved with formic acid by placing the dried peptide pins onto a microtiter plate with each well filled with 0.2 ml of 70% formic acid (Sigma Chemical) and incubating them for 20 h at 37°C in a sealed container (12). The solvent was removed by vacuum evaporation, and dried residues were resuspended in phosphate-buffered saline (PBS), pH 7.4. These preparations were used in the T-cell proliferation assay. Each of two control decapeptides which have difficult coupling residues was recovered at a concentration of up to 10 µg following formic acid cleavage. Only 1 of 131 decapeptides derived

from the 75-kDa sequence contained an internal D-P-G formic acid cleavage site.

To determine whether rabbit antisera contained antibodies to the 75-kDa protein, 11 rabbit antiserum specimens were tested in an immunoblot analysis. Figure 1 shows that the 75-kDa protein is one of the three major antigens recognized by rabbit antisera. We selected six rabbit antiserum specimens on the basis of their strong immunoblot binding and assayed their immunoreactivity with the decapeptide homologs in a solid-phase peptide enzyme-linked immunosorbent assay (Fig. 2). The number of decapeptides reactive with antibody ranged from 10 in rabbit number 6 to 50 in rabbit number 5 (both immunized with L2) (Fig. 2A). Collectively, 75 of 131 decapeptides were recognized by at least one antiserum specimen. On the basis of the titer (as determined by arbitrary optical density [OD] units at a dilution of 1:200) (Fig. 2C) and frequency of reactivity (number of rabbit antiserum specimens recognizing the decapeptide) (Fig. 2B), two peptides (numbers 33 and 80) were defined as immunodominant.

To determine whether immunogenic sequences were surface accessible to antibody binding on native organisms, pooled antisera were preadsorbed with EBs. The immunoreactivity of pooled antisera prior to adsorption was similar to that of individual antisera (compare Fig. 3A with Fig. 2D). Pooled antisera were preadsorbed with either viable or acetone-fixed EBs from serovar B (Fig. 3B) or serovar C (Fig. 3C). Most anti-peptide antibodies were removed or significantly reduced by adsorption with acetone-fixed EBs from either serovar B or C, indicating that the immunogenic regions of the 75-kDa sequence are conserved between these two serovars. Antibodies specifically binding to decapeptides 33 and 80 were selectively adsorbed by EBs of either serovar, suggesting that these two sequences are also surface exposed on EBs of both serovars. HeLa cell debris alone or fixed with acetone failed to remove any anti-peptide antibodies (Fig. 3D). Pooled sera from three nonimmunized rabbits failed to significantly bind to 75-kDa protein decapeptide homologs (Fig. 3E).

Subsequently, the same set of decapeptides was subjected to formic acid cleavage to generate free decapeptides for use in a T-cell proliferation assay. Female BALB/c mice, 7 to 8

* Corresponding author.

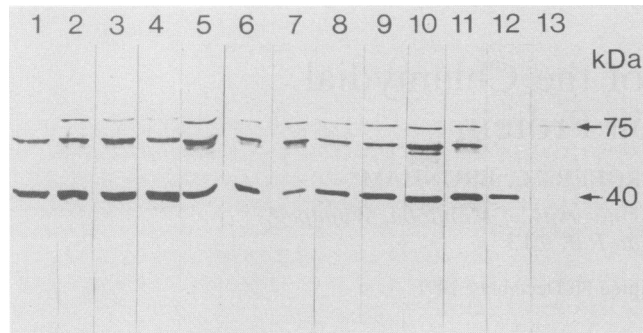


FIG. 1. Serovar B EBs were subjected to sodium dodecyl sulfate-12% polyacrylamide gel separation, and the protein bands were transferred to nitrocellulose membranes. Each strip was blotted with one antiserum specimen at a dilution of 1:200. Lanes 1 through 7, blotted with antiserum R₁ anti-B to R₇ anti-B; lane 8, blotted with R₁ anti-C; lane 9, blotted with R₅ anti-C; lane 10, blotted with R₁ anti-L2; lane 11, blotted with R₂ anti-L2; lane 12, stained with major outer membrane protein-specific monoclonal antibody E-4 (kindly provided by L. De la Maza, University of California, Irvine); lane 13, stained with a 75-kDa protein-specific monoclonal antibody (kindly provided by I. Maclean, University of Manitoba, Winnipeg, Canada).

weeks old, were immunized with serovar B EBs emulsified with an equal volume of complete Freund adjuvant (CFA), chlamydial recombinant 75-kDa protein (4) mixed with an equal volume of CFA, or PBS alone (pH 7.4) mixed with CFA. Ten to 14 days after immunization, the draining popliteal lymph nodes were collected, single-cell suspensions were made, and T cells were enriched by nylon wool filtration (9). T cells (4×10^5) in 100 μ l of complete medium were added to each microtiter well which already contained 1×10^5 thioglycolate-elicited irradiated peritoneal macrophages as antigen-presenting cells and approximately 1.5 μ g of cleaved decapeptides. Cultures were performed in duplicate, and results were expressed as the stimulation index. Figure 4 shows the T-cell responses to 131 decapeptide homologs of chlamydial 75-kDa protein sequences. A single decapeptide (peptide number 57 with the sequence Q-P-F-I-T-I-D-A-N-G) significantly stimulated both recombinant 75-kDa protein (Fig. 4A) and whole chlamydial EB-primed BALB/c lymph node T cells (Fig. 4B). None of the 131 peptides stimulated a significant proliferation response when T cells primed with CFA alone were used (Fig. 4C).

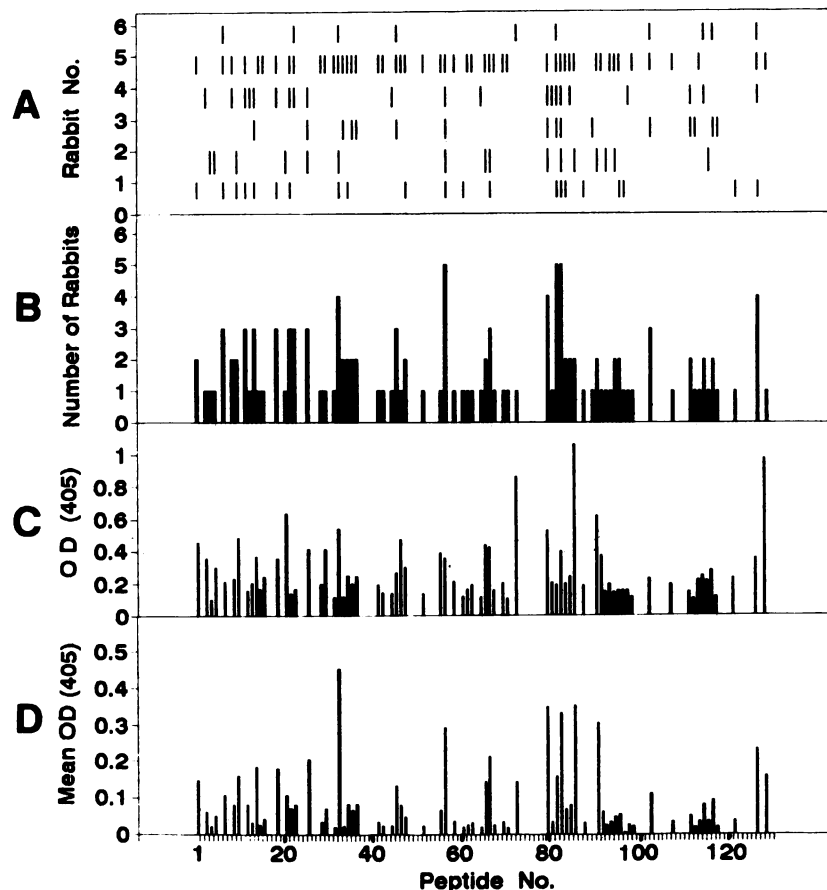


FIG. 2. Profiles of immunogenic responses as a function of all 75-kDa protein decapeptides. Antibody recognition of the chlamydial 75-kDa protein is measured by the reactivity of rabbit antisera to decapeptide analogs of the 75-kDa protein sequence. Each parameter is plotted as the partially overlapped decapeptide number. (A) Reactivity pattern specific to each of six rabbit antiserum specimens. Rabbit antiserum number 1 is from R₂ anti-B, number 2 is from R₅ anti-B, number 3 is from R₁ anti-C, number 4 is from R₅ anti-C, number 5 is from R₁ anti-L2, and number 6 is from R₂ anti-L2. Response to any decapeptide is indicated with a short vertical bar. (B) Frequency of the immunogenic response given by the number of rabbit antiserum specimens that react with each decapeptide; (C) individual titer (as measured in arbitrary OD units) or, in cases in which more than one serum specimen reacted, the geometric mean titer of the antisera; (D) geometric mean OD units obtained by considering the total number of rabbit antiserum specimens investigated. The OD value from a negative-reaction antiserum was taken as zero.

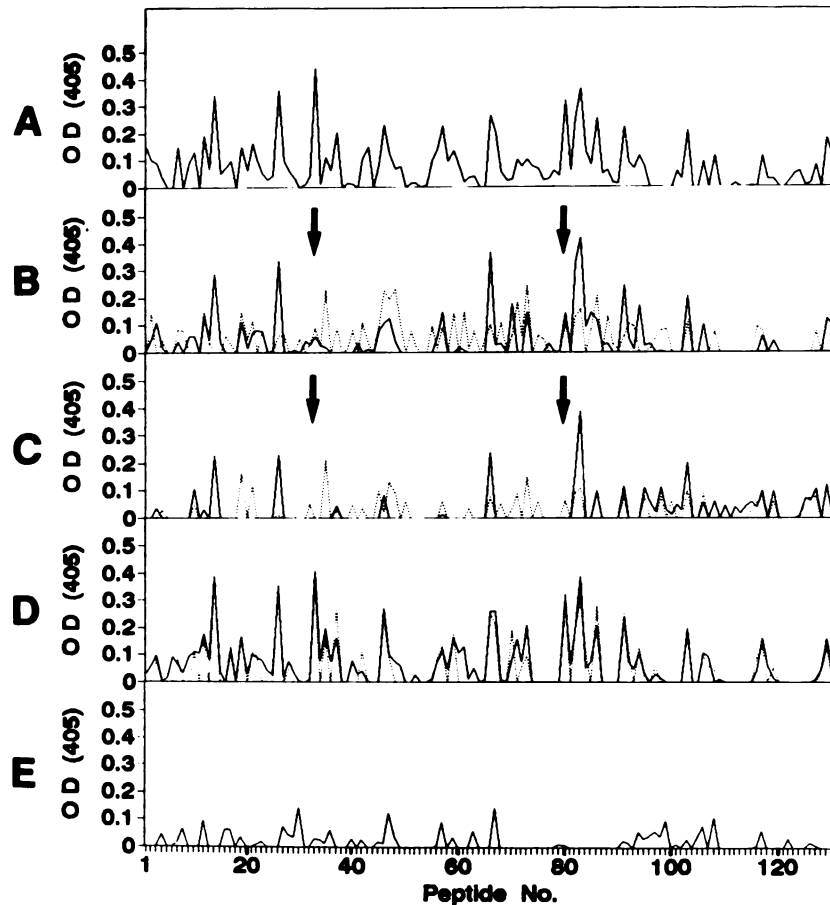


FIG. 3. Pooled antisera from six rabbits (at equal ratios) were assayed for their reactivity with 131 decapeptides (A) or preabsorbed with live (solid line) or acetone-fixed (dotted line) EBs from serovar B (B) or C (C) or HeLa cell debris (D). Arrows indicate absorbed peaks (B and C). (E) Reactivity of pooled rabbit sera from three normal rabbits with the decapeptides.

The potential biological relevance of the 75-kDa chlamydial protein is suggested by its large degree of sequence similarity to members of the hsp70 family (3) and its preferential transcription during heat stress of chlamydial organisms (5). Like other hsp70 proteins, the chlamydial 75-kDa protein may assist in the export of outer membrane proteins and remain loosely associated with these proteins after export. This hypothesis accords with the following observations: antibody to the 75-kDa protein coprecipitates a 40-kDa protein which may be the major outer membrane protein (5); the 75-kDa protein is principally distributed within the cytoplasm of reticulate bodies (1); and certain antigenic sites of the 75-kDa protein are surface exposed on EBs (present study).

The 75-kDa chlamydial protein frequently elicits immune responses in naturally infected humans (2) and was found to be highly immunogenic, constituting one of the three principal chlamydial antigens recognized by experimentally immunized outbred rabbits. At least 75 of a potential 131 decapeptides from the protein were found to be immunogenic, and immunogenic regions appeared to be randomly distributed throughout the entire sequence. This is noticeably different from the immunogenic profile of the major outer membrane protein (13-15). Two peptides, located at decapeptides 33 (G-R-I-A-G-L-D-V-K-R) and 80 (S-C-G-I-E-T-C-G-G-V), were identified as immunodominant. These two epitopes

also appeared to be surface exposed, as demonstrated by the adsorption experiment.

We compared the inferred amino acid sequences of the three reported chlamydial 75-kDa protein sequences with the bovine hsp70 sequence (3). The bovine protein is composed of two domains: an amino-terminal fragment containing ATPase activity and a carboxy terminus apparently functioning as a protein recognition domain. The ATPase fragment has been crystallized, and its three-dimensional structure has been elucidated (6). The chlamydial 75-kDa protein shows high sequence homology with bovine hsp70, and the surface-exposed B-cell epitope (G-R-I-A-G-L-D-V-K-R) may be located near the ATP hydrolytic site if the chlamydial protein has structural similarity to the bovine hsp70 ATPase domain.

We also used synthetic peptides to map potential T-cell sites on chlamydial hsp70. Van der Zee et al. (12) reported that the multiple short peptides could be detached from their solid phase by incorporation of a formic acid cleavage site at the C terminus of each peptide to generate free peptides which could subsequently be used for mapping T-cell epitopes. By this approach, a single peptide was found to be able to stimulate a significant proliferation response of chlamydial antigen-primed T cells but not of control T cells. It may be that few T-cell sites exist on this protein because of extensive sequence homology with a host protein.

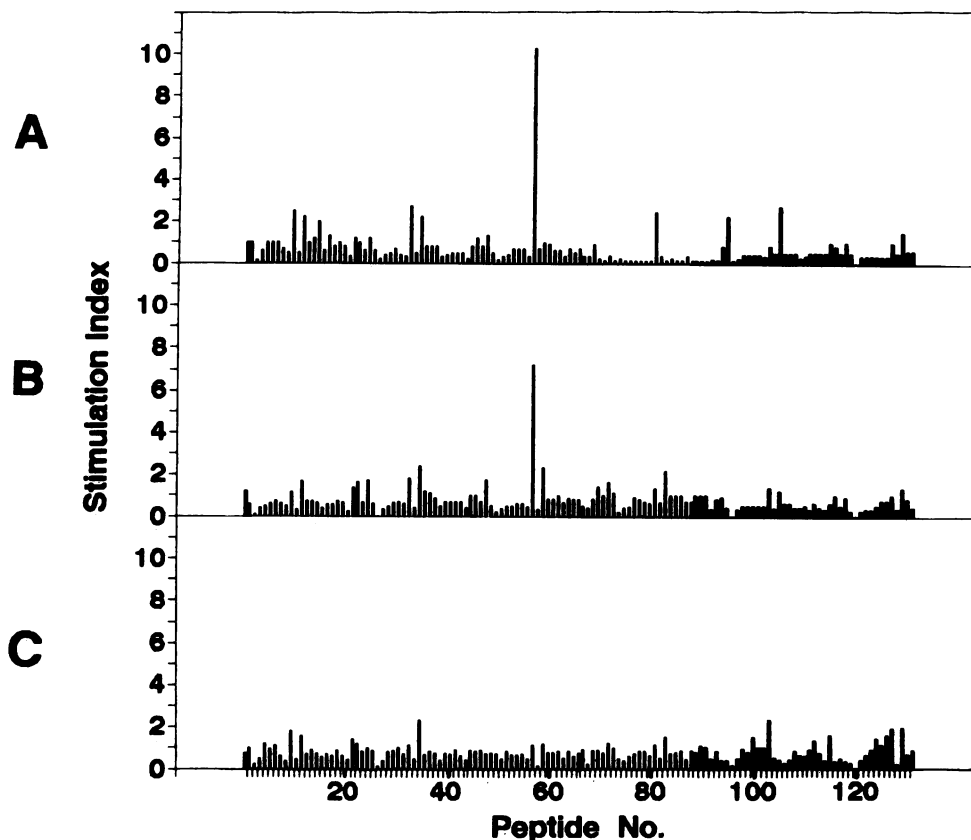


FIG. 4. Formic acid-cleaved decapeptides stimulate *in vitro* proliferation of primed lymph node T cells with recombinant 75-kDa protein (A), serovar B EBs (B), or CFA alone (C). The results are plotted for each peptide number.

In summary, the modified pepscan method is a convenient technique for analysis of protein immunogenicity, allowing for evaluation of both B- and T-cell epitopes with a single synthesis. Application of this technique to resolve the immunogenicity of the chlamydial 75-kDa protein provided potentially useful information for evaluating its potential in vaccine development.

This work was supported by a grant from the Edna McConnell Clark Foundation and from the Medical Research Council of Canada.

REFERENCES

- Birkelund, S., A. G. Lundemose, and G. Christiansen. 1990. The 75-kilodalton cytoplasmic *Chlamydia trachomatis* L2 polypeptide is a DnaK-like protein. *Infect. Immun.* **58**:2098–2104.
- Brunham, R. C., R. Peeling, I. Maclean, J. McDowell, K. Persson, and S. Osser. 1987. Postabortal *Chlamydia trachomatis* salpingitis: correlating risk with antigen-specific serological responses and with neutralization. *J. Infect. Dis.* **155**:749–755.
- Chappell, T., B. B. Konforti, S. L. Schmid, and J. E. Rothman. 1987. The ATPase core of a clathrin uncoating protein. *J. Biol. Chem.* **262**:746–751.
- Danilition, S. L., I. W. Maclean, R. Peeling, S. Winston, and R. C. Brunham. 1990. The 75-kilodalton protein of *Chlamydia trachomatis*: a member of heat shock protein 70 family? *Infect. Immun.* **58**:189–196.
- Engel, N. J., J. Pollack, E. Perara, and D. Granem. 1990. Heat shock response of murine *Chlamydia trachomatis*. *J. Bacteriol.* **172**:6959–6972.
- Flaherty, K., C. Deluca-Flaherty, and D. B. McKay. 1990. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature (London)* **346**:623–628.
- Geysen, M. N., J. S. Rodda, J. T. Mason, G. Tribbick, and G. P. Schoofs. 1987. Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods* **102**:259–274.
- Kornak, J. M., C.-C. Kuo, and L. A. Campbell. 1991. Sequence analysis of the gene encoding the *Chlamydia pneumoniae* DnaK protein homolog. *Infect. Immun.* **59**:721–725.
- Lee, C. K., B. Singh, M. A. Barton, A. Procyshyn, and M. Wong. 1979. A simple reliable system for studying antigen-specific murine T cell proliferation. *J. Immunol. Methods* **25**:159–170.
- Maclean, I. W., R. W. Peeling, and R. C. Brunham. 1988. Characterization of *Chlamydia trachomatis* antigens with monoclonal and polyclonal antibodies. *Can. J. Microbiol.* **34**:141–147.
- Peeling, R., I. Maclean, and R. C. Brunham. 1984. *In vitro* neutralization of *Chlamydia trachomatis* with monoclonal antibody to an epitope on the major outer membrane protein. *Infect. Immun.* **46**:484–488.
- Van der Zee, D., W. V. Eden, R. J. Moloen, A. Moordzij, and J. Embden. 1989. Efficient mapping and characterization of a T cell epitope by the simultaneous synthesis of multiple peptides. *Eur. J. Immunol.* **19**:43–47.
- Zhong, G., and R. C. Brunham. 1990. Immunoaccessible peptide sequences of the major outer membrane protein from *Chlamydia trachomatis* serovar C. *Infect. Immun.* **58**:3438–3441.
- Zhong, G., and R. C. Brunham. 1991. Antigenic determinants of the chlamydial major outer membrane protein resolved at a single amino acid level. *Infect. Immun.* **59**:1141–1147.
- Zhong, G., R. E. Reid, and R. C. Brunham. 1990. Mapping antigenic sites on the major outer membrane protein of *Chlamydia trachomatis* with synthetic peptides. *Infect. Immun.* **58**:1450–1455.