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Human Antibody Responses to a Chlamydia-Secreted Protease Factor

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We have previously identified a chlamydia-secreted protein (designated chlamydial proteasome/protease-like activity factor, or CPAF) in the cytosol of chlamydia-infected cells. Although CPAF is known to degrade host transcription factors required for major histocompatibility complex antigen expression in cultured cells, it is not clear whether CPAF is produced and maintains similar functions in humans infected with chlamydial organisms. We now report that CPAF does not preexist in chlamydial organisms and that CPAF synthesis requires live organism replication in cultured cells. Mice inoculated with live, but not mice inoculated with dead, chlamydial organisms produced a strong antibody response to CPAF, correlating CPAF-specific antibody production with CPAF synthesis in animals. Sera from women diagnosed with *Chlamydia trachomatis* cervicitis displayed higher levels of antibodies to CPAF than to either chlamydial major outer membrane protein or heat shock protein 60, suggesting that CPAF is both produced and immunogenic during human chlamydial infection.

Chlamydia trachomatis urogenital tract infection, if left untreated, can lead to severe complications, including pelvic inflammatory diseases, ectopic pregnancy, and infertility (25, 30, 31). It has been hypothesized that chlamydia-induced diseases are largely due to host inflammatory responses provoked by chlamydial interactions with host cells (27, 31). The ability of chlamydiae to survive in the infected cells for long periods of time and to transmit between cells is thought to be a major contributor to pathogenic inflammation (22, 31)

A typical chlamydial infection starts with endocytosis of an infectious elementary body (EB) into an epithelium cell of mammalian hosts. Within 2 h, the EB-containing vacuole arrives in the peri-Golgi region and acquires Golgi apparatus-derived lipids (15, 29), and by 8 h, the EB differentiates into a noninfectious but metabolically active reticulate body (RB) that can undergo replication. By 24 h, the replicating RBs start to differentiate back into EBs. The mature EBs are finally released extracellularly to infect the adjacent cells. The entire replication cycle occurs within an enlarged cytoplasmic endosome (called inclusion) modified from the initial vacuole and is completed within 2 to 3 days in cell culture systems (14). However, chlamydial growth in humans following a natural infection is thought to take much longer (22). Given the fact that RBs are noninfectious and easy to break, it is to the benefit of chlamydial organisms to maintain the integrity of the infected cells during chlamydial replication. To secure a safe environment for intracellular replication and survival, chlamydiae have evolved multiple strategies for both preventing phagolysosomal fusion (10, 29) and protecting infected cells from the host defense system (7, 11–13, 38–40). For example, chlamydia-infected cells display reduced levels of major histocompatibility complex (MHC) antigens on the surface (39, 40), which may allow the infected cells to avoid immune recognition and attack. Members of our laboratory have recently identified a chlamydia-secreted protein, designated chlamydial proteasome/protease-like activity factor (CPAF), in the cytosol of the chlamydia-infected cells (38). CPAF is synthesized as a proenzyme and processed to form a functional intramolecular dimer (8, 9, 38). Most importantly, CPAF is both necessary and sufficient for degrading the host transcriptional factors RFX5 and USF-1, required for MHC gene activation (38), which may provide a molecular explanation for chlamydia-induced inhibition of MHC antigens. All of these studies, however, have been carried out in cultured cells. It is not known whether CPAF is produced and maintains similar functions in humans following chlamydial infection. The focus of the present study was to address whether CPAF is produced in vivo.

MATERIALS AND METHODS

Chlamydial infection. *C. trachomatis* serovar D (kindly provided by Cho-Chou Kuo at the University of Washington, Seattle) was used to infect HeLa cells (American Type Culture Collection, Manassas, Va.) as described elsewhere (44). The infection was permitted for various periods of time, as indicated in discussions of individual experiments, at a multiplicity of infection of 1 or as indicated in discussions of individual experiments. At the end of infection, the culture samples were either fixed for immunofluorescence staining or lysed to generate whole-cell lysates for other assays as described below. For animal infection, serovar D organisms, either live or after UV inactivation, were inoculated intranasally into mice at a dose equivalent to one million inclusion forming units/mouse as described elsewhere (17, 20). Both female BALB/c and C57BL mice, 5 to 7 weeks of age, were inoculated (The Jackson Laboratory, Bar Harbor, Maine). The mice were inoculated four times biweekly, and 10 to 14 days after the final inoculation, mice were sacrificed for collecting blood. The mouse sera were used for measuring chlamydial protein-specific antibodies. The mouse use protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

Cloning chlamydial genes and expressing chlamydial fusion proteins. The open reading frames coding for major outer membrane protein (MOMP), heat shock protein 60 (HSP60), and CPAF from the *C. trachomatis* serovar D genome (http://violet.berkeley.edu:4231/index.html; http://www.stdgen.lanl.gov/) were cloned into pGEX vectors (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) and expressed as fusion proteins with glutathione *S*-transferase (GST)

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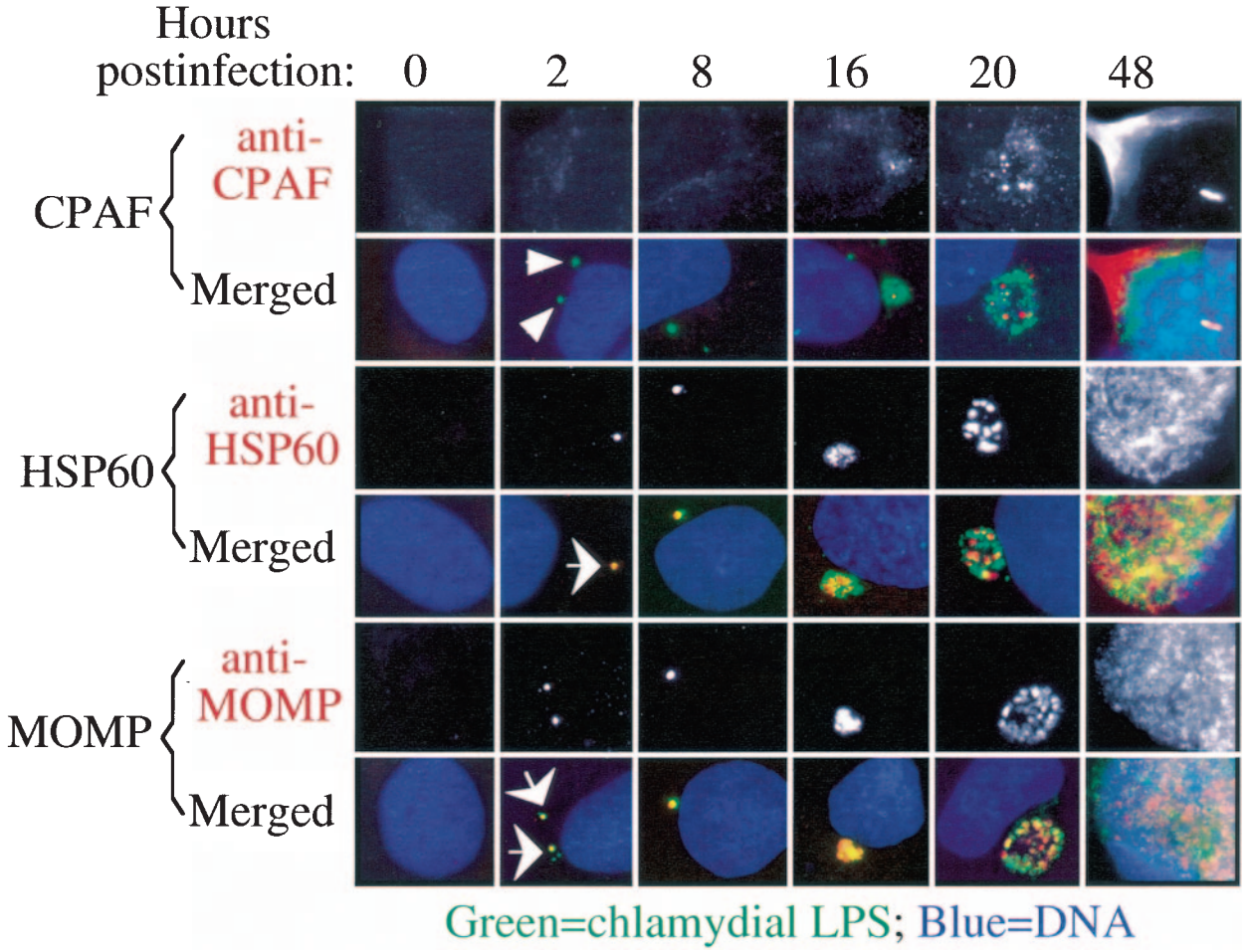

FIG. 1. Production of CPAF in chlamydia-infected cells. HeLa cells with or without *C. trachomatis* serovar D organism infection at a multiplicity of infection of 1 (~50% infection rate) were processed at various time points (in hours) after infection (as indicated on top of the figure) for triple immunofluorescence staining. DNA was labeled with Hoechst dye (blue), and chlamydial organisms were labeled with an anti-LPS MAb (green). The chlamydial antigens CPAF, HSP60, and MOMP were labeled with corresponding antibodies and are displayed both in single-color (gray) and triple-color (red) images. CPAF was not detectable until 16 h after infection. Arrowheads indicate chlamydial organisms labeled with the anti-LPS antibody alone without colocalization with CPAF while arrows indicate colocalization (yellow) of LPS and HSP60 or MOMP to the same chlamydial organisms. CPAF was secreted into the host cell cytosol at the late stages of infection while both HSP60 and MOMP remained inside chlamydial inclusions throughout the course of infection. Note that CPAF was not detected at 2 and 8 h, and CPAF was not associated with chlamydial organisms even when CPAF became detectable 16 h after infection.

fused to the N terminus of the chlamydial proteins. Expression of the fusion proteins designated GST-CPAF, GST-MOMP, and GST-HSP60 was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; Invitrogen, Carlsbad, Calif.), and the fusion proteins were extracted by lysing the bacteria via sonication in a Triton X-100 lysis buffer (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 75 U of aprotinin/ml, 20 μM leupeptin, and 1.6 μM pepstatin). After a high-speed centrifugation to remove debris, the fusion protein-containing supernatants were either directly used in various assays or further purified with glutathione-conjugated agarose beads (Pharmacia). The bead-bound fusion proteins were also used to isolate chlamydial antigen-specific antibodies from human sera (see below).

Human sera and purification of chlamydial antigen-specific human antibodies. Human sera were collected from women seen in the Project SAFE research clinic in San Antonio, Texas, who were diagnosed with *C. trachomatis* cervical infections. Women enrolled in this 5-year follow-up study were screened annually for sexually transmitted infections, including chlamydial infection. The diagnosis was based on the detection of *C. trachomatis*-specific nucleic acids in endocervical secretions by a PCR-based ligase chain reaction method without distinguishing the serotypes of the organisms (LCX; Abbot Laboratories, North Chicago, Ill.). The sera were collected at the time of clinic visits and stored in aliquots at −20°C. The 10 human sera used in the present study were randomly selected from the freezer without referring to the clinical manifestations of these patients. For measuring the antibody titers, the 10 sera, either individually or after being pooled together at an equal ratio, were serially diluted and applied to the corresponding assays as described below. For purifying or isolating chlamydial antigen-specific human antibodies, the pooled human sera were allowed to react with the chlamydial fusion proteins GST-CPAF, GST-HSP60, and GST-MOMP immobilized on glutathione-agarose beads. The bead-bound human antibodies were eluted with a high-salt solution (500 mM NaCl). After diluting the salt in the eluates with water, the eluted human antibodies were used to stain chlamydia-infected HeLa cells as described below. It is worth noting that the high-salt elution condition used in this experiment eluted only human antibodies from the glutathione beads without affecting the binding of GST fusion proteins to glutathione conjugated to agarose beads. Free glutathione is usually required for competing off the GST fusion proteins, and in the absence of the free reduced glutathione, a high-salt condition (used in this experiment) alone is not sufficient for disrupting the interactions between GST and glutathione.

ELISA. Either mouse or human sera were titrated against chlamydial GST fusion proteins by enzyme-linked immunosorbent assays (ELISA) as described elsewhere (42, 43), except that the GST fusion proteins were immobilized on the 96-well ELISA microplates (Pierce, Rockford, Ill.) via the interactions between the GST and glutathione precoated on the microplates. Briefly, bacterial lysates

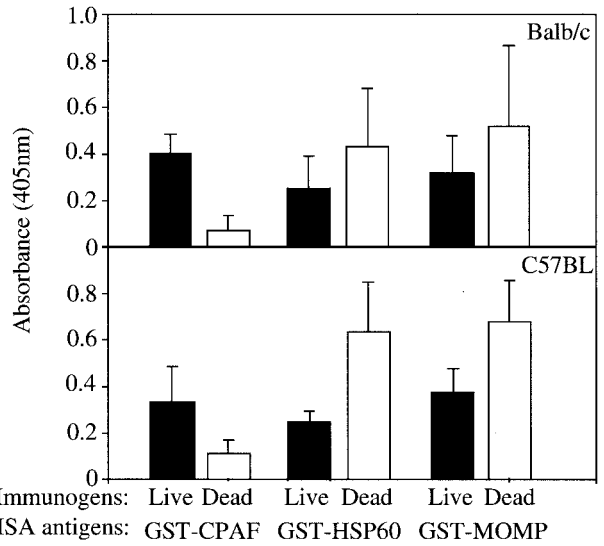

FIG. 2. Production of CPAF antibodies in mice inoculated with live, but not mice inoculated with dead, chlamydial organisms. Sera from groups of BALB/c (top panel) and C57BL (bottom) mice inoculated with either live (black bars) or dead (white bars) chlamydial organisms were analyzed for levels of antibodies to chlamydial proteins in an ELISA with the corresponding GST-chlamydia fusion proteins as antigens, as indicated at the bottom of the figure. The results were expressed as mean OD values ± standard deviations. All mouse sera were diluted 1:100. Four to five mice were included in each group. There is a statistically significant difference in levels of antibodies to GST-CPAF between mice inoculated with live and dead organisms in both BALB/c ($P < 0.01$) and C57BL ($P < 0.05$) mice. Statistical analysis was done with a two-tailed Student t test (http://faculty.vassar.edu/lowry/tu.html). The lysates made from bacteria expressing GST alone were used as negative antigen controls. None of the serum samples showed any significant binding to the control antigens (data not shown).

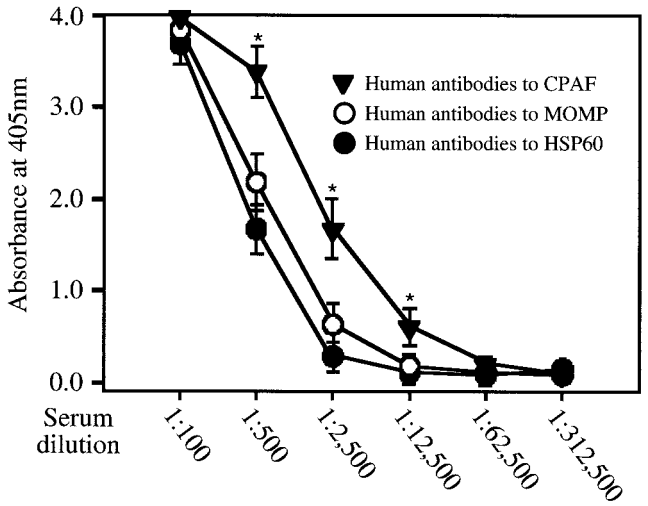

FIG. 3. Quantitation of chlamydial protein-specific antibodies in human sera by ELISA. Sera from 10 human patients diagnosed with *C. trachomatis* urogenital tract infection were examined in an ELISA for levels of antibodies to the fusion proteins GST-CPAF, GST-MOMP, and GST-HSP60. Individual sera were fivefold serially diluted, as indicated along the bottom of the figure, and the results were expressed as mean OD values ± standard deviation. Asterisks indicate statistically significant differences in OD values between CPAF and MOMP antibodies ($P < 0.01$ at 1:500 and 1:2,500; $P < 0.05$ at 1:12,500). Statistical analysis was done as described in the legend to Fig. 2. The lysates made from bacteria expressing GST alone were used as negative antigen controls. None of the serum samples showed any significant binding to the control antigens (data not shown).

containing the GST fusion proteins were directly added to the glutathione plates. After being washed to remove excess fusion proteins and being blocked with 2.5% nonfat milk (in phosphate-buffered solution), sera were diluted as indicated in discussions of the individual experiments and added to the antigen-immobilized microplates. The serum antibody binding was detected with an HRP (horseradish peroxidase)-conjugated goat anti-mouse or -human immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) in combination with the soluble substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulforic acid) diammonium salt (ABTS; Sigma, St. Louis, Mo.) and quantitated by reading the absorbance (optical density [OD]) at 405 nm with a microplate reader (Molecular Devices Corporation, Sunnyvale, Calif.).

Immunofluorescence staining assays. The *C. trachomatis* serovar D-infected HeLa monolayers grown on coverslips were fixed and permeabilized for antibody staining as previously described (13, 41). For monitoring chlamydial protein expression during infection in cultured cells, a triple staining procedure was used. DNA was labeled with Hoechst dye (blue; Sigma), and chlamydial organisms were labeled with an anti-LPS (lipopolysaccharide) monoclonal antibody (MAb) (clone MB5H9, murine IgG3 [mIgG3]; unpublished data) that is visualized with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG3 (green; Caltag Laboratories, Burlingame, Calif.). The chlamydial protein antigens were labeled with a MAb to CPAF (clone 100a, mIgG1) (38) or HSP60 (clone BC7.1, mIgG1; unpublished data) or with a rabbit antiserum to MOMP (unpublished data), followed by visualization with the corresponding secondary antibodies conjugated with Cy3 (red), including a goat anti-mouse IgG1 for both CPAF and HSP60 and a goat anti-rabbit IgG for MOMP (both conjugate antibodies from Caltag). The images were acquired under an AX-70 fluorescence microscope (Olympus, Seattle, Wash.) with SimplePCI software for the single color (gray) and merged for tricolor images. For assessing the binding of the fusion protein-purified human antibodies (see above) to endogenous chlamydial antigens in the infected cells, we used a double immunofluorescence staining method. The DNA was stained blue with the Hoechst dye as described above. The purified human antibody binding to corresponding endogenous chlamydial antigens was visualized with a Cy2-conjugated goat anti-human IgG (green; Jackson). As staining controls, chlamydial antigen-specific mouse antibodies (MAb 100a for CPAF, MAb BC7.1 for HSP60, and a mouse antiserum for MOMP) were added to parallel culture samples. The mouse antibody binding was visualized with a Cy2-conjugated goat anti-mouse IgG (green; Jackson).

Immunoprecipitation and Western blot assays. The immunoprecipitation and Western blot assays were carried out as described elsewhere (33, 37). Briefly, human sera were conjugated to protein G or A agarose beads (Pharmacia), and the antibody-bead complexes were mixed with either bacterial lysates that contain the desired chlamydial fusion proteins or lysates made from chlamydia-infected HeLa cells. After being washed, the beads were pelleted and the precipitates were resolved on a sodium dodecyl sulfate (SDS)-polyacrylamide gel for Western blot analysis. As antigen controls, the lysates were directly loaded onto parallel gel wells. After electrophoresis, the proteins were transferred to nitrocellulose membranes and the blots were detected with antibodies specific to known chlamydial proteins (MAb 100a to CPAF, MAb BC7.1 to HSP60, and a mouse antiserum to MOMP). Primary antibody binding was detected with an HRP-conjugated goat anti-mouse IgG and visualized with an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). The Western blot assay was also used to titrate the pooled human sera for their reactivities with chlamydial fusion proteins GST-CPAF, GST-MOMP, and GST-HSP60. Each of these purified fusion proteins was loaded with equal amounts of SDS-polyacrylamide gel in multiple sets. One set was stained with brilliant blue R250 (Sigma) for visualizing the total amount of protein in each lane, and the rest of the sets were transferred onto a nitrocellulose membrane for assessing human antibody binding to the chlamydial fusion proteins, which was visualized by using an HRP-conjugated goat anti-human IgG and ECL as described above.

RESULTS

CPAF is synthesized and secreted during chlamydial organism replication in cultured cells. Members of our laboratory previously showed that CPAF was not detected in purified

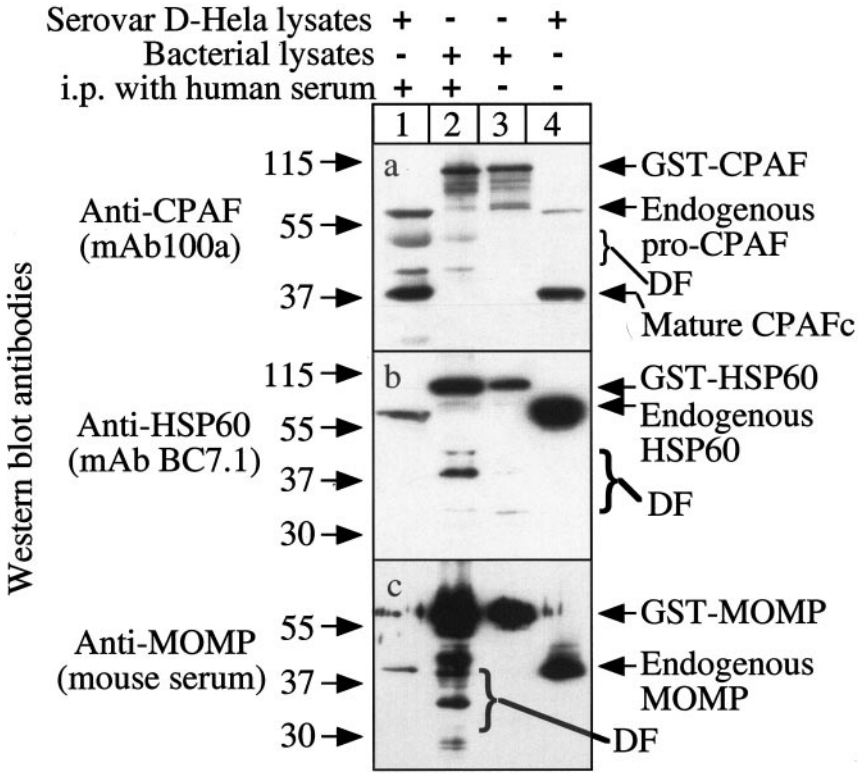

FIG. 4. Human antibody recognition of chlamydial GST fusion and endogenous antigens. Antibodies in the pooled human sera were immobilized onto protein G- and protein A-agarose beads, and the bead complexes were used to react with chlamydial antigens in either *C. trachomatis* serovar D-infected HeLa cell lysates (lane 1) or bacterial lysates (lane 2) containing GST-CPAF (a), GST-HSP60 (b), or GST-MOMP (c). The bead-bound antigens were loaded onto an SDS-polyacrylamide gel for Western blot detection with specific antibodies to CPAF (MAb 100a) (a), HSP60 (MAb BC7.1) (b), or MOMP (mouse antiserum) (c). As antigen loading controls, the bacterial (lane 3) and chlamydia-infected HeLa (lane 4) lysates (without prior precipitation) were directly loaded onto the parallel gel wells. Note that the human sera efficiently precipitated both GST fusion and endogenous chlamydial proteins. DF, degradation fragments; i.p., immunoprecipitation; +, present; −, absent.

chlamydial organisms but in chlamydia-infected whole-cell lysates (38). In the present study, we monitored CPAF synthesis and secretion during chlamydial infection in cultured cells by an immunofluorescence assay (Fig. 1). HeLa cells infected with *C. trachomatis* serovar D organisms for various periods of time were triply labeled with a DNA dye (blue) and an antibody against chlamydial LPS (green) in combination with antibodies to one of the following molecules: CPAF, HSP60, or MOMP (red). We found that CPAF protein was not detectable until 16 h after infection, although chlamydial organisms were clearly detected with the LPS antibody at both 2 and 8 h. By 20 h after infection, obvious CPAF-containing vesicles were detected in the infected cells and most of the CPAF signal was in the host cell cytosol by 48 h. This observation is consistent with a previous finding that the CPAF gene is activated late in the infection cycle (3) and supports the conclusions that CPAF does not exist in mature organisms (38) but is synthesized and secreted only during chlamydial organism replication (16). As controls, the chlamydial MOMP and HSP60 were also monitored throughout the infection course. Both MOMP and HSP60 were detected throughout the infection, and both proteins were associated with the chlamydial organisms and remained inside chlamydial inclusions, which is consistent with the knowledge that both HSP60 and MOMP are structural elements of chlamydial organisms.

Production of CPAF-specific antibodies in mice is dependent on CPAF synthesis by live chlamydial organisms. Both BALB/c and C57BL mice were inoculated with either live or inactivated chlamydial organisms, and serum antibodies to CPAF, HSP60, and MOMP were measured (Fig. 2). We found that mice infected with live chlamydial organisms produced obvious antibody responses to all three antigens, CPAF, HSP60, and MOMP. However, mice immunized with UV-inactivated chlamydial organisms failed to produce any significant levels of antibodies to CPAF, although the titers of antibodies to both HSP60 and MOMP were very high in these mice. Similar results were produced in both BALB/c (top panel) and C57BL (bottom panel) mice. As negative controls, sera from three BALB/c mice that were sham inoculated were also measured against these fusion proteins and no significant reactivity was detected between the control mouse sera and any of the fusion proteins (data not shown).

CPAF-specific antibodies are predominantly produced in humans infected with *C. trachomatis*. Having established that CPAF antibody production is dependent on CPAF synthesis in animals, we next detected the presence of CPAF-specific an-

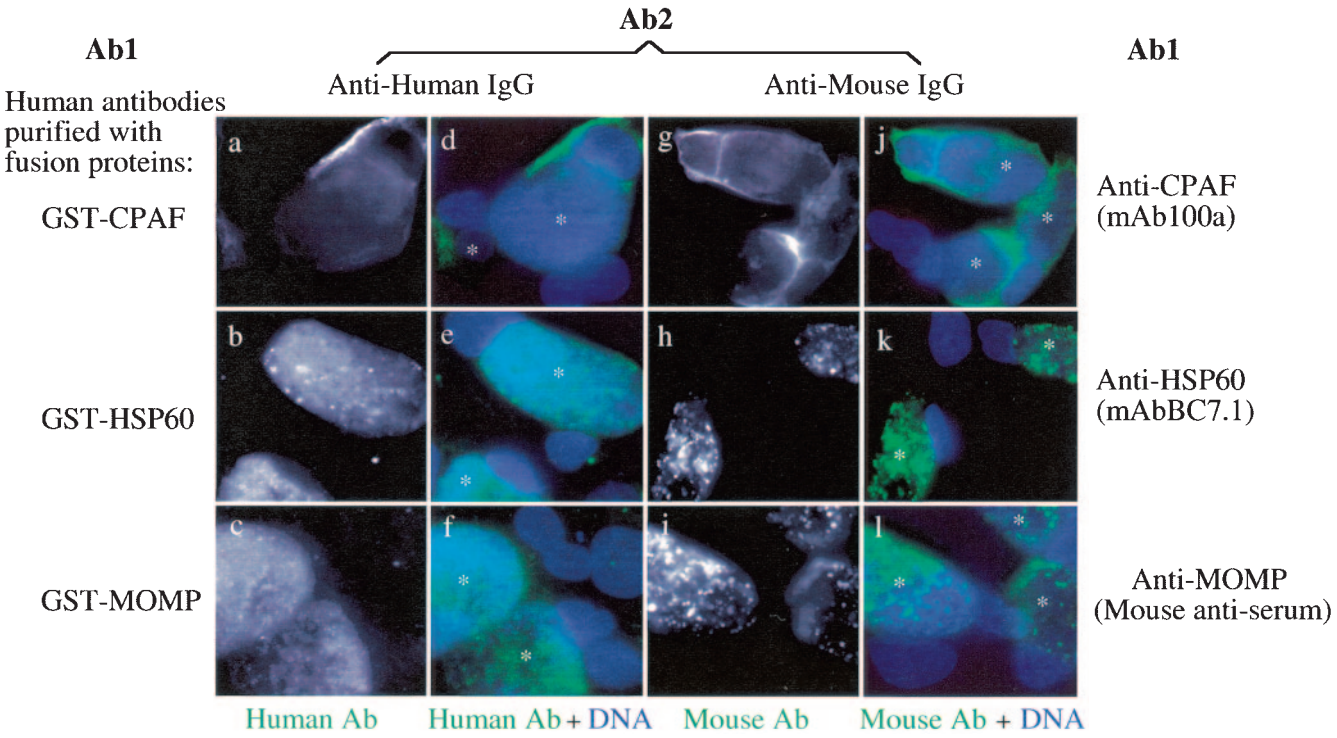

FIG. 5. Detection of endogenous chlamydial proteins in chlamydia-infected cells by human antibodies purified with chlamydial GST fusion proteins. The chlamydial GST fusion proteins (including GST-CPAF, GST-HSP60, and GST-MOMP, as indicated on the left of the figure) were immobilized onto glutathione-agarose beads, and each fusion protein bead complex was used to purify the corresponding antibodies from the pooled human sera. The fusion protein-purified human antibodies were used to stain HeLa cells infected with *C. trachomatis* serovar D organisms for 72 h for CPAF (a and d), HSP60 (b and e), and MOMP (c and f). Parallel cultures were stained with specific antibodies to CPAF (clone 100a) (g and j), HSP60 (BC7.1) (h and k), or MOMP (mouse antiserum) (i and l) as staining controls. The first antibody binding was revealed by either fluorescein isothiocyanate-conjugated goat anti-human IgGs (a to f) or mouse IgGs (g to l) and was displayed as either single-color (gray) (a to c and g to i) or dual-color (green) (d to f and j to l) images. The DNA was costained with the Hoechst dye (blue). Note that GST-CPAF-purified human antibodies (Ab) stained cytosolic signals similarly to the CPAF-specific MAb 100a.

tibodies in human sera as a way to indirectly evaluate CPAF production in humans infected with chlamydial organisms. Sera from 10 women with *C. trachomatis* cervicitis were analyzed for antibodies to CPAF, MOMP, and HSP60 by an ELISA which used the corresponding GST fusion proteins as antigens (Fig. 3). We found that all patients produced an overwhelming antibody response to CPAF. The level of antibodies to CPAF was significantly higher than those to MOMP or HSP60. Sera from eight individuals negative for chlamydial nucleic acid detected by the PCR-based ligase chain reaction and antibody measured by an immunofluorescence assay with the infected cells as antigens were used as negative controls. None of the control sera showed any significant binding to any of the chlamydial fusion proteins (data not shown). The finding that CPAF was more immunodominant than MOMP and HSP60 in chlamydia-infected individuals was surprising to us, since both MOMP and HSP60 have traditionally been considered the most immunodominant antigens during chlamydial immunization and infection of mice (2, 18, 26, 32, 35, 36, 43) and humans (4, 5, 21, 24). To further verify that the antibodies measured in the ELISA were specific, we used the human sera to precipitate either GST-chlamydia fusion proteins from *E. coli* bacterial lysates or endogenous chlamydial antigens from the infected HeLa cell lysates and detected the precipitates with mouse antibodies known to specifically recognize CPAF, MOMP, or HSP60 on a Western blot (Fig. 4). We found that the human sera efficiently precipitated both the GST fusion proteins and endogenous chlamydial CPAF, MOMP, and HSP60 (panels a, b, and c, respectively), suggesting that the binding of human serum to the fusion proteins coated on the ELISA plates was specific and that the fusion proteins mimicked the conformation of the corresponding endogenous proteins required for human antibody binding. We indeed found that human antibodies purified with the GST fusion proteins recognized the corresponding endogenous chlamydial proteins in chlamydia-infected cells (Fig. 5). For example, the GST-CPAF-purified human antibodies mainly detected a cytosolic signal in the infected cells (panels a and d), a pattern similar to that detected by the known CPAF-specific MAb 100a (panels g and j). As predicted, the GST-HSP60- and GST-MOMP-purified human antibodies mainly detected chlamydial inclusions in the infected cells (panels b, e, c, and f). Finally, the human antibody recognition of the GST-chlamydia fusion proteins was confirmed on a Western blot (Fig. 6). We found that the pooled human sera recognized all three GST-chlamydia fusion proteins well but had the highest titer to CPAF, consistent with the ELISA results (Fig. 3). The above observations together demonstrate that humans produce a dominant antibody response to CPAF, suggesting that CPAF is synthesized during human chlamydial infection.

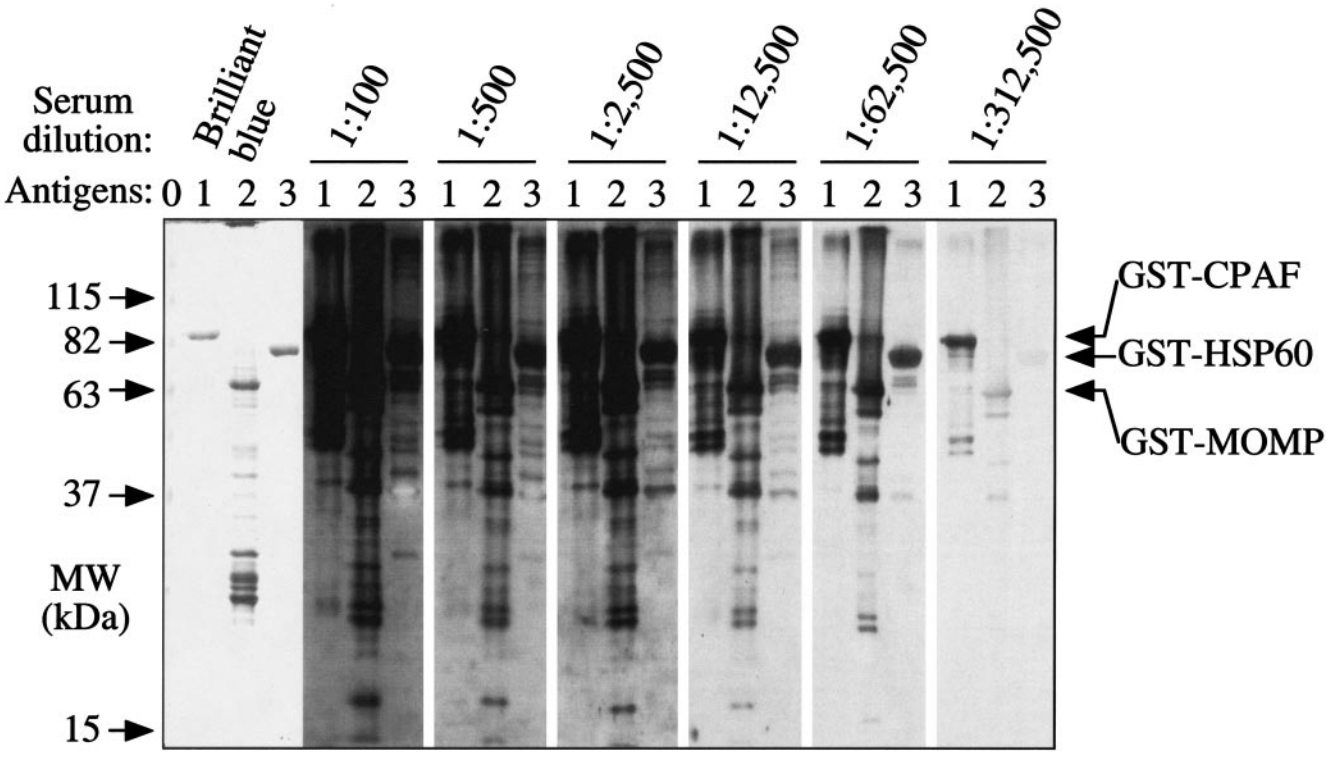

FIG. 6. Detection of chlamydial protein-specific antibodies in human sera by Western blotting. The chlamydial GST fusion proteins, including GST-CPAF (lane 1), GST-MOMP (lane 2), and GST-HSP60 (lane 3), were resolved on an SDS-polyacrylamide gel and transferred onto nitrocellulose membranes for measuring human antibody binding to the corresponding chlamydial proteins. The human sera were pooled from 10 patients and subjected to fivefold serial dilution (as indicated at the top of the figure). The primary antibody binding was visualized with an HRP-conjugated goat anti-human IgG and ECL. The GST-CPAF band was obviously detected after 1:312,500 dilution of the human sera while the GST-MOMP and GST-HSP60 were no longer or minimally detected at the same serum dilution. The leftmost panel is an image of the gel stained with brilliant blue dye for total amounts of protein loaded to each lane. Lane 0 was loaded with a prestained molecular mass marker.

DISCUSSION

Although CPAF has been extensively characterized in cell culture systems, the present work is the first to evaluate CPAF synthesis and CPAF antibody production in humans. To understand the importance of CPAF in chlamydial pathogenesis, it is essential to determine whether CPAF is produced during chlamydial infection in humans. However, we have not been able to directly detect CPAF protein from human samples despite our efforts in this regard. We are now providing the indirect, yet compelling, evidence that CPAF is produced in humans during chlamydial infection. First, CPAF does not preexist in chlamydial organisms, and CPAF production requires chlamydial replication. CPAF was not detected in purified organisms (38) and became detectable only in the infected cultures 16 h after infection (Fig. 1). The CPAF gene is known to be activated late in the infection cycle (3). These facts have formed the basis for us to use CPAF antibody production to indirectly evaluate CPAF synthesis in vivo. Second, we have demonstrated that the production of CPAF-specific antibody is dependent on CPAF synthesis after live organism infection in animals. Mice inoculated with live, but not those infected with dead, chlamydial organisms produced significantly high levels of antibodies to CPAF (Fig. 2). Third, humans infected with chlamydia produced a dominant antibody response to CPAF. The level of antibodies to CPAF in human sera is significantly higher than that to either MOMP or HSP60 (Fig. 3 and 6). It is important to point out that the high titers of CPAF antibodies detected in human sera may be contributed to by many factors, including the immunogenicity of the CPAF protein sequence, the amount of CPAF produced during chlamydial infection, and the constant stimulation of host immune systems by the continuously synthesized CPAF. Although we cannot completely exclude the possibility that low levels of preexisting CPAF proteins can be directly passed from the male into the female along with the chlamydial organisms, it is unlikely that the preexisting CPAF can survive the extensive protease activities in vaginal mucus and induce a robust CPAF antibody response such as that detected in the women in this study. The fact that human antibodies purified with GST-CPAF recognized the endogenous CPAF secreted by live chlamydial organisms into the cytosol of the infected cells (Fig. 5) further suggests that the human CPAF antibodies prefer the conformation of newly synthesized CPAF and are likely induced by CPAF synthesized during infection. Together, the above observations and analyses overwhelmingly support the conclusion that CPAF is synthesized during chlamydial infection in humans.

A surprising finding of the present study is that women infected urogenitally with *C. trachomatis* produced a significantly higher level of antibodies to CPAF than to MOMP or

HSP60. Both MOMP and HSP60 have been traditionally considered the immunodominant antigens during chlamydial infection (2, 4, 5, 18, 21, 24, 26, 28, 32, 35, 36, 43). The seemingly conflicting observations are not likely due to variations in detection methods. This is because both conformation-dependent (including ELISA and precipitation assays) and -independent (Western blotting) approaches used in the present study consistently showed a dominant antibody response to CPAF (Fig. 3 and 6). The so-called immunodominance is relative to the antigens being compared in a given study, and CPAF has never been included in the antigenicity and immunogenicity studies with either MOMP or HSP60. Many previous studies have been conducted for analyzing chlamydial protein antigenicity and immunogenicity, and many different experimental methods were used in these studies, including Western blotting (6, 28), ELISA (1), and the microimmunofluorescence assays pioneered by Wang et al. (34). However, in all of these assays, either purified chlamydial organisms, selected chlamydial proteins or protein fragments, or chlamydial protein-derived peptides were used as antigens, and none of these antigens contained the chlamydia-secreted CPAF. The present study is the first to directly compare CPAF with MOMP and HSP60 for antibody responses in humans. We have thus identified CPAF as a novel immunodominant antigen in humans. Clearly, it is necessary to analyze more patient samples with more chlamydial antigens to accurately assess the immunodominance of CPAF during chlamydial infection in humans. It is also worth noting that antibody responses to a given antigen are often serotype specific if the antigen displays sequence variations between serotypes. For example, the MOMP sequence varies between serotypes, and MOMP-specific antibodies are known to recognize MOMPs from homologous serovars with the highest titers. On the other hand, CPAF sequences are almost 100% identical among all human *C. trachomatis* serotypes sequenced so far, and CPAF-specific antibodies are expected to react with CPAF from all serotypes equally well. Both MOMP and CPAF from serovar D were used in the present study. Although serovar D is known to be one of the prevalent serotypes in the United States, we may still need to include MOMPs from many other urogenital serotypes in the antigenicity analysis. In this way, the relative levels of immunodominance of MOMP and CPAF can be fairly compared, since we often do not know what serotypes actually infect the women from whom sera are collected. In addition to the assessment of relative immunodominance, it will also be interesting to determine the biological and immunological significance of human antibody responses to CPAF, which should be possible since human antibody responses to MOMP were correlated with protective immunity while those to HSPs correlated with immunopathology (5, 19, 23). At this moment, it is not clear what roles CPAF antibodies may play in chlamydial infection, since CPAF is not part of the chlamydial organisms. We hypothesize that the CPAF-specific secretary IgA antibodies may be able to neutralize CPAF function intracellularly, which may allow the infected cells to restore the ability to present chlamydial peptides to T cells.

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