Monoclonal Antibodies Define Genus-Specific, Species-Specific, and Cross-Reactive Epitopes of the Chlamydial 60-Kilodalton Heat Shock Protein (hsp60): Specific Immunodetection and Purification of Chlamydial hsp60

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Ocular and urogenital tract infections with *Chlamydia trachomatis* can progress to chronic inflammatory diseases that produce blindness and tubal infertility. The pathophysiology of these chronic disease conditions is thought to be immunologically mediated, and the chlamydial 60-kDa heat shock protein (hsp60) has been implicated as a major target antigen that stimulates the immunopathological response. The lack of chlamydial hsp60 antibodies and purified hsp60 has severely restricted studies to define more thoroughly the role of this protein in the immunopathogenesis of chlamydial disease. We produced a panel of antichlamydial hsp60 monoclonal antibodies (MAbs) and defined their specificities by immunoblotting against lysates of *C. trachomatis*, *C. psittaci*, and six other genera of bacteria. Three patterns of anti-hsp60 immunoreactivity were observed: chlamydial species specific, chlamydial genus specific, and cross-reactive. The epitopes recognized by these MAbs were localized within the primary amino acid sequence of hsp60 by immunoblotting against recombinant amino-terminal truncated hsp60 fusion polypeptides and then precisely mapped by use of overlapping synthetic peptides. The majority of the MAbs mapped to either the amino or the carboxyl termini of hsp60. Epitopes defining all three MAb reactivities mapped within amino-terminal residues 6 to 16. Genus-specific hsp60 MAbs mapped to epitopes located within this region and to residues 17 to 28 and 177 to 189. Antichlamydial hsp60 MAbs stained inclusions as effectively as MAbs specific for the major outer membrane protein. Homogeneous preparations of full-length recombinant chlamydial hsp60 and amino-terminal truncated recombinant hsp60 polypeptides were obtained by immunoabsorption chromatography with an hsp60 MAb reactive to the carboxyl terminus of the protein. Thus, the antichlamydial MAbs described here should be extremely useful for the specific immunodetection of hsp60 in tissues from individuals having different disease manifestations and for the purification of hsp60 or truncated hsp60 polypeptides for use in serologic and lymphocyte proliferation assays. The availability of these MAbs will facilitate studies to define more precisely the role of hsp60 in the immunopathogenesis of chlamydial disease.

Chlamydia trachomatis infects the ocular and genital mucosal epithelium and commonly produces asymptomatic or acute self-limiting infections, such as uncomplicated conjunctivitis, urethritis, or cervicitis. Acute infections occasionally progress to chronic conditions characterized by severe inflammatory responses that subsequently lead to blindness or genital tract disease. Infections of the lower genital tract in women can ascend to infect fallopian tubes, resulting in acute or chronic salpingitis. These infections can subsequently cause infertility or ectopic pregnancy by tubal blockage (39). Trachoma, a chronic inflammatory disease of the eye caused by a persistent or recurrent conjunctival infection with *C. trachomatis*, is the leading cause of preventable blindness in the world (20).

The pathogenetic events that culminate in trachoma and chlamydial salpingitis are not well understood; however, an immunopathological basis for chlamydial disease has been proposed (14, 30). Data from experimental animal models of chlamydial infection and from studies of human chlamydial disease suggest that a hypersensitivity response to a genus-common antigen might contribute to the disease process (11, 14, 15, 40). We identified and characterized a 57-kDa chlamydial protein that invoked a mononuclear cellular inflammatory response when placed on the conjunctival surface in guinea pigs that had recovered from a primary ocular chlamydial infection (28). Sequence analysis of the gene encoding the 57-kDa protein revealed that this protein was a member of the family of widely conserved 60-kDa heat shock proteins (hsp60) (27). Although the precise involvement of chlamydial hsp60 in stimulating immunopathogenetic responses in human chlamydial disease has not been determined, correlations between immune responsiveness to a ca. 60-kDa protein and the severe sequelae of chlamydial disease have been demonstrated in women with pelvic inflammatory disease, ectopic pregnancy, and tubal factor infertility and in patients with postchlamydial reactive arthritis (4, 9, 18, 22, 38).

Studies to define more precisely the role of hsp60 in eliciting the immunopathology associated with chronic chlamydial disease are certainly needed. A major obstacle in these studies is the lack of chlamydial hsp60 probes. We produced and characterized a panel of antichlamydial hsp60 monoclonal antibodies (MAbs) and identified several that

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recognize hsp60 epitopes unique to the genus *Chlamydia*. These MAbs should be useful for the immunodetection of chlamydial hsp60 in infected tissues and for immunoaffinity purification of native or recombinant chlamydial hsp60, which can then be used as antigens in in vitro antibody and T cell assays. By selection of the appropriate MAb, recombinant fragments can be purified and used for epitope mapping studies of T cell and antibody responses.

MATERIALS AND METHODS

Organisms. The *C. trachomatis* serovars A/Har-13 and L2/LGV-434 (hereafter referred to as serovars A and L2, respectively) and *C. psittaci* GPIC (a strain causing guinea pig inclusion conjunctivitis) were grown in HeLa cells, and elementary bodies were purified by discontinuous density centrifugation in Renograffin (E. R. Squibb and Sons, Princeton, N.J.) (7). *Escherichia coli* JM109 and *Salmonella typhimurium* SL3261 were grown in Luria broth at 37°C. *Neisseria gonorrhoeae* MS11, *Campylobacter jejuni*, *Coxiella burnetti* Nine Mile, and *Borrelia burgdorferi* B31 were obtained from R. Belland, W. Cieplak, D. Hackstadt, and P. Rosa, respectively (Rocky Mountain Laboratories, Hamilton, Mont.). Recombinant clones expressing *C. trachomatis* serovar A hsp60 [JM109(pTA571)] and *C. psittaci* GPIC hsp60 [JM109(pGP57)] were described previously (27, 29).

SDS-PAGE, electrophoretic transfer, and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% polyacrylamide gels as described by Dreyfuss et al. (13), except that samples were solubilized in Laemmli sample buffer (24). Samples contained approximately 0.5 to 1.0 mg of protein per ml, and 20 μl was loaded for electrophoresis. Electrophoretic transfer and processing of immunoblots were performed as described previously (43). Detection of non-protein A-binding antibodies was facilitated by using affinity-purified rabbit anti-mouse immunoglobulin G (IgG) (Organon Teknika Corp., Durham, N.C.).

Production of MAbs. Recombinant clones JM109(pGP57) and JM109(pTA571) were grown overnight in Luria broth containing 250 μg of carbenicillin per ml. The bacteria were washed twice with 50 mM phosphate-buffered saline (PBS) (pH 7.2) and solubilized in Laemmli sample buffer, and polypeptides were separated by SDS-PAGE. SDS-PAGE-separated recombinant serovar A and strain GPIC hsp60 were cut from the gels and electroeluted (8). BALB/c mice were immunized intraperitoneally with 50 μg of recombinant serovar A or strain GPIC hsp60 emulsified in complete Freund adjuvant. Three weeks later, mice were given a booster immunization of antigen in incomplete Freund adjuvant, and cell fusions were performed 4 days later, as described previously, with splenic lymphocytes and NS-1 myeloma cells (21). Hybridomas producing anti-hsp60 MAbs were identified by immunoblotting with culture supernatants. MAb immunotypes were determined by immunodiffusion (Ouchterlony) with class- and subclass-specific rabbit antisera (Litton Bionetics, Inc., Kensington, Md.). All hybridomas selected secreted MAbs of the IgG1 subclass.

Preparation of amino-terminal truncated recombinant hsp60 polypeptides. Five amino-terminal truncated recombinant hsp60 polypeptides were prepared as LacZ α peptide fusions. The recombinant plasmid containing the *C. trachomatis* serovar A *hyp* operon (pTA571) was purified by Qiagen chromatography in accordance with the manufacturer's recommended procedure (Qiagen, Inc., Chatsworth, Calif.). The 4-5 kb chlamydial DNA insert of pTA571 was

cut from the plasmid by digestion with *Kpn*I and *Sal*I endonucleases, purified following agarose gel electrophoresis, and used as the DNA template in the polymerase chain reactions for the production of sequential 5′ deletions of *hypB*. Five 5′ forward primers (FP1, 5′-TTCTAGAGG TCGCTAAAAACATTAA-3′; FP2, 5′-TTCTAGATACA GAAGGATTACGCAA-3′; FP3, 5′-TTCTAGACTCTAGCT ACTTCGCAAC-3′; FP4, 5′-TTCTAGACATTAGCGAAG AGTTGGG-3′; and FP5, 5′-TTCTAGAACATGCTACAAT CGCTGC-3′) and one reverse primer (RP1, 5′-GGCAT GCGGAATCTAAAAAAAGCCT-3′) were selected and synthesized such that the amplified gene fragments could be inserted in frame with the gene encoding the LacZ α peptide. Amplification, cloning, and expression of the amino-terminal truncated *hypB* genes were performed by previously described methods (32). The polymerase chain reaction products were digested with *Sph*I and *Xba*I and purified by agarose gel electrophoresis and phenol extraction. The purified gene fragments were ligated into *Sph*I-*Xba*I-digested, alkaline phosphatase-treated pUC18. *E. coli* JM109 was transformed with the recombinant plasmids (10), and recombinant clones were selected by growth on Luria broth agar plates containing carbenicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and isopropyl-β-D-thiogalactopyranoside. Recombinant clones were tested for the expression of amino-terminal truncated hsp60 polypeptides by SDS-PAGE and immunoblotting with polyclonal rabbit anti-hsp60 sera as described above. The 5′-truncated *hypB* sequence in the recombinant plasmids was confirmed by restriction mapping and by sequencing of splice regions and the first 100 nucleotides of the 5′ and 3′ ends of the DNA insert (Sequenase; United States Biochemical Corp., Cleveland, Ohio). The five recombinant clones selected expressed fusion polypeptides consisting of 14 amino-terminal residues of the LacZ α peptide followed by various lengths of amino-terminal truncated chlamydial hsp60.

Peptide synthesis and epitope mapping of anti-hsp60 MAbs. Peptides were synthesized by use of a commercially available kit in accordance with the manufacturer's recommended procedures (epitope scanning kit; Cambridge Research Biochemicals, Wilmington, Del.). Octapeptides representing the entire *C. trachomatis* serovar A hsp60 sequence and overlapping by 7 amino acid residues were synthesized and used as the solid-phase antigen in an enzyme-linked immunosorbent assay (ELISA) to map MAb-reactive determinants. Peptides of 12 residues representing amino acid residues 1 to 100 of the serovar A hsp60 sequence and overlapping by 11 residues beyond this sequence were also synthesized. Epitope mapping of anti-hsp60 MAbs was performed by a pin ELISA, in accordance with the manufacturer's recommendations (Cambridge Research Biochemicals), with alkaline phosphatase-conjugated rabbit anti-mouse IgG (γ chain specific; Zymed Laboratories, Inc., South San Francisco, Calif.) and Sigma 104 substrate (Sigma Chemical Co., St. Louis, Mo.).

Indirect immunofluorescence assay. HeLa cell monolayers were infected with *C. trachomatis* serovar L2 and fixed with methanol 24 h after infection. Methanol-fixed cell monolayers were incubated for 45 min with either an anti-hsp60 MAb (hybridoma culture supernatant) or protein A-purified anti-major outer membrane protein (MOMP) MAb DIII-A3, which reacts with a subspecies-specific determinant located in variable domain IV of the MOMP (42). The monolayers were washed twice with PBS and incubated for 45 min with fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin. Monolayers were washed twice to remove non-
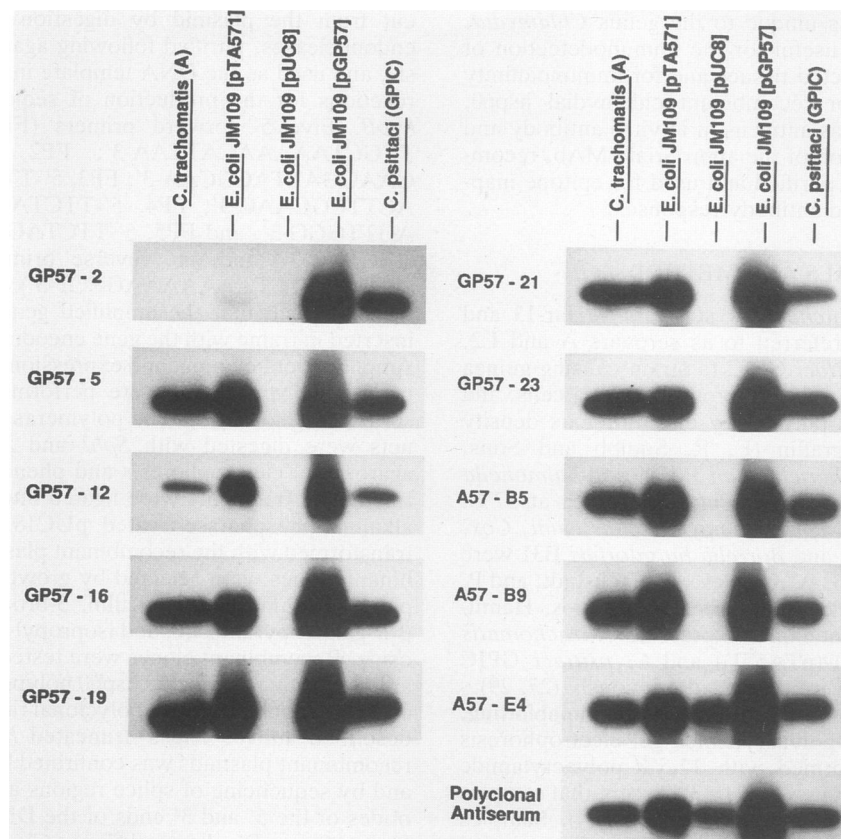
FIG. 1. Identification of anti-hsp60 MAbs by SDS-PAGE and immunoblotting. Ten MAbs were obtained from mice immunized with purified recombinant *C. psittaci* GPIC or *C. trachomatis* serovar A hsp60 and designated "GP57-" or "A57-," respectively. Immunoblotting of whole-cell lysates of *C. trachomatis* serovar A and *C. psittaci* GPIC elementary bodies, recombinant *E. coli* expressing either serovar A [JM109(pTA571)] or GPIC [JM109(pGP57)] hsp60, and *E. coli* was used to screen for MAb reactivity. Polyclonal anti-hsp60 serum was included as a positive control and has been described previously (28).

bound antibody, and inclusions were viewed by fluorescence microscopy.

Immunoaffinity purification of recombinant chlamydial hsp60. An immunoaffinity column was prepared by use of the antichlamydial hsp60 MAb A57-B9. The MAb was purified by passing a hybridoma supernatant through a protein G affinity column in accordance with the manufacturer's recommendations (Zymed Laboratories). The anti-hsp60 affinity column was prepared by covalently cross-linking the purified MAb to Sepharose-protein G as described previously (16). Chlamydial hsp60 was purified from recombinant clone JM109(pTA571), which expresses *C. trachomatis* serovar A hsp60 (29). The recombinant clone was grown overnight in Luria broth supplemented with 250 μg of carbenicillin per ml. Bacteria were pelleted by centrifugation, washed three times with PBS, resuspended in PBS, and disrupted by sonication. The insoluble material was removed by centrifugation at $100,000 \times g$ for 30 min. The supernatant was removed and mixed 1:1 with 2% *n*-octyl-β-D-glucopyranoside (OGP) (1% final concentration). The supernatant was passed through the anti-hsp60 affinity column, the column was washed with 50 mM PO_4 containing 0.5 M NaCl and 1% OGP, and hsp60 was eluted with 100 mM glycine-HCl (pH 2). Fractions were analyzed by SDS-PAGE and Coomassie blue staining. Fractions containing hsp60 were pooled, dialyzed overnight against 3 liters of 10 mM PO_4 (pH

7.2), and lyophilized. Lyophilized hsp60 was reconstituted with distilled H_2O.

RESULTS

Specificity of antichlamydial hsp60 MAbs. Ten hybridomas producing antichlamydial hsp60 MAbs were characterized. Seven were from mice immunized with recombinant GPIC hsp60 (GP57-2, GP57-5, GP57-12, GP57-16, GP57-23, GP57-21, and GP57-19), and three were from mice immunized with recombinant serovar A hsp60 (A57-B5, A57-E4, and A57-B9). MAbs were screened initially by immunoblotting of hybridoma supernatants against whole-cell lysates of *C. trachomatis*, *C. psittaci*, *E. coli*, and *E. coli* expressing recombinant *C. trachomatis* or *C. psittaci* hsp60. Eight MAbs reacted with *C. psittaci* and *C. trachomatis* hsp60, one (MAb GP57-2) reacted primarily with GPIC hsp60, and one (MAb A57-E4) reacted with *C. psittaci*, *C. trachomatis*, and *E. coli* hsp60 (Fig. 1). The nine *C. trachomatis*-reactive anti-hsp60 MAbs reacted with the hsp60 of all 15 *C. trachomatis* serovars (data not shown). Six genera of gram-negative bacteria were probed by immunoblotting to determine more thoroughly the specificities of the MAbs. Six of the MAbs cross-reacted with hsp60 from other procaryotes (Fig. 2). A57-E4, the most broadly cross-reactive MAb, reacted with *Chlamydia*, *E. coli*, *S. typhimurium*, *N. gonorrhoeae*,
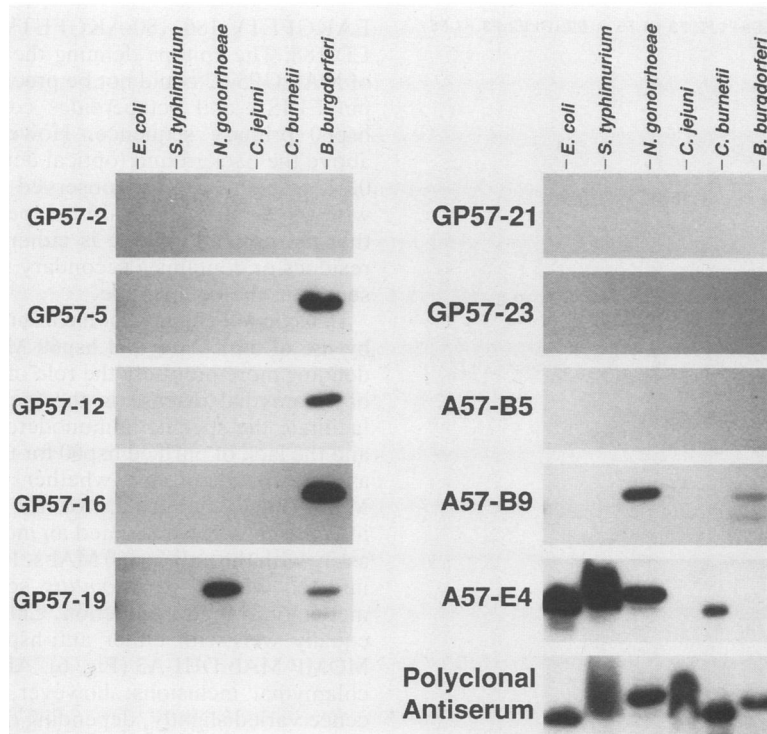
FIG. 2. Cross-reactivity of MAbs with procaryotic hsp60. Whole-cell lysates of *E. coli*, *S. typhimurium*, *N. gonorrhoeae*, *C. jejuni*, *C. burnetii*, and *B. burgdorferi* were probed with MAbs or polyclonal rabbit anti-hsp60 serum. MAbs GP57-5, GP57-12, GP57-16, GP57-19, A57-B9, and A57-E4 and polyclonal antiserum reacted with heterologous hsp60, whereas MAbs GP57-2, GP57-21, GP57-23, and A57-B5 did not react with any of the nonchlamydial hsp60 tested and likely recognized chlamydia-specific regions of hsp60.

and *C. burnetii* hsp60. The other cross-reactive MAbs showed limited genus cross-reactivity. None of the MAbs reacted with eucaryotic hsp60 (HeLa 229 cells) in immunoblotting (data not shown). On the basis of this survey of heterologous hsp60, MAbs reactive to chlamydial species-specific and genus-specific hsp60 epitopes as well as broadly cross-reactive epitopes were obtained.

Localization of MAb epitopes in regions of the hsp60 primary sequence. *E. coli* transformed with recombinant plasmids containing *lacZ-hypB* gene fusions expressed sequential amino-terminal truncated forms of chlamydial hsp60, and the clones were designated F1 to F5 (Fig. 3). All fusion polypeptides contained the first 14 amino acid residues of the LacZ α peptide followed by various lengths of
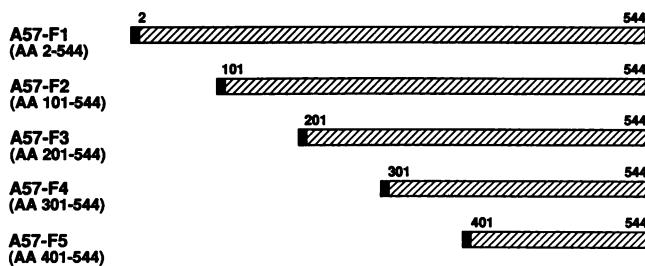

FIG. 3. Diagrammatic representation of LacZ-hsp60 fusion polypeptides. Five recombinant *E. coli* clones (designated F1 to F5) that expressed fusion polypeptides containing the first 14 amino acid (AA) residues of the LacZ α peptide (■) followed by selected lengths of amino-terminal truncated chlamydial hsp60 (▨) were prepared. Gene fusions were confirmed by DNA sequencing.

amino-terminal truncated hsp60. To determine the approximate locations of MAb determinants in the primary amino acid sequence, we probed whole-cell lysates of the recombinant clones by immunoblotting (Fig. 4). The migration of the fusion polypeptides of F1 to F5 in a 12.5% polyacrylamide gel approximated their respective calculated molecular weights of 59,500, 48,800, 38,100, 27,200, and 16,300. MAbs GP57-5, GP57-12, GP57-16, GP57-23, and A57-B5 reacted with epitopes located between amino acid residues 2 and 100 of hsp60, epitopes recognized by MAbs GP57-21 and A57-E4 were located between residues 101 and 200, and MAbs GP57-19 and A57-B9 reacted with the carboxyl terminus of hsp60 (residues 401 to 544). MAb A57-E4 reacted with the fusion polypeptides of F1 and F2; however, because this MAb cross-reacted with *E. coli* hsp60, an immunoreactive 60-kDa polypeptide was observed with all recombinants (F1 to F5). The epitope recognized by the *C. psittaci*-specific MAb GP57-2 was localized to the amino terminus by reaction of the MAb with a recombinant MalE-GPIC hsp60 fusion polypeptide containing amino-terminal amino acid residues 2 to 97 of GPIC hsp60 (data not shown).

Epitope mapping of MAbs. For precise mapping of the MAb epitopes, the MAbs were reacted in a pin ELISA with overlapping synthetic peptides corresponding to the primary amino acid sequence of *C. trachomatis* serovar A hsp60. Six MAbs reacted with linear epitopes located at the amino terminus of hsp60, in a region showing substantial sequence heterogeneity among the various hsp60 (Fig. 5). The epitopes recognized by GP57-5, GP57-12, GP57-16, and GP57-23 contained a common heptameric sequence, 8-YNEEARK-14, but differed in the optimal linear sequence recognized by the antibody paratope. A57-B5 reacted with a
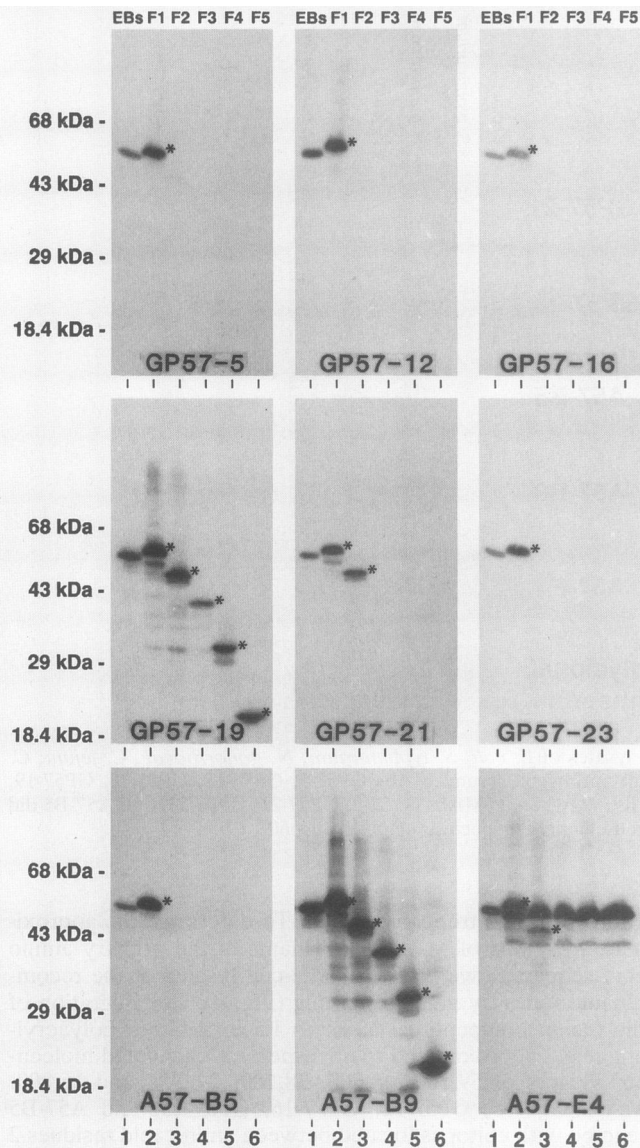
FIG. 4. Localization of MAb epitopes in regions of the hsp60 primary sequence. For determination of the approximate locations of MAb epitopes in the primary sequence of hsp60, whole-cell lysates of recombinant clones expressing amino-terminal truncated hsp60 fusion polypeptides were probed by immunoblotting with MAbs. Lanes: 1, *C. trachomatis* serovar A elementary bodies; 2 to 6, recombinant clones F1 to F5, respectively. MAbs used as the probes are indicated at the bottom of the blots. Immunoreactive fusion polypeptides are identified by asterisks. MAb A57-E4 cross-reacted with *E. coli* hsp60; thus, an immunoreactive 60-kDa polypeptide was observed in all lanes.

linear epitope of 12 amino acid residues at the amino terminus in a region of sequence heterogeneity, and A57-E4 reacted with a hexameric peptide located in a region of sequence identity among the hsp60. GP57-19 and A57-B9 reacted with an identical 6-amino-acid linear epitope located at the carboxyl terminus of hsp60. The paratope of MAb GP57-21 spanned the sequence 177-VEEAKGFETVLDV-189 and reacted strongly with octapeptides 177-VEEA KGFE-184, 178-EEAKGFET-185, and 182-GFETVLDV-189 but weakly with the intervening octapeptides 179-

EAKGFETV-186, 180-AKGFETVL-187, and 181-KGFETV LD-188. The epitope defining the species-specific reactivity of MAb GP57-2 could not be precisely mapped by use of the pin ELISA and octapeptides corresponding to the GPIC hsp60 primary sequence. However, an ELISA reaction above the background (optical densities at 405 nm, 0.15 and 0.04, respectively) was observed when GP57-2 was reacted with the 9-NEDARKK-16 octapeptide. These data suggest that the optimal epitope is either larger than 8 amino acid residues or contains a secondary structure that is not represented in the octapeptide.

Detection of chlamydial inclusions and purification of hsp60 by use of antichlamydial hsp60 MAbs. A major obstacle in defining more precisely the role of hsp60 in the pathogenesis of chlamydial disease is the lack of reagents that would facilitate the specific immunodetection of chlamydial hsp60 and the lack of purified hsp60 for use in in vitro immunologic assays. To determine whether the antichlamydial hsp60 MAbs might be useful for the detection of chlamydia-infected cells, we evaluated an indirect immunofluorescence assay with the anti-hsp60 MAbs. HeLa cell monolayers were infected with *C. trachomatis* serovar L2 and fixed with methanol 24 h after infection. Chlamydial inclusions stained equally well with either anti-hsp60 MAb A57-B9 or anti-MOMP MAb DIII-A3 (Fig. 6). All anti-hsp60 MAbs stained chlamydial inclusions; however, the intensity of fluorescence varied slightly, depending on which MAb and serovar were used. Furthermore, staining of chlamydial inclusions with either anti-hsp60 MAbs or the Syva MicroTrak direct specimen test reagent (anti-MOMP) (Syva Co., Palo Alto, Calif.) was comparable.

Study of the in vitro T cell responses to chlamydial hsp60 necessitates obtaining detergent-free homogeneous preparations of hsp60. The recombinant expressing chlamydial hsp60, JM109(pTA571), is a good source of antigen, but the presence of *E. coli* hsp60 precludes chlamydia-specific analysis. Therefore, we chose the chlamydial hsp60-reactive, non-*E. coli* hsp60-reactive MAb A57-B9 to prepare an immunoaffinity column for the purification of recombinant chlamydial hsp60. Chlamydial hsp60 was efficiently purified with the A57-B9 affinity column (Fig. 7). Approximately 1 to 2 mg of hsp60 could be purified from a single elution of a 5-ml affinity column. Furthermore, the recombinant LacZ-hsp60 truncated fragments reported here were also purified with the A57-B9 affinity column, and similar yields were obtained (data not shown). The homogeneous detergent-free preparations of chlamydial hsp60 and its recombinant truncated fragments will facilitate the analysis of the antibody and T cell responses to hsp60 of individuals having different chlamydial disease conditions and allow for the localization of immunoreactive determinants.

DISCUSSION

Heat shock proteins, which belong to families of widely conserved proteins found in procaryotes and eucaryotes, are commonly immunodominant antigens recognized following infection with many bacterial and protozoan pathogens. hsp60 of bacterial pathogens have been shown to elicit antibody and T cell responses. These immune responses are either protective or immunopathological, depending on the pathogen and experimental system. It was previously proposed that the cellular immune response to hsp60 is pathogenetic and contributes to the severe sequelae, such as blindness, infertility, and arthritis, that follow a chlamydial infection (26). Inoculation of purified hsp60 onto the con-

	GP57-2	GP57-5	GP57-12
C. trachomatis (A)	..E.....	6-IKYNEEARKK-19	8-YNEEARKK-15
C. trachomatis (L2)	..E.....	....	....
C. psittaci (GPIC)	9-NEDARKKI-16	.....D....	...D....
E. coli	GN...V.M	V.FGND..V.	FGND..V.
B. burgdorferi	......SL	.YF..D...S	F..D...S
C. burnetii	SH.VLHAM	L.FSH.VLHA	FSH.VLHA
M. tuberculosis	D.E..RGL	.A.D....RG	.D....RG
Hu mitochondrial P1	GA...ALM	V.FGAD..AL	FGAD..AL

	GP57-16	GP57-23	A57-B5
C. trachomatis (A)	8-YNEEARK-14	6-IKYNEEARKKI-16	17-QKGVKTLAEAVK-28
C. trachomatis (L2)	....	....	....
C. psittaci (GPIC)	...D...	.....D.....	H...........
E. coli	FGND..V	V.FGND..V.M	LR..NV..D...
B. burgdorferi	F..D...	.YF..D...SL	LS..EK.SN...
C. burnetii	FSH.VLH	L.FSH.VLHAM	SR..EV..N...
M. tuberculosis	.D....R	.A.D....RGL	ER.LNA..D...
Hu mitochondrial P1	FGAD..A	V.FGAD..ALM	LQ..DL..D..A

	A57-E4	GP57-21	A57-B9 GP57-19
C. trachomatis (A)	117-KRGIDK-122	177-VEEAKGFETVLDV-189	517-LTTEAL-522
C. trachomatis (L2)	......	....	......
C. psittaci (GPIC)	......	....	......
E. coli	......	..DGT.LQED...	I...CM
B. burgdorferi	.K...H	...S.T.D.TIS.	....CA
C. burnetii	......	..DGS.L.NA.E.	I...CM
M. tuberculosis	....E.	...SNT.GLQ.EL	V
Hu mitochondrial P1	R..VML	.KDG.TLNDE.EI	T.A.VV

FIG. 5. Comparison of the primary amino acid sequences of procaryotic and eucaryotic hsp60 and identification of MAb epitopes. Epitopes were identified by reacting MAbs in a pin ELISA with peptides corresponding to the primary amino acid sequence of C. trachomatis serovar A hsp60. The primary amino acid sequences of other procaryotic and eucaryotic hsp60 are given for comparison (9, 17, 19, 27, 29, 34, 36, 37). Hu, human. Dots indicate amino acid identity.

junctivae of animals that have recovered from a primary ocular chlamydial infection results in a delayed-type hypersensitivity response (27, 28). This response is characterized by a cellular infiltrate of the submucosal conjunctival epithelium consisting primarily of mononuclear lymphocytes and macrophages and resembles the histological profile of conjunctivae from humans with chronic trachoma (1, 5, 31).

Experimental findings in animal models of ocular chlamydial infection led to our proposing that an immune response to hsp60 contributes to the pathogenesis of chlamydial disease. It is not known whether hsp60 cause immunopathology in humans, but correlative evidence exists between antibody responsiveness to a ca. 60-kDa protein and the development of chronic inflammatory sequelae (4, 9, 18, 22, 38). Wager et al. observed a serologic correlation between immune responsiveness to hsp60 and the chronic inflammatory sequelae associated with human genital tract infections (38). The authors proposed that perhaps women who have chlamydial pelvic inflammatory disease and who develop chronic inflammatory sequelae (infertility, ectopic pregnancy) are those with anti-hsp60 antibodies. Indeed, a detailed and quantitative analysis of the serologic response to hsp60 of individuals with different chlamydial disease manifestations (acute and chronic) is needed to understand this relationship more thoroughly. With the purified hsp60 and hsp60 recombinant fragments described here, a quantitative ELISA can be performed to determine whether a correlation exists between severe disease and hsp60 titer or perhaps an antibody response to a particular hsp60 determinant. We have begun a detailed analysis of the human serologic response to chlamydial hsp60. Our preliminary data indicate that sera from patients with chlamydial urogenital tract infections and from patients with reactive arthritis of presumed chlamydial etiology have prominent IgG1 antibody responses to the amino and carboxyl termini and to an internal region of the hsp60 polypeptide (41). In a recent study of women with pelvic inflammatory disease and ectopic pregnancy, the anti-hsp60 antibody response was localized to the carboxyl half of hsp60 by immunoblot analysis (9). Our preliminary results corroborate the finding that the carboxyl terminus is immunogenic but indicate that other regions are also recognized during human chlamydial infections.

As an initial step in defining the antigenic structure of chlamydial hsp60, we mapped linear B cell epitopes recognized by anti-hsp60 MAbs. Most epitopes were localized to the amino and carboxyl termini. The epitopes for five of the MAbs (GP57-2, GP57-5, GP57-12, GP57-16, and GP57-23) contained >60% charged amino acids and were located in a region of the polypeptide predicted to be a B cell site. Chlamydial species- and genus-specific as well as cross-reactive epitopes were also located in this region. Although the epitope for the species-specific MAb GP57-2 could not be precisely mapped with octapeptides, weak but specific reactivity was observed with the 9-NEDARKKI-16 octapeptide. If the optimal epitope for this MAb is larger, then the amino acid difference at position 17 between C. psittaci (histidine) and C. trachomatis (glutamine) may be important in conferring the species specificity of this MAb. A comparison of the amino acid sequence of the MAb epitopes with aligned amino acid sequences of other procaryotic and eucaryotic hsp60 is consistent with the observed reactivities of the other MAbs (chlamydia specific and broadly cross-reactive) (Fig. 5). The chlamydia-specific MAbs tend to react with large epitopes (≥11 residues) located in regions of sequence heterogeneity, whereas the epitopes for the more broadly cross-reactive MAbs (A57-E4, A57-B9, and GP57-19) are smaller (6 residues) and are located in regions of sequence identity. However, the ubiquity of hsp60 and the sequence identity among members of the hsp60 family preclude the
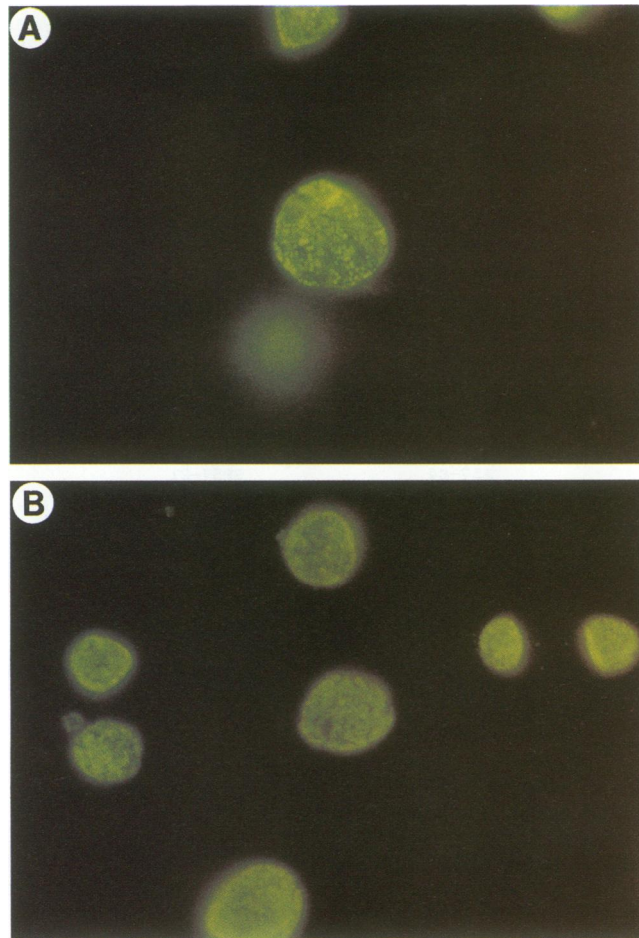
FIG. 6. Immunofluorescence of chlamydial inclusions with anti-hsp60 MAbs. HeLa cell monolayers were infected with *C. trachomatis* serovar L2 and fixed with methanol 24 h after infection. Fixed monolayers were reacted with either A57-B9 (anti-hsp60) (A) or DIII-A3 (anti-MOMP) (B), washed, incubated with fluorescein-conjugated goat anti-mouse immunoglobulin, and visualized by fluorescence microscopy. The exposure time for each photomicrograph was identical.

complete analysis of the genus- and species-specific reactivities claimed.

Repeated mucosal infections or the development of a persistent chlamydial infection are likely sources of antigen for the chronic inflammatory response associated with trachoma, salpingitis, and arthritis. Data from studies evaluating diagnostic procedures for trachoma provide indirect evidence that persistent infections occur in vivo. Chlamydial antigen and DNA were detected in conjunctival specimens of many individuals with active trachoma, even though chlamydiae could not be isolated by culturing (33). Those results did not appear to be due to the insensitivity of the culture methods and suggest that chlamydiae might be present in a noncultivatable, persistent state. The in vitro growth of chlamydiae in the presence of penicillin G or gamma interferon results in a persistent chlamydial infection from which infectious chlamydiae are not culturable (6, 12, 23, 25, 35). We used the anti-hsp60 MAbs in an indirect immunofluorescence assay to detect chlamydiae in persistently infected cells. Normal chlamydial inclusions stained equally well with anti-hsp60 and anti-MOMP MAbs (Fig. 6). Conversely, aberrant chlamydial inclusions, such as those induced by in vitro growth of chlamydiae in the presence of

penicillin G or gamma interferon, were easily detectable with anti-hsp60 MAbs but were nearly undetectable with anti-MOMP or antilipopolysaccharide MAbs (3). We do not know whether gamma interferon induces similar persistent infections in vivo, but gamma interferon is present locally in secretions of patients with mucosal chlamydial infections (2). These results suggest that if similar persistent infections occur in vivo, then anti-hsp60 MAbs may be useful for the identification of chlamydiae in tissues believed to be noninfected on the basis of negative isolation and nonreactivity with anti-MOMP MAbs.

Delineation of the epitopes recognized by both antibodies and T cells will be important in understanding the immunopathological conditions caused by infection with *C. trachomatis*. The MAbs described here will be extremely useful reagents for such studies. Their use as probes for the detection of hsp60 in tissues from patients with different chlamydial disease manifestations and as ligands for affinity purification of hsp60 for use in in vitro T cell and antibody assays will help to define more precisely the role of these antigens in the immunopathology of chlamydial disease.
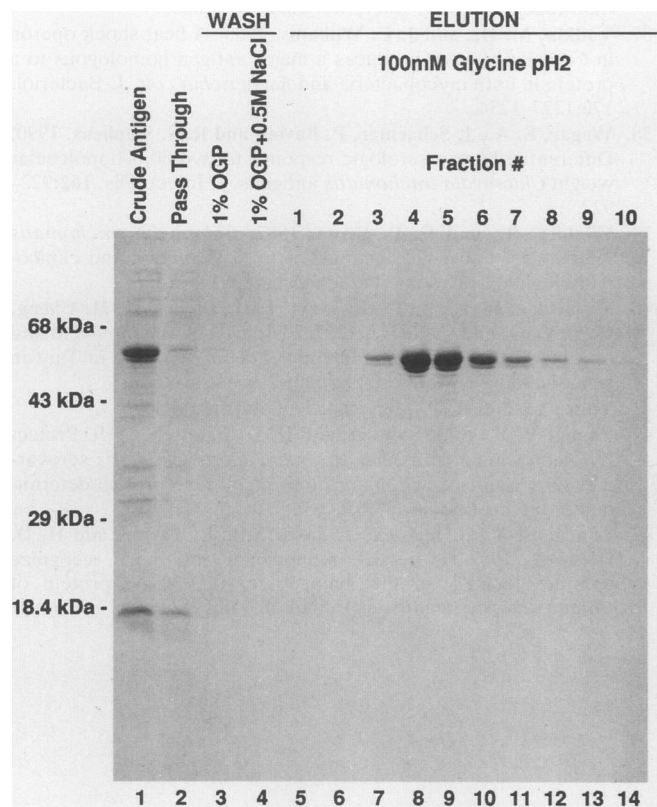
FIG. 7. Immunoaffinity purification of recombinant chlamydial hsp60 (see Materials and Methods for details). Shown is a Coomassie blue-stained SDS-polyacrylamide gel of a typical purification experiment. Lanes: 1, crude antigen extract added to the affinity column; 2, crude antigen preparation after passage through the affinity column; 3, column eluate after a wash with 1% OGP; 4, column eluate after a wash with 1% OGP containing 0.5 M NaCl; 5 through 14, 1-ml fractions obtained following elution with 100 mM glycine-HCl (pH 2).

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