Inhibition of Proteolytic Activity of a Chlamydial Proteasome/Protease-Like Activity Factor by Antibodies from Humans Infected with *Chlamydia trachomatis*

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We have previously shown that individuals infected with *Chlamydia trachomatis* can develop a robust antibody response to a chlamydia-secreted protein (designated chlamydial proteasome/protease-like activity factor [CPAF]). We now report that sera collected from these infected individuals neutralized the proteolytic activity of CPAF. Depletion of the serum sample with CPAF proteins to remove the CPAF-specific antibodies effectively blocked the neutralization, whereas similar depletion with the HSP60 proteins failed to do so. We further demonstrated that the CPAF central region covering residues 200 to 338 was predominantly recognized by the human neutralization antibodies. The significance of the CPAF neutralization antibodies generated in chlamydia-infected individuals is discussed.

Urogenital tract infection with *Chlamydia trachomatis* is a leading cause of sexually transmitted bacterial diseases in the United States. However, the pathogenic mechanisms remain unclear, and there are no effective vaccines for preventing *C. trachomatis* infections and/or the associated infection-induced diseases. It is thought that the chlamydia-induced diseases are largely due to host inflammatory responses provoked by chlamydial interactions with host cells (8, 10). *Chlamydia* has evolved various strategies for protecting the infected cells from the host defense system (2, 5–7, 13–15). A chlamydia-secreted protein designated chlamydial proteasome/protease-like activity factor (CPAF) was identified in the cytosol of the chlamydia-infected cells (13). CPAF was both necessary and sufficient for degrading host transcriptional factors, including RFX5 required for major histocompatibility complex gene activation (13), which may provide a molecular explanation for chlamydial evasion of host immune recognition. Interestingly, humans infected with *C. trachomatis* can develop a robust antibody response to CPAF (9). However, the significance of the human anti-CPAF antibodies in chlamydial pathogenesis and host immunity against chlamydial infection is unknown. The focus of the current study is to evaluate whether the human anti-CPAF antibodies can neutralize the CPAF proteolytic activity since it is the CPAF proteolytic activity that may aid in chlamydial evasion of host immune recognition.

A cell-free degradation assay that has been previously established for measuring CPAF activity (3, 4, 13) was used to assess the ability of the human sera to block CPAF degradation of RFX5 (Fig. 1). A nuclear extract (containing RFX5 [15]) from normal HeLa cells was used as the sub-

strate, and a cytosolic fraction (L2S100; containing functional CPAF [13]) made from *C. trachomatis* L2-infected HeLa cells was used as the enzyme source. After the substrate and enzyme mixtures were incubated at 37°C for 1 h, the residual RFX5 was detected with a rabbit anti-RFX5 antibody on a Western blot as described previously (15). L2S100 degraded RFX5, and the degradation was inhibited by lactacystin, a proteasome inhibitor known to be able to inhibit CPAF, but not by the solvent dimethyl sulfoxide alone (lanes 1 to 4), indicating that the assay was suitable for measuring CPAF-specific proteolytic activity as previously demonstrated (3, 13, 15). Sera collected from individuals diagnosed with *C. trachomatis* urogenital infection were found to contain high titers of antibodies to CPAF (9). Five such anti-CPAF positive sera were pooled together, and the pooled sera were tested for their ability to neutralize CPAF proteolytic activity by using the cell-free degradation assay. Three anti-CPAF negative sera collected from individuals with or without *C. trachomatis* urogenital infection were also pooled as the controls. When the L2S100 was incubated with the pooled anti-CPAF positive human sera prior to reacting with the substrate, the RFX5 degradation was inhibited (Fig. 1, lanes 5 and 6). Further dilution of the anti-CPAF positive sera removed the inhibitory effect (lane 7). As controls, the mouse monoclonal antibodies 100a and 54b did not show any significant inhibitory effects on the RFX5 degradation (lanes 12 and 13), a finding which is consistent with previous observations that neither 100a nor 54b neutralized CPAF activity, although these monoclonal antibodies bind to CPAF well (3, 13). A more relevant control is that the pooled anti-CPAF negative human sera failed to affect the RFX5 degradation activity of L2S100 (lanes 8 and 9) regardless of dilution. These observations have demonstrated that sera from individuals infected with *C. trachomatis* can neutralize the ability of CPAF to degrade RFX5.

To further evaluate whether the CPAF-specific antibodies in the human sera are indeed responsible for the neutral-

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FIG. 1. Neutralization of CPAF-mediated degradation of RFX5 in a cell-free assay. A nuclear extract (NE; containing RFX5) from normal HeLa cells was used as the substrate and a cytosolic fraction (L2S100; containing functional CPAF) made from *C. trachomatis* L2-infected HeLa cells as the enzyme source. After the enzyme-substrate reaction, the residual RFX5 was detected with a rabbit anti-RFX5 antibody on a Western blot. Lactacystin is a proteasome inhibitor known to be able to inhibit CPAF. Dimethyl sulfoxide is the solvent for dissolving lactacystin. For neutralization of CPAF activity, 5 μ l of the pooled anti-CPAF positive human sera after various dilutions, the monoclonal antibodies (mAbs 100a and 54b; $\sim$ 100 μ g of IgG per ml), the pooled anti-CPAF negative sera, or phosphate-buffered saline buffer was used to mix with 5 μ l of L2S100. The mixtures were incubated for 30 min incubation at room temperature before being added to the nuclear extract substrate. It is clear that the pooled anti-CPAF positive sera after 1:100 dilution blocked the RFX5 degradation activity of CPAF, whereas the pooled anti-CPAF negative sera failed to do so even at a 1:20 dilution.

ization activity, we used glutathione *S*-transferase (GST)–CPAF fusion proteins immobilized onto glutathione-conjugated beads to remove the CPAF-specific antibodies from the serum samples and evaluated the neutralization ability of the remaining serum samples in the cell-free degradation assay. The same anti-CPAF positive and negative human sera tested in Fig. 1 were used in the depletion-preabsorption experiment. As shown in Fig. 2, the cell-free assay worked well in measuring CPAF activity (lanes 1 to 4). Although the pooled anti-CPAF positive sera effectively neutralized the RFX5 degradation activity (lane 5), the residual serum sample after depletion with the GST-CPAF fusion proteins completely lost the neutralization ability (lane 6). As a depletion antigen control, GST-HSP60 fusion protein was used to deplete the same pooled anti-CPAF positive sera. The depletion with GST-HSP60 fusion proteins failed to remove the serum neutralization ability (lane 7). Consistent with what was shown in Fig. 1, the pooled anti-CPAF negative sera did not affect the RFX5 degradation by CPAF (lane 8). These observations have confirmed that the CPAF-specific antibodies in the CPAF-positive human sera are required for the neutralization of CPAF degradation activity.

We further mapped the immunoreactivity of the anti-CPAF positive human sera with 14 CPAF fragments in the form of GST fusion proteins (4) both in an enzyme-linked immunosorbent assay (ELISA) (Fig. 3A) and by Western blot assay (Fig. 3B) as described previously (9, 12). Both the ELISA and Western blot assays consistently showed that a strong antibody reactivity was detected with the CPAF fragments 1-283, 1-488, 1-525, 9-609, 60-609, 136-609, 186-609, 242-609, and 284-609, in addition to the full-length 1-609. In contrast, the fragments

1-100, 1-200, 488-609, 387-609, and 338-609 were only minimally recognized by the human sera. These results suggest that the immunodominant region of CPAF is located between residues 200 and 338.

Finally, we used these CPAF fragments to further determine the CPAF regions targeted by the human neutralization antibodies in a depletion-preabsorption experiment as

FIG. 2. CPAF-specific antibodies are required for the human sera to neutralize CPAF function. The pooled anti-CPAF positive human sera were diluted at 1:100, and 5 μ l of the diluted sera was used for neutralization. For antibody depletion, GST-CPAF or GST-HSP60 fusion proteins immobilized onto glutathione-conjugated beads were incubated with the pooled anti-CPAF positive human sera for 30 min at room temperature. After the beads were pelleted, the remaining supernatants were used for neutralization as described above. Note that depletion with GST-CPAF but not GST-HSP60 completely blocked the neutralization effect. The pooled anti-CPAF negative sera failed to alter the CPAF-mediated degradation of RFX5 (lane 8).

FIG. 3. Reactivity of human sera with CPAF fragments. A total of 14 CPAF deletion fragments in the form of GST fusion proteins were reacted with the pooled anti-CPAF positive human sera in both ELISA (A) and Western blot (B) analyses. For ELISA, the GST fusion proteins and GST alone (shown along the x axis) were immobilized onto a glutathione-coated microplate, and the 10-fold serially diluted human serum samples (see legend inside the figure) were added to the plates. The human antibody binding to antigens was visualized with a goat anti-human IgG conjugate plus a colorimetric substrate. The binding intensity was detected by measuring the optical density at 405 nm. The optical density values shown along the y axis represent the mean values of duplicates from two independent experiments. For Western blotting, the CPAF GST fusion proteins, along with other control GST fusion proteins including MOMP, HSP60 (CT110), and ORF CT112, and 574-encoded proteins from serovar D genome (as indicated on top of the figure) were loaded onto polyacrylamide sodium dodecyl sulfate gels. After electrophoresis separation, one set of the gels was directly stained with Coomassie blue dye to visualize the amount of protein in each lane (subpanel a), and the rest were transferred onto nitrocellulose membrane for detection with the pooled anti-CPAF positive human sera at various dilutions as indicated along the left side of the figure (subpanels b to d). The primary antibody binding was visualized with a secondary antibody enzyme conjugate by using standard enhanced chemiluminescence.

described in Fig. 2. Shown in Fig. 4A are the human antibody neutralization patterns with or without preabsorption with CPAF fragments. Again, the human antibodies neutralized CPAF degradation activity (lane 5), and the antibody neutralization was removed by preabsorption with the full-length GST-CPAF (lane 6) but not the control GST-HSP60 proteins (lane 7). These results validated the current preabsorption experiment. More importantly, preabsorption with the CPAF fragments that displayed a strong reactivity with human sera, including CPAF fragments 1-283, 1-488, 1-525, 9-609, 60-609, 136-609, 186-609, 242-609, and 284-609 (see Fig. 3), significantly removed the neutralization ability of the human antibodies. However, the preabsorption with the minimally reactive CPAF fragments, including CPAF fragments 1-100, 1-200, 488-609, 387-609, and 338-609 (see

Fig. 3), failed to block the human antibody neutralization. It is clear that the immunoreactive CPAF regions are also predominantly targeted by the human neutralization antibodies (Fig. 4B).

Although we have comprehensively analyzed the human serum immunoglobulin G (IgG) antibody neutralization of CPAF degradation activity, the significance of the human CPAF neutralization antibodies in chlamydial pathogenesis and immunity is still unclear. Since CPAF is believed to play an important role in chlamydial evasion of host defense through its proteolytic activity, the human antibody neutralization of CPAF proteolytic activity may be beneficial to host defense against chlamydial infection. However, CPAF is confined within the infected host cells when it degrades host transcription factors. Since serum IgG anti-

FIG. 3—Continued.

bodies cannot get inside host cells, it is hard to imagine how the CPAF-specific antibodies in the human sera contribute to the inhibition of the intracellular CPAF activity. In addition, CPAF is not in the infectious form of chlamydial organisms (13). It is unlikely that the CPAF neutralization antibodies can directly affect the chlamydial organism infectivity. Furthermore, various previous studies have demonstrated a lack of correlation of the IgG neutralizing antibodies against other chlamydia-encoded proteins, such as MOMP, with protection against chlamydial challenge infection (11).

Nevertheless, the fact that the human serum antibodies can neutralize CPAF proteolytic activity suggests that humans have the ability to make antibodies with the correct fine specificities for neutralizing CPAF function. The next

step is to test whether the CPAF-specific secretory IgA antibodies from the urogenital tract of individuals infected with *C. trachomatis* can also neutralize the CPAF proteolytic activity. Since secretory IgA (sIgA) can access to intracellular environment, we hypothesize that the CPAF-specific neutralization sIgA antibodies may play a role in host defense against chlamydial infection by neutralizing CPAF function intracellularly. With the inhibition of CPAF function, the infected cells may be able to restore their ability to express major histocompatibility complex antigens and to present chlamydial peptides to T cells. This hypothesis is consistent with the observation that the level of chlamydia-specific sIgA antibodies in human urogenital tracts was inversely correlated with the number of chlamydial organisms recovered from the urogenital tracts (1).

FIG. 4. Blockade of human antibody neutralization with CPAF fragments. (A) This experiment was carried out as described for Fig. 2 except that CPAF fragments were used to preabsorb the pooled anti-CPAF positive human sera (lanes 1 to 14 and lanes 17 to 23) in addition to the various control reactions (lanes 1 to 5) and full-length CPAF (lane 6) and HSP60 (lane 7) preabsorption. The pooled negative serum sample was used as a neutralization control (lane 24). It is clear that the CPAF fragments dominantly reactive to human sera significantly blocked the human antibody neutralization (lanes 10 to 12 and lanes 18 to 23), whereas the minimally reactive fragments failed to do so (lanes 8, 9, 13, 14, and 17). In addition to the typical human immunoglobulin heavy chain bands (huIgH), there were also other unknown bands migrating above the huIgH bands in some reactions (?). (B) Both the immune reactivity and the ability to block human antibody neutralization of CPAF fragments were summarized. Based on the reactivity and neutralization blockade patterns, it was proposed that the CPAF region covering residues 200 and 338 was dominantly recognized by human neutralization antibodies.

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