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Profiling of Human Antibody Responses to *Chlamydia trachomatis* Urogenital Tract Infection Using Microplates Arrayed with 156 Chlamydial Fusion Proteins

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The available chlamydial genome sequences have made it possible to comprehensively analyze host responses to all chlamydial proteins, which is essential for further understanding of chlamydial pathogenesis and development of effective chlamydial vaccines. Microplates arrayed with 156 *Chlamydia trachomatis* fusion proteins were used to evaluate antibody responses in women urogenitally infected with *C. trachomatis*. Based on both the antibody recognition frequency and titer, seven chlamydial antigens encoded by open reading frames (ORFs) CT089, CT147, CT226, CT681, CT694, CT795, and CT858, respectively, were identified as relatively immunodominant; six of these are encoded by hypothetical ORFs. Antibody binding to these chlamydial fusion proteins was blocked by *C. trachomatis*-infected but not by normal HeLa cell lysates or irrelevant bacterial lysates. These results have revealed novel immune-reactive chlamydial antigens, not only indicating that the hypothetical ORF-encoded proteins are expressed during chlamydial infection in humans but also providing the proof of principle that the fusion protein-based approach can be used to profile human immune responses to chlamydial infection at the whole-genome scale.

Infection of the urogenital tract with *Chlamydia trachomatis* is a leading cause of sexually transmitted bacterial diseases worldwide (23) and is also linked to cervical carcinoma (22, 37). Although *C. trachomatis* infection is sensitive to antibiotic treatment, many infected individuals do not seek treatment, due to the fact that acute chlamydial infection can be asymptomatic or cause only mild discomfort, and thus they become vulnerable to developing persistent infections (4, 11, 34, 56), leading to severe complications such as ectopic pregnancy and infertility (28). Therefore, further understanding of the mechanisms of chlamydial pathogenesis and development of effective preventive strategies are urgently needed. Although previous studies have correlated host immune responses to the major outer membrane protein (MOMP) and heat shock proteins (HSPs) with chlamydial protective immunity and pathogenic responses, respectively (1, 2, 7, 15, 31–33, 35, 37, 39, 40, 52, 58–61, 64), neither MOMP nor HSP immune responses can account for the overall protective immunity or pathogenic responses induced during chlamydial infection. This is because these antigenicity studies were either focused on a few preselected antigens or based on analysis of denatured proteins or peptides. A comprehensive analysis of all chlamydial antigens at the whole-genome scale in an unbiased assay system is required in order to fully determine the antigenic basis of host protective and pathogenic responses to chlamydial infection.

The available chlamydial genome sequences (42, 51) have made it possible to perform a comprehensive analysis of the antigenicities and immunogenicities of all chlamydial proteins. Our ultimate goal is to develop a protein array assay at a whole-genome scale that can be used to compare the antigenicities and immunogenicities of all chlamydial proteins. To provide the proof of principle, we evaluated the recognition of 156 chlamydial fusion proteins by antibodies from 15 patients with *C. trachomatis* urogenital infections in the current study. Although the number of proteins analyzed here is only a fraction of the number of proteins encoded by the entire chlamydial genome, seven immune-reactive antigens have already been revealed, five of which represent novel antigens discovered for the first time, demonstrating that the fusion protein approach is useful for profiling antibody responses to chlamydial infection at the whole-genome scale.

MATERIALS AND METHODS

Chlamydial infection. *C. trachomatis* serovar D (kindly provided by Cho-Chou Kuo, University of Washington, Seattle) was used to infect HeLa cells (ATCC, Manassas, VA) as described elsewhere (24). Infection was allowed to proceed for various periods of time as indicated for individual experiments at a multiplicity of infection of 1 or as indicated for individual experiments. At the end of infection, the culture samples were either fixed and permeabilized for immunofluorescence staining or lysed to produce whole-cell lysates for precipitation or Western blot assays.

Cloning of chlamydial genes and expression of chlamydial proteins. A total of 156 open reading frames (ORFs) were selected from the *C. trachomatis* serovar D genome sequence (http://www.stdgen.lanl.gov). These ORFs are distributed across the entire genome, with representatives in every genome sector. Although no particular programs were used to selectively include or exclude any particular gene classes, the 156 ORFs are mainly composed of hypothetical genes. The 156 ORFs from the serovar D genome plus MOMP genes from eight other *C. trachomatis* serovar genomes were cloned into a pGEX vector system (Amersham Biosciences Corp., Piscataway, NJ). This vector system allows the protein of interest to be expressed as a fusion protein with glutathione *S*-transferase (GST) fused to the N terminus of the chlamydial protein (12, 45, 63). Protein expression was induced with isopropyl-β-D-thiogalactoside (IPTG; Invitrogen,

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Carlsbad, CA). To ensure that each fusion protein is produced with adequate quantities of full-length fusion proteins, induction of fusion protein expression was individually optimized using the following variables: IPTG concentration (0.1 to 5 mM), starting number of bacteria (optical density [OD], 0.5 to 1.5), incubation temperature (10°C to 37°C), and time (0.5 h to overnight). After protein induction, bacteria were harvested via centrifugation. The bacterial pellets were resuspended in a Triton lysis buffer (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 75 IU/ml of aprotinin, 20 μM leupeptin, and 1.6 μM pepstatin in PBS [phosphate-buffered saline at pH 7.5]) and were lysed by short pulses of sonication on ice. After a high-speed centrifugation to remove debris, bacterial lysates were aliquoted and stored at −80°C. The quality of the expressed fusion proteins was assessed by purifying the fusion proteins from a portion of the lysates using glutathione-conjugated agarose beads (Amersham Biosciences Corp.). The fusion proteins were checked on sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with a Coomassie blue dye (Sigma). Bacterial lysate samples that showed a prominent band at the expected molecular weight position were used for the subsequent microplate array assays.

Arraying chlamydial proteins onto microplates precoated with glutathione. The bacterial lysates containing the fusion proteins were added to glutathione-coated 96-well microplates (Pierce, Rockford, IL) at a 1:10 dilution in PBS with a total volume of 200 μl/well. The plates were incubated overnight at 4°C to allow GST fusion proteins to bind to the glutathione immobilized on the plate. To minimize differences in the quantity of fusion proteins captured on the plates between lysate samples, an excessive amount of each fusion protein was used to saturate the glutathione-coated assay plates. We found that 20 μl bacterial lysate per well was sufficient for saturating the assay plate if the amount of full-length fusion protein precipitated from the 20-μl bacterial lysate was visible on an SDS gel after Coomassie blue staining (data not shown). After two washes with PBS–0.05% Tween (Sigma) and blocking with 2.5% milk in PBS (2.5 g of nonfat dry milk in 100 ml PBS) at room temperature for 1 h, the plates were ready for use.

Use of microplates arrayed with chlamydial fusion proteins to detect human antibodies. Human sera were collected from women seen in the Project SAFE research clinic in San Antonio, Tex., who had been 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 detection of *C. trachomatis*-specific nucleic acids in endocervical secretions using a ligase chain reaction method without distinguishing the serotypes of the organisms (Abbott LCX; Abbot Laboratories, Chicago, IL). The sera were collected at the time of clinic visits and stored in aliquots at −20°C. The human sera used in the current study were from the initial visit. An institutional review board exempt permit is in place for the current study. A total of eight sera from healthy female individuals without *C. trachomatis* infection were used as negative controls. To minimize the detection of cross-reactive antibodies (human sera may contain antibodies reactive with bacterial antigens that potentially contaminate the microplate wells during the fusion protein array), all serum samples were preabsorbed with bacterial lysates. The bacterial lysates were made in the same way as the fusion protein-containing lysates (see "Cloning of chlamydial genes and expression of chlamydial proteins" above) except that XL1-Blue bacteria transformed with the pGEX-6p-2 vector plasmid alone were used. Note that the bacterial lysates used for preabsorption contain free GST. After preabsorption, both the serum samples from patients and those from healthy individuals were titrated for their ability to recognize chlamydial antigens in an immunofluorescence assay. Although the patient sera displayed high antibody titers (>1:1,000) in recognizing chlamydial antigens, the healthy sera did not show any significant binding to the chlamydial antigens (<1:20). For the microplate array assay, the preabsorbed serum samples were diluted in PBS containing 10% fetal calf serum and applied to the microplates with the bound fusion proteins for 2 h at room temperature. After a wash, alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in combination with the substrate *p*-nitrophenylphosphate (Sigma) was used to visualize the primary antibody binding. The human antibody binding to chlamydial fusion proteins was quantitated by reading the absorbance (OD) at 405 nm with a microplate reader (Molecular Devices, Ramsey, MN). In some assays, the human antibody samples were also preabsorbed with lysates made from either HeLa cells alone or *C. trachomatis* serovar D-infected HeLa cells at 4°C overnight in addition to the bacterial lysate absorption.

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blotting were carried out as described previously (13, 14, 54, 62, 63). For immunoprecipitation, human sera were bound to protein G/A agarose beads (Amersham Biosciences Corp.) and the bead complexes were used to precipitate bacterial lysates containing the desired chlamydial fusion proteins or chlamydia-infected HeLa cell lysates containing endogenous chlamydial proteins. The precipitates were resolved in an SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The blots were detected with antibodies specific to individual chlamydial proteins (monoclonal antibody 100a to chlamydial protease-like activity factor [CPAF], a mouse antiserum to MOMP, and another mouse antiserum to CT089) as previously described (13, 45, 63). The primary antibody binding was detected by a goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories). For Western blotting, which was used to confirm the reactivity of human serum antibodies with chlamydial fusion proteins in the present study, the purified chlamydial fusion proteins were resolved in the SDS gel and transferred to nitrocellulose membranes. The preabsorbed human serum samples, after the appropriate dilution as indicated for individual experiments, were applied to the nitrocellulose membranes. Human antibody binding was detected with a goat anti-human IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories). A standard enhanced chemiluminescence (ECL) detection system was used to visualize antibody detection (54).

Immunofluorescence assay. *C. trachomatis*-infected HeLa monolayers grown on coverslips for various times as indicated for individual experiments were processed for antibody staining as previously described (24, 57). For monitoring human serum absorption efficiency, human antibody samples that had or had not been absorbed previously with uninfected or chlamydia-infected HeLa cell lysates as described above were added to the coverslips. The primary antibody binding was visualized with a Cy2-conjugated goat anti-human IgG. The Hoechst DNA dye (Sigma) was used to visualize both host nuclei and chlamydial inclusions. For localization of endogenous chlamydial antigens, antibodies against individual chlamydial proteins raised in mice with the corresponding chlamydial fusion proteins were used in combination with a rabbit anti-chlamydial heat shock protein as primary antibodies. The reactivities of the two primary antibodies were visualized with Cy3-conjugated goat anti-mouse and Cy2-conjugated goat anti-rabbit antibodies together with the Hoechst dye. Images were acquired under an Olympus (Seattle, WA) AX-70 fluorescence microscope using SimplePCI software (Olympus) as previously described (17, 45).

RESULTS

Development of a microplate assay using chlamydial fusion proteins. A total of 156 chlamydial proteins (Table 1) were selected to establish a microplate-based protein array assay. The 156 chlamydial proteins were expressed as GST fusion proteins. The quality of the chlamydial fusion proteins was monitored on an SDS-polyacrylamide gel. As an example, 26 representative fusion proteins induced under a single protein expression condition were examined on an SDS gel stained with Coomassie blue (Fig. 1A). In most cases, a dominant band migrating at the expected molecular weight was purified from the corresponding bacterial lysates by using glutathione-conjugated agarose beads, indicating that the GST fusion proteins are readily captured from the bacterial lysates by the immobilized glutathione. For the fusion proteins with obvious degradation and/or contaminated bands (GST-CT101, -CT119, -CT141, -CT449, and -CT618), we further optimized the expression conditions so that a dominant full-length band was produced in each of these samples.

To evaluate whether the GST-chlamydia fusion proteins can be recognized by human antibodies generated during natural chlamydial infection, we reacted the human antibodies with protein G/A-agarose beads and used the bead complexes to precipitate either the bacterial lysates containing the GST-chlamydia fusion proteins or chlamydia-infected HeLa cell lysates containing the endogenous chlamydial antigens (Fig. 1B). The human antibodies precipitated both the recombinant and endogenous chlamydial proteins CT089 (Fig. 1Ba), MOMP (Fig. 1Bb), and CPAF (Fig. 1Bc), suggesting that the fusion proteins can be used to detect human antichlamydial antibodies.

TABLE 1. *C. trachomatis* ORFs expressed as GST-chlamydia fusion proteins

ORF no.[a]	*C. trachomatis* gene
1	CT001, hypothetical
2	CT005, hypothetical
3	CT006, hypothetical
4	CT011, hypothetical
5	CT018, hypothetical
6	CT021, hypothetical
7	CT036, hypothetical
8	CT049, hypothetical
9	CT056, hypothetical
10	CT058, hypothetical
11	CT085, hypothetical
12	CT088, *sycE*
13	CT089, *lcrE*
14	CT101, hypothetical
15	CT110, *groEL1*
16	CT111, *groES*
17	CT112, *pepF*
18	CT113, *clpB*
19	CT115, *incD*
20	CT116, *incE*
21	CT117, *incF*
22	CT118, *incG*
23	CT119, *incA*
24	CT133, hypothetical
25	CT134, hypothetical
26	CT135, hypothetical
27	CT141, *secA*
28	CT147, hypothetical
29	CT149, hydrolase?
30	CT151, hypothetical
31	CT153, hypothetical
32	CT154, hypothetical
33	CT155, hypothetical
34	CT161, hypothetical
35	CT162, hypothetical
36	CT163, hypothetical
37	CT164, hypothetical
38	CT165, hypothetical
39	CT171, *trpA*
40	CT173, hypothetical
41	CT174, hypothetical
42	CT181, hypothetical
43	CT191, hypothetical
44	CT192, hypothetical
45	CT195, hypothetical
46	CT196, hypothetical
47	CT214, hypothetical
48	CT223, *inc*
49	CT224, hypothetical
50	CT225, hypothetical
51	CT226, hypothetical
52	CT227C, hypothetical
53	CT228, hypothetical
54	CT229, *inc*
55	CT232, *incB*
56	CT233, *incC*
57	CT260, hypothetical
58	CT266, hypothetical
59	CT277, hypothetical
60	CT286, *clpC*
61	CT288, hypothetical
62	CT296, hypothetical
63	CT300, hypothetical
64	CT309, hypothetical
65	CT321, *secE*
66	CT324, hypothetical
67	CT338, hypothetical
68	CT341, *dnaJ*
69	CT343, endopeptidase
70	CT344, *lon*
71	CT345, hypothetical
72	CT351, hypothetical
73	CT357, hypothetical
74	CT358, hypothetical
75	CT365N, hypothetical
76	CT372, hypothetical
77	CT375, D-aa dehydrogenase[b]
78	CT383, hypothetical
79	CT384, hypothetical
80	CT395, *grpE*
81	CT396, *dnaK*
82	CT406, hypothetical
83	CT407, *dksA*
84	CT421, hypothetical
85	CT422, metalloproteas
86	CT425, hypothetical
87	CT427, hypothetical
88	CT431, *clpP*
89	CT442, *crpA*
90	CT446, *euo*
91	CT449, hypothetical
92	CT457C, *yebC*
93	CT473, hypothetical
94	CT474, hypothetical
95	CT482, hypothetical
96	CT484, hypothetical
97	CT493C, *polA*
98	CT546, hypothetical
99	CT548, hypothetical
100	CT560, hypothetical
101	CT565, hypothetical
102	CT569, hypothetical
103	CT571N, *gspE*
104	CT572, *gspD*
105	CT573, hypothetical
106	CT574, *pepP*
107	CT576, *lcrH*
108	CT577, hypothetical
109	CT604, *groEL2*
110	CT606, hypothetical
111	CT611, hypothetical
112	CT618, hypothetical
113	CT627, *yceA*
114	CT638, hypothetical
115	CT647, hypothetical
116	CT648, hypothetical
117	CT654, hypothetical
118	CT657, hypothetical
119	CT659, hypothetical
120	CT668, hypothetical
121	CT670, hypothetical
122	CT671, hypothetical
123	CT676, hypothetical
124	CT681, *ompA*
125	CT694, hypothetical
126	CT700, hypothetical
127	CT701, *secA2*
128	CT705, *clpX*
129	CT706, *clpP2*
130	CT712, hypothetical
131	CT717, *ftil*
132	CT718, hypothetical
133	CT724, hypothetical
134	CT728, hypothetical
135	CT733, hypothetical
136	CT734, hypothetical
137	CT739C, *ftsK*
138	CT741, hypothetical
139	CT753, hypothetical
140	CT755, *groEL3*
141	CT716, hypothetical
142	CT764, hypothetical
143	CT768, hypothetical
144	CT779, hypothetical
145	CT789b, hypothetical
146	CT795, hypothetical
147	CT814, hypothetical
148	CT814.1, hypothetical
149	CT820, *fstY*
150	CT823C, *htrA*
151	CT825C, hypothetical
152	CT845, hypothetical
153	CT847, hypothetical
154	CT849, hypothetical
155	CT850, hypothetical
156	CT858, *cpaf*

[a] Renumbered from 1 to 156 for the convenience of the present study. The suffix N or C after the ORF designation indicates either the N terminus or the C terminus of the ORF expressed as a GST fusion protein. Both the ORFs and the protein designations are taken from the website http://www.stdgen.lanl.gov/.

[b] D-aa, D amino acid.

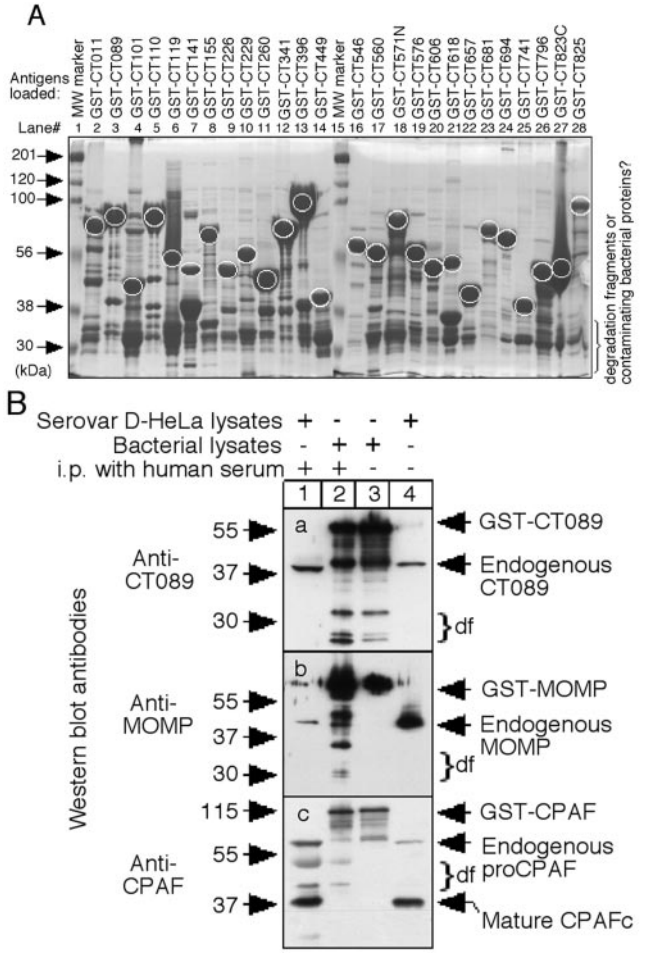

FIG. 1. Quality of GST-chlamydia fusion proteins. (A) GST fusion proteins were precipitated with glutathione-agarose beads from bacterial lysates, and the precipitates were resolved on an SDS gel. The gel was stained with Coomassie blue dye. This method was routinely used to check the quality of each fusion protein. Shown on the gel are 26 GST fusion proteins expressed under a single induction condition as examples. The expected full-length fusion proteins are circled in white. (B) The abilities of the GST-chlamydia fusion proteins to be recognized by human antibodies were further assessed by immunoprecipitation plus Western blotting. Serum antibodies from patients with *C. trachomatis* urogenital tract infections were used to precipitate either bacterial lysates containing GST fusion proteins (lane 2) or chlamydia-infected HeLa lysates containing endogenous chlamydial proteins (lane 1). The precipitates were detected on a Western blot with antibodies specific to either CT089 (a), MOMP (b), or CT858 (CPAF) (c). As antigen controls, both the bacterial (lane 3) and chlamydia-infected HeLa (lane 4) lysates were loaded directly onto the gel. Note that both GST-chlamydia fusion proteins and endogenous chlamydial proteins were effectively precipitated by the human serum antibodies.

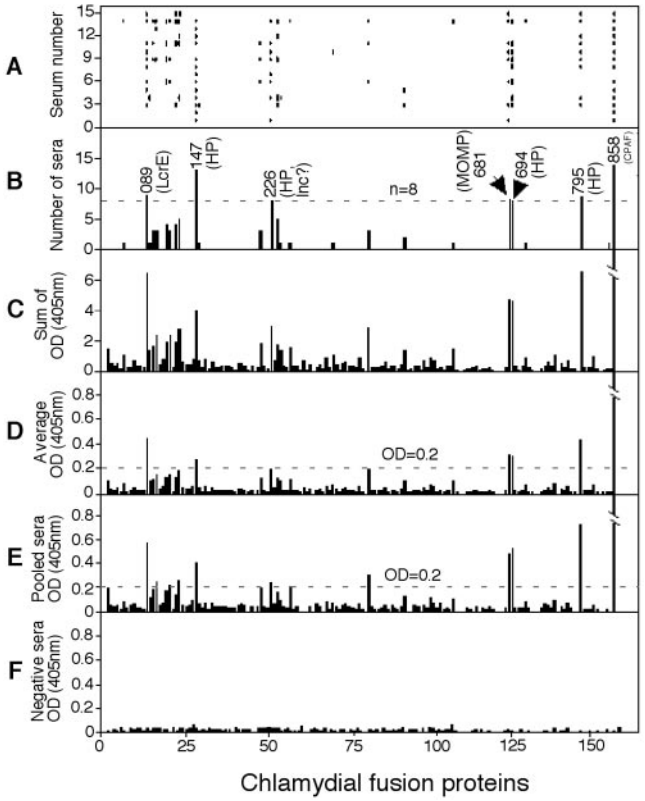

FIG. 2. Reactivities of human antibodies with 156 chlamydial fusion proteins. (A) Fifteen serum antibodies from patients (numbers on *y* axis) were individually diluted at 1:500 and reacted with 156 GST-chlamydia fusion proteins immobilized onto microplates (*x* axis) in an ELISA. Each positive reaction is marked with a vertical bar. (B) The number of sera that reacted with each fusion protein was plotted. Fusion proteins that reacted positively with eight or more serum samples are marked with their corresponding ORF names. (C) The cumulative ODs of the 15 sera to each fusion protein were added up. (D) Average ODs were calculated. (E) The 15 sera were pooled at an equal ratio, the pooled sera after 1:200 dilution were measured against the 156 fusion proteins, and the raw ODs were plotted. (F) Pooled sera from eight healthy individuals after 1:200 dilution were reacted with the 156 fusion proteins. Horizontal dashed lines in each panel indicate cutoff values for determination of immunodominant antigens.

Identification of immune-reactive antigens recognized by human antibodies. Microplates arrayed with 156 chlamydial fusion proteins were used to measure the reactivities of 15 sera from women urogenitally infected with *C. trachomatis* (Fig. 2). The binding of a given human serum to a given fusion protein with an OD four times above the background was determined as positive (Fig. 2A). Differences in the number of chlamydial fusion proteins recognized by different human serum samples were observed. For example, serum 14 recognized 18 of the 156 chlamydial fusion proteins, while serum 2 recognized only 2. Although each serum displayed a unique reactivity pattern in terms of the types of chlamydial fusion proteins, many of the 15 sera recognized the same fusion proteins. The number of human sera that positively recognized a given fusion protein is defined as the recognition frequency (Fig. 2B). Chlamydial proteins recognized at a higher frequency are considered to be more immunodominant during chlamydial infection. Based on the criteria used in previous antigenicity analyses (19, 20, 65), we considered the chlamydial fusion proteins that were recognized by 8 or more of the 15 human serum samples to be the dominant antigens. Seven out of the 156 chlamydial proteins meet this requirement: CT089 (an LcrE homologue, recognized by 9 human sera), CT147 (a hypothetical protein, recognized by 13 sera), CT226 (a hypothetical protein, recognized by 8 sera), CT681 (MOMP, recognized by 8 sera), CT694 (a hypothetical protein, recognized by 8 sera), CT795 (a hypo-

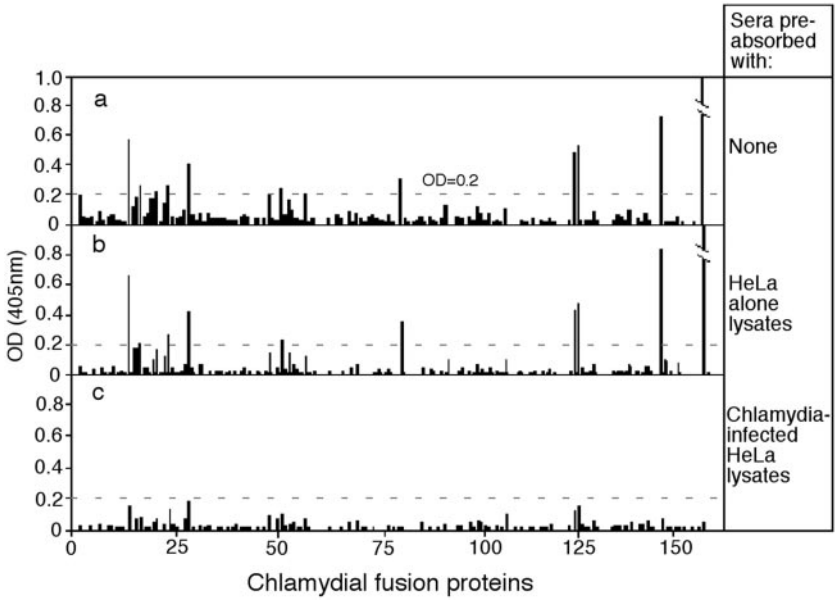

FIG. 3. Effects of absorption with chlamydial antigens on human antibody reactivities with the 156 fusion proteins. Pooled human sera from 15 patients were absorbed with or without uninfected (HeLa alone) or chlamydia-infected HeLa cell lysates prior to being reacted with the 156 fusion proteins in an ELISA. The ability of the pooled sera to react with chlamydial antigens was completely blocked by absorption with chlamydia-infected (c) but not with uninfected (b) HeLa cell lysates.

thetical protein, recognized by 9 sera), and CT858 (CPAF, recognized by 14 sera). Since immunodominance of a given protein is also affected by antibody titers, we further compared the titers of antibodies reactive to each chlamydial fusion protein. The raw ODs measured between each serum antibody and chlamydial fusion protein were used to represent the antibody titers, which are summarized in Fig. 2C (accumulative ODs from all 15 antibody samples) and Fig. 2D (average ODs). Interestingly, the seven fusion proteins that were recognized by human antibodies with the highest frequency also maintained the highest accumulative and average ODs. We next pooled the 15 human sera at an equal ratio and reacted the pooled sera with the 156 fusion proteins (Fig. 2E). The raw ODs obtained with the pooled human serum samples were similar to the average ODs obtained with the individual samples, indicating that the pooled serum samples can be used to measure the overall reactivity of the individual human sera. As a negative control, we pooled sera from eight healthy individuals without chlamydial infection and similarly measured the reactivity of the pooled negative serum samples with the chlamydial fusion proteins (Fig. 2F). No significant reactivity was found (none of the ODs was near or above 0.2). By considering both the recognition frequency and the titer, we determined that the fusion proteins recognized by >50% of the human antiserum samples with a raw OD significantly above background (with an average OD equal to or above 0.2) were relatively immunodominant antigens under the current assay conditions. The same seven antigens recognized by eight or more human serum samples—CT089 (LcrE), CT147, CT226, CT681 (MOMP), CT694, CT795, and CT858 (CPAF)—also meet the new requirement for immunodominant antigens.

To confirm that the antibody binding to the arrayed fusion proteins is specific to chlamydial antigens, we carried out an additional absorption experiment using the endogenous chlamydial proteins. The pooled patient sera were absorbed with either uninfected (Fig. 3b) or chlamydia-infected (Fig. 3c) HeLa cell lysates before the sera were reacted with the fusion proteins arrayed on the plate. The antibody binding to all seven immune-reactive fusion proteins was completely removed by absorption with the chlamydia-infected (Fig. 2c) but not the uninfected (Fig. 2b) HeLa cell lysates.

Antigenicity titration of the identified immunodominant antigens. The pooled sera from either the 15 patients or the 8 healthy individuals were serially diluted and analyzed against the seven immune-reactive antigens and eight other MOMPs by both a fusion protein array enzyme-linked immunosorbent assay (ELISA) (Fig. 4A) and a Western blot assay (Fig. 4B). The ODs decreased as the pooled positive serum samples were diluted from 1:100 to 1:12,500 (Fig. 4Aa), suggesting that the chlamydial protein-specific antibodies were not saturated under these dilutions. The GST-CPAF fusion protein was significantly recognized by the pooled positive sera at a dilution of 1:12,500, CT795 and CT089 at 1:2,500, and CT147, CT226, CT694, and various MOMPs at 1:500, demonstrating that CPAF, CT795, and CT089 are more immunodominant than MOMP regardless of the types of MOMPs analyzed. Comparing the nine MOMPs, higher ODs were obtained with MOMPs from serovars B, Ba, D, E, and L2, all of which belong to the *C. trachomatis* subspecies B complex, suggesting that the 15 patients were predominantly infected with B complex serovars, most likely serovars D and E, in agreement with the epidemiological finding that both serovars D and E are among the most prevalent *C. trachomatis* serotypes in individuals with sexually transmitted chlamydial infections (3, 9, 29, 30, 49). The GST-alone control was not significantly recognized at any dilution. The pooled negative sera from eight normal individuals dis-

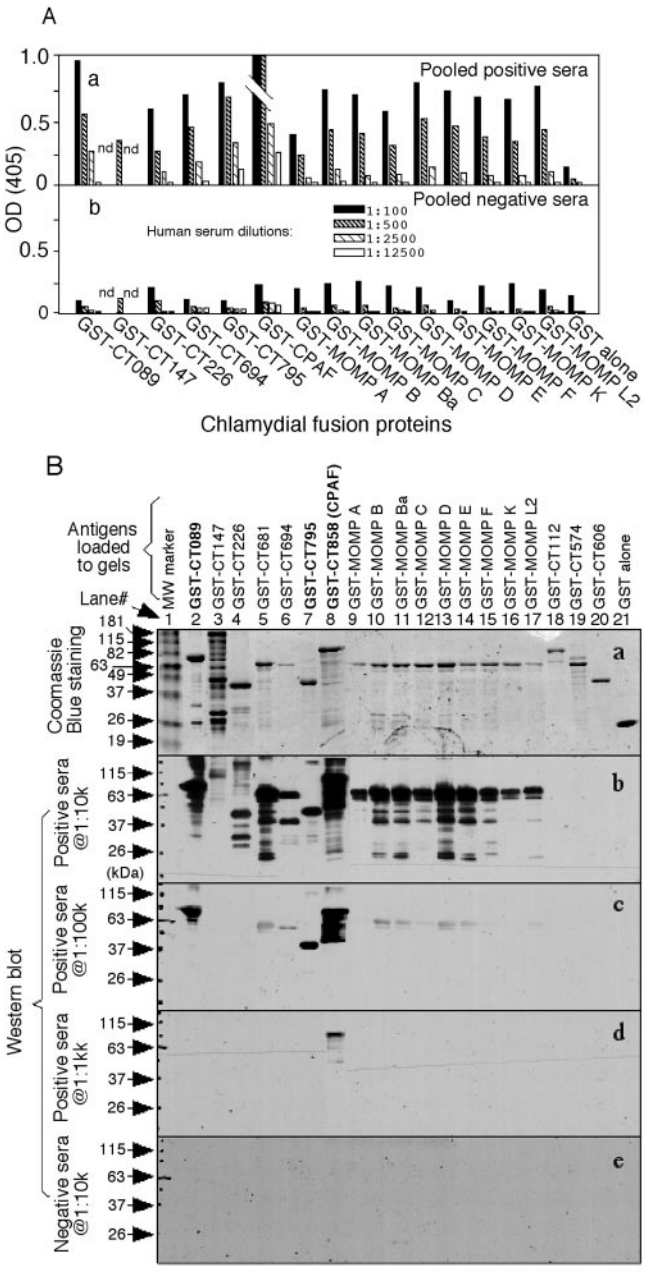

FIG. 4. Antigenicity analysis of the immunodominant antigens. The antigenicities of the seven immunodominant antigens and of MOMPs from eight other *C. trachomatis* serovars were further analyzed using different dilutions of the pooled human sera, as indicated, by both a fusion protein array ELISA (A) and a Western blot assay (B). Pooled sera from the 15 patients (positive sera) (Aa, Bb, Bc, and Bd) or from 8 healthy individuals without chlamydial infection (negative sera) (Ab and Be) were used. (A) In the ELISA, the positive sera displayed significant recognition of GST-CPAF at 1:12,500, of CT795 and CT089 at 1:2,500, and of CT147, CT226, CT694, and various MOMPs at 1:500. Comparing the nine MOMPs, higher ODs were obtained with MOMPs from serovars B, Ba, D, E, and L2. The GST-alone control was not significantly recognized at any dilution. The pooled negative sera from eight healthy individuals displayed minimal levels of reactivity even at the 1:100 dilution. nd, not detected. (B) In the Western blot assay, the purified fusion proteins were loaded onto an SDS gel and visualized using Coomassie blue staining (a). Human antibody binding to the chlamydial fusion proteins was detected with a goat anti-human IgG conjugate and visualized using ECL. The positive sera recognized CPAF (lane 8) at a 1:1,000,000 dilution (d), CT795 (lane 7) and CT089 (lane 2) at 1:100,000 (c), and the rest of the chlamydial fusion proteins at 1:10,000 (b). The control fusion proteins CT112 (lane 18), CT574 (lane 19), CT606 (lane 20), and GST alone (lane 21) were not detected regardless of the serum dilution. The pooled negative sera displayed no detectable reactivity with the chlamydial fusion proteins (e). k, thousand; kk, million.

played a minimal level of reactivity even at a 1:100 dilution (Fig. 4Ab). The above results, obtained with varying dilutions of human serum samples, not only confirmed the observations presented in Fig. 2 but, more importantly, provided a more detailed analysis of the relative antigenicities of the immunodominant antigens and of the various MOMPs.

A Western blot assay was used to confirm the above observations (Fig. 4B). The same seven immunodominant fusion proteins, together with eight other MOMPs and several control proteins, were used as antigens. A dominant full-length fusion protein band migrating at the expected molecular weight position was identified for each fusion protein sample. When the antigens were detected on the Western blot, the pooled positive serum samples recognized CPAF (Fig. 4B, lane 8) at a 1:1,000,000 dilution (Fig. 4Bd), CT795 (lane 7) and CT089 (lane 2) at 1:100,000 (Fig. 4Bc), and the rest of the chlamydial fusion proteins at 1:10,000 (Fig. 4Bb). The control fusion proteins CT112 (Fig. 4B, lane 18), CT574 (lane 19), CT606 (lane 20), and GST alone (lane 21) were not detected regardless of the serum dilution. Among the nine MOMPs, the pooled patient sera preferentially recognized MOMPs from the B complex serovars, including B, Ba, D, E, and L2. The pooled negative sera displayed no detectable reactivity with the chlamydial fusion proteins at 1:10,000 (Fig. 4Be). These Western blot results were largely consistent with the ELISA data shown in Fig. 4A.

Characterization of the immune-reactive antigens. To evaluate the expression patterns and determine the locations of the endogenous proteins, we generated antibodies against each of the newly identified immune-reactive antigens and used these antibodies to track the endogenous proteins in chlamydia-infected cultures (Fig. 5). As a control, MOMP was detected completely overlapping with intravacuolar organisms at both early (8-h) and late (48-h) stages of infection, while CPAF was detected only in the infected-cell cytosol at the late infection stage. The hypothetical protein CT226 was detected on the inclusion membrane at the late infection stage but was not detectable at the early stage. The hypothetical protein CT147 was detected during the entire infection course, inside the inclusion at the early infection stage and in the peripheral region of the inclusion at the late stage. The hypothetical protein CT795 was expressed early and appeared to be restricted to some but not all inclusions, while CT694 was detected only at the late stage of infection within the inclusion (mostly overlapping with chlamydial organisms). Finally, CT089 was detected throughout the infection cycle overlapping with the intravacuolar chlamydial organisms, an expression/localization pattern similar to that of MOMP.

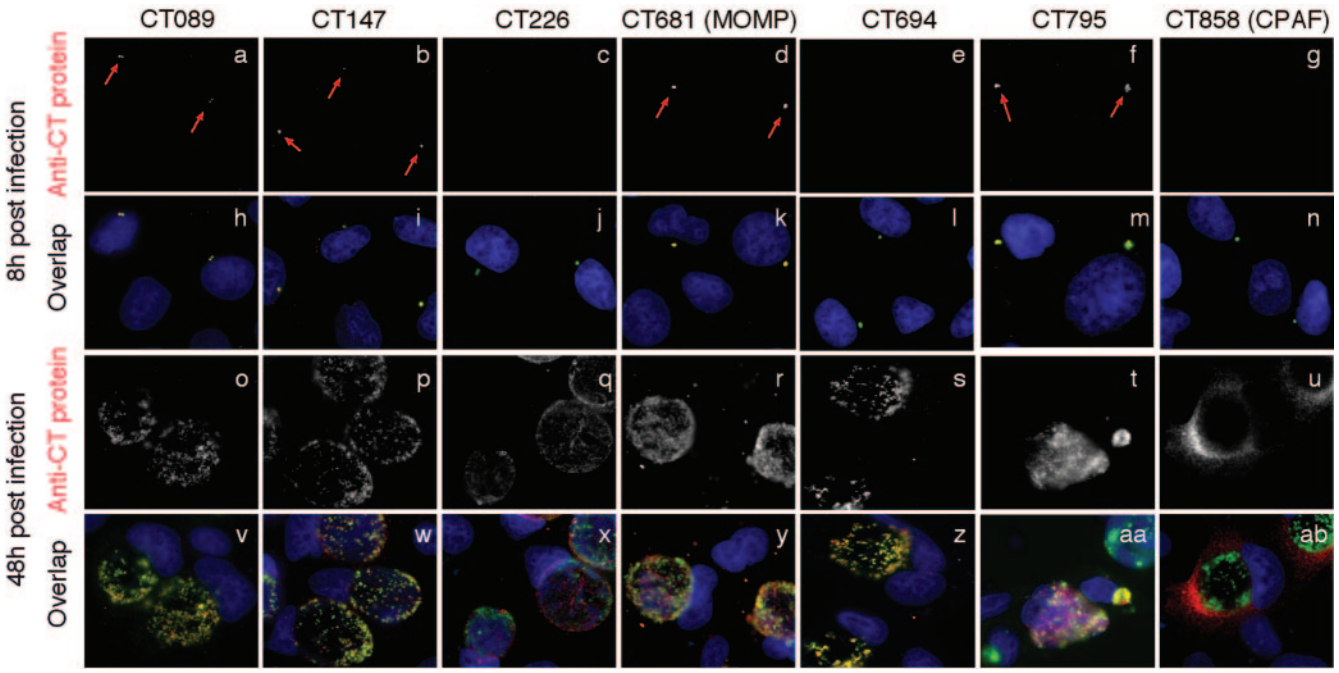

FIG. 5. Characterization of the immune-reactive antigens. *C. trachomatis*-infected HeLa monolayers grown on coverslips for 8 or 48 h were processed for antibody staining. Mouse antibodies against CT089 (a, h, o, and v), CT147 (b, i, p, and w), CT226 (c, j, q, and x), MOMP (d, k, r, and y), CT694 (e, l, s, and z), CT795 (f, m, t, and aa), and CPAF (g, n, u, and ab) in combination with a rabbit anti-chlamydial heat shock protein were used as primary antibodies. The reactivities of the primary antibodies were visualized with Cy3-conjugated goat anti-mouse (red) and Cy2-conjugated goat anti-rabbit (green) antibodies. The Hoechst DNA dye was used to visualize host nuclei (blue). Single-color images were acquired under an Olympus AX-70 fluorescence microscope with SimplePCI software (Olympus, Seattle, WA) (a to g and o to u) and overlaid to form tricolor images (h to n and v to ab).

DISCUSSION

A comprehensive analysis of human immune responses to chlamydial infection at the whole-genome scale is essential to the search for effective chlamydial vaccines and to determination of the molecular basis of chlamydial pathogenesis. Although the availability of chlamydial genome sequences has stimulated a great deal of interest in using the genome information to search for vaccine candidates and pathogenic determinants (21, 27, 50), experimental work carried out in this direction has achieved only limited success. This is mainly because it has been difficult to develop a genome-scale assay with each antigen detected at similar amounts and in an unbiased fashion. Assays based on the resolution of endogenous antigens from chlamydial organisms in 2-dimensional gels have numerous problems, including the lack of equal representation of each antigen in the organisms and denaturation of the antigens. In the current study, we have produced 156 chlamydial fusion proteins and developed a subgenomic protein array assay, in which each protein is arrayed at similar amounts and in solution, to analyze human antibody responses to chlamydial infection, which has led us to identify novel immunodominant antigens. This subgenomic array has provided the proof of principle that development of a whole-genome fusion protein array for comprehensive analysis of host immune responses to chlamydial infection is both necessary and feasible in order to search for immunodominant antigens.

Using the subgenomic-scale fusion protein ELISA, we identified seven immune-reactive antigens, including CPAF (CT858), CT795, CT089, MOMP (CT681), CT694, CT226, and CT147. Although both CPAF and MOMP have been shown to be immunogenic in humans (2, 45), it is not known whether the remaining five chlamydial antigens are immune reactive in individuals urogenitally infected with *C. trachomatis*. The current report presents the first experimental evidence for the immunogenicity of the five chlamydial proteins in humans. These five immunogenic proteins are encoded by hypothetical ORFs in the chlamydial genome. CT089 is a putative regulatory molecule for the type III pathway (designated LcrE). CT147 is an early-expressed protein that shares homology with stretches of amino acid sequences in EEA1 (early endosomal antigen) (5); however, its function is not clear. CT226 is encoded by an ORF located within a cluster of genes that are predicted to code for inclusion membrane proteins. Our current report presents the experimental evidence confirming the inclusion membrane localization of CT226. Both CT694 and CT795 are chlamydia-specific hypothetical proteins with no significant homology with any other known sequences.

It is not clear why these 7 proteins are relatively more immunodominant among the 156 chlamydial proteins assayed in the same system. In a cell culture system, all of the seven proteins are expressed, although at different time points, and each has its unique distribution pattern, including associating with the organisms or being secreted to inclusion membranes or host cell cytosol. It is likely that the protein expression and/or distribution patterns are not the major determining factors for the relative immunodominance of these antigens. Regardless of the precise

mechanisms that govern these proteins' immunodominance, a more important question is whether immune responses to these immunodominant antigens can be correlated with either pathogenesis or protective immunity. Obviously, a large patient pool with a well-defined clinical categorization has to be analyzed in order to establish any meaningful correlations. The fact that antibody responses, especially the responses of urogenital secretory IgA antibodies, to MOMP (6, 10, 58, 59) have been correlated with host protective immunity suggests that it is possible to correlate antigen-specific host responses with host clinical outcomes. It is obvious that animal models are required in order to further determine the relative roles of the antigens identified in chlamydial pathogenesis and immunity.

Surprisingly, none of the heat shock proteins (CT110, CT111, CT604, and CT755) was dominantly recognized by the human serum samples in the current assay, although human antibody responses to these heat shock proteins have been reported previously (1, 7, 44, 60). This discrepancy is probably due to multiple reasons, including the fact that all human sera used in the current study were preabsorbed with bacterial lysates. Due to the high homology of chlamydial heat shock proteins with other bacterial heat shock proteins, the bacterial lysate preabsorption may have effectively removed the cross-reactive antibodies that are able to recognize both chlamydial and other bacterial heat shock proteins. In addition, human antibody responses to chlamydial heat shock proteins have been detected mainly in individuals associated with patients who suffer from more-chronic chlamydial disease conditions, such as tubal scarring and infertility (1). The lack of anti-chlamydial heat shock protein antibodies in the 15 patients in this study may be due to the fact that these patients were at the acute stage of infection when the serum samples were collected (data not shown).

Interestingly, the detailed antigenicity analysis of the seven immune-reactive antigens revealed that CT089, CT795, and CT858 (CPAF) were consistently more immunodominant than MOMP under the current assay conditions. However, MOMP has been widely considered the most immunogenic antigen of chlamydial organisms (2, 7, 40, 52, 65) and has been extensively studied as a vaccine candidate (16, 25, 26, 38, 53, 64). The apparent discrepancy may be due to the following reasons: First, many previous studies used Western blotting and peptide ELISAs to analyze chlamydial antigenicity. In these assays, antigen conformations are dramatically different from their native conformations; therefore, these assays often fail to detect the conformation-dependent antibodies. In the current assay, each chlamydial protein is arrayed onto microplates in solution, and the fusion protein antigens immobilized on the plate are efficiently recognized by human antibodies. Although one should not expect the fusion proteins to maintain the same conformation as that of the endogenous proteins, the soluble GST-chlamydia fusion proteins may represent a significant improvement in preserving the structures required for human antibody recognition. Second, none of the previous studies compared the antigenicities of all proteins encoded by the chlamydial genome. Instead, only a few selected antigens, such as MOMP and HSPs, were analyzed. In the current study, we have extended the antigenicity analysis to 156 chlamydial proteins. Although the 156 proteins represent only a small fraction of proteins encoded by the entire chlamydial genome, they have provided us the opportunity to discover new antigens beyond MOMP. Third, in attempts to compare all chlamydial proteins, many previous studies have used purified chlamydial organisms as the source of antigens (8, 41, 44, 46, 47, 55), which led to biased results. This is because chlamydial proteins are not equally represented in chlamydial organisms. Chlamydial gene expression is regulated during chlamydial growth, with some genes expressed early and some late (5, 36). The differential gene expression and the different protein half-lives can dramatically affect the amounts of proteins associated with chlamydial organisms at the time when the organisms are harvested. Constitutively expressed proteins such as MOMP, with a long half-life, are much more abundant than other antigens in the organisms. The antigen abundance alone can dramatically increase the detection sensitivity of the MOMP-specific antibodies. Furthermore, some chlamydial antigens exist only in either reticulate bodies or elementary bodies (48). More importantly, there are chlamydial proteins that are not even associated, or only minimally associated, with the purified organisms, although these proteins are amply produced during natural infection. These include CPAF, which is secreted into host cell cytosol (63), and Inc proteins, which are secreted to inclusion membranes (18, 43). Obviously, antibodies to these proteins are not even detectable when the purified organisms are used as antigens. Therefore, it is not possible to accurately analyze the antigenicities of all chlamydial proteins by using endogenous antigens from chlamydial organisms. To solve these problems, we have tested a fusion protein array assay, in which each of the chlamydial proteins is more or less equally represented, to analyze human immune responses to chlamydial infection, which has led us to discover new antigens that are even more immunodominant than MOMP.

Finally, it is also worth noting that the fusion protein approach has its own limitations, including the potentially insufficient purity of the full-length fusion proteins and differences in conformation between native and fusion proteins. These limitations may lead to an underestimation of immune-reactive antigens and even to biased results. For example, the recombinant MOMP in the form of a GST fusion protein may have a conformation different from that of native MOMP, and this difference can contribute to the reduced immune reactivity of human antibodies with GST-MOMP fusion proteins assayed in the current study. In fact, antigenicity analysis of bacterial membrane proteins has always been a challenge due to the difficulties in maintaining the native conformation of the membrane-anchored proteins in heterologous systems. One strategy for minimizing this problem is to express the extracellular domains of membrane proteins with or without conformational constraints. Alternatively, an immunoprecipitation assay may be used to directly analyze antibody reactivity with metabolically labeled endogenous membrane proteins.

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