**NOTES**

*Clostridium perfringens* Antigens Recognized by Broiler Chickens Immune to Necrotic Enteritis

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Little is known about immunity to necrotic enteritis (NE) in chickens. A recent study of broiler chickens showed that protection against NE was associated with infection-immunization with virulent but not with avirulent *Clostridium perfringens*. In the current study, six secreted antigenic proteins unique to virulent *C. perfringens* that reacted to serum antibodies from immune birds were identified by mass spectrophotometry; three of these proteins are part of the VirR-VirS regulon.

Necrotic enteritis (NE) is an economically important enteric disease of chickens caused by *Clostridium perfringens* (8). Although vaccination offers an alternative approach to antimicrobial drugs in control of the disease, little is known about immunity to NE. However, there is the suggestion that the alpha-toxin, a phospholipase C exoenzyme, is an important immunogen (1, 13, 21). A recent study showed that alpha-toxin is not essential in the pathogenesis of NE (10). Nevertheless, the importance of alpha-toxin or any other protein in immunity to NE in chickens remains to be demonstrated.

A recent study from this laboratory showed that it was possible to immunize broiler chickens successfully against NE (23). The immunizing ability was associated with virulent but not with avirulent strains. This study showed that some alpha-toxin-minus mutants also successfully immunized chickens against infection (23). The conclusion from this study was that a factor(s) other than alpha-toxin could provide immunity to infection. The objective of the current study was to identify proteins unique to virulent, protective *C. perfringens* that reacted to serum and intestinal antibodies from previously infection-immunized birds (23) by use of Western blotting and mass spectrometry techniques.

Four strains of *C. perfringens* (CP1, CP4, CP5, and CP6) used in this study are clinical isolates from field cases of NE. Strains CP1 and CP4 are virulent, and CP5 and CP6 avirulent, isolates, as assessed by their abilities to cause NE (23). *Clostridium perfringens* cells were grown anaerobically in an empirically formulated medium (tryptic soy broth [Difco] 50%, nutrient broth [Difco] 25% and peptone water [Difco] 25%) for 24 h at 37°C, and the cells and culture supernatant were collected thereafter. The cells were lysed by eight freeze-thaw cycles with liquid nitrogen to obtain whole-cell proteins. The culture supernatant was dialyzed and concentrated by use of 10-kDa cutoff Amicon filters (Millipore Inc., Billerica, MA) to obtain secreted proteins. The protein concentration was determined using a PlusOne 2-D Quant kit (Amersham Biosciences, San Francisco, CA). The protein contents of concentrated secreted and whole-cell protein samples were 3 to 4 mg/ml. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis under reducing conditions, 100 μg of protein sample was applied.

The secreted and whole-cell proteins were separated by one-dimensional SDS-PAGE in a 12.5% acrylamide gel under reducing and nonreducing conditions (11). The gels were visualized by Coomassie R-250 staining. The secreted and whole-cell protein profiles of the virulent, protective (CP4) and avirulent, nonprotective (CP5) strains are shown in Fig. 1A and B, respectively. Three Coomassie-stained secreted protein bands of 33, 37, and 52 kDa (Fig. 1A) and three whole-cell protein bands of 34, 55, and 117 kDa (Fig. 1B) were unique to CP4. The 37- and 52-kDa secreted proteins unique to CP4 were later identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and perfringolysin O, respectively, since these were among those proteins that reacted strongly to immune serum (Fig. 2A; Table 1). The proteins from the gel were transferred to a nitrocellulose membrane of 0.45-μm pore size (Bio-Rad Laboratories) by use of a Hoefer tank buffer system (Amersham Biosciences) followed by reaction with primary antibodies (serum or intestinal washing) at 1:1,000 and 1:500 dilutions, respectively. Serum (source of immunoglobulin Y [IgY]) used in this study was pooled from broiler chickens immune to virulent *C. perfringens* challenge in infection-immunization experiments (23). The pooled small intestinal washings made from these birds by use of phosphate-buffered saline were dialyzed, concentrated, and used as the source of primary antibody (IgA and IgY) in Western blotting and neutralization experiments. Anti-chicken IgY (heavy plus light chains) and anti-chicken IgA were used as secondary antibodies at 1:2,000 and 1:1,000 dilutions, respectively. Specific immunoreactive protein bands were visualized using an alkaline phosphatase-conjugated substrate kit (Bio-Rad Laboratories). Several protein bands from strain CP4 showed reactivity to immune se-
rum, but similar reactivity was not observed for secreted proteins from CP5 (Fig. 2A). This lack of reactivity was also observed when secreted proteins from avirulent strain CP6 were reacted with immune serum. Secreted proteins from another virulent strain, CP1, showed reactivity similar to that seen for CP4. Two CP4-secreted protein bands, a 115-kDa band that reacted strongly to serum and a 10-kDa band that reacted to both serum and intestinal antibodies, could not be accurately traced on the Coomassie-stained gel because of doublet bands. The secreted protein bands of CP4 that showed reactivity to immune serum were consistently reactive in multiple gels run at different times. Although there was little reactivity of CP4- and CP5-secreted proteins to intestinal IgA, the reactivity of these secreted proteins to intestinal IgY was similar to that of Western blots done with immune serum (Fig. 3B). Therefore, it seems that both intestinal and serum IgY antibodies are important in immunity to this infection. No differences in the whole-cell protein reactivities to serum or intestinal washings between virulent and avirulent strains were observed, suggesting that the trait of immune protection against NE lies in the secreted components of virulent *C. perfringens*.

Six immunoreactive secreted proteins unique to virulent strains, of which five were highly antigenic (Fig. 2A), were identified in the parallel-run Coomassie-stained gels by use of the coordinates of molecular-weight-marker bands and the distance of migration. The gels from the centers of these bands were excised, in-gel digested, and identified by mass spectrometric techniques, namely, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS/MS). The peptide masses and sequence data from MS analysis were searched against the National Center for Biotechnology Information (NCBI) protein database by MS-Fit (http://prospector.ucsf.edu/prospector/4.0.7/html/msfit.htm) and Matrix-Science Mascot search (http://www.osc.edu/hpc/software/apps/mascot.shtml) to identify the protein that had the highest homology percentage match. Of the six antigenic secreted proteins unique to the two virulent, immunoprotective strains identified by MS (Fig. 2A; Table 1), three (perfringolysin O, fructose 1,6 biphosphate aldolase [FBA], and elongation factor G [EF-G]) are regulated by the VirR-VirS virulence regulon of *C. perfringens* (4). In addition to virulence genes, this regulon controls genes involved in energy metabolism, such as those encoding FBA and NAD-dependent β-hydroxybutyryl coenzyme A dehydrogenase, as well as others that may be indirectly involved in bacterial virulence (4, 9, 19). It therefore seems possible that the marked difference in the immunoreactivities of the secreted proteins between the two virulent and the two avirulent strains is the result of a mutation in this regulatory region in the avirulent strains and that the quantity of these proteins produced is too low to be detected in SDS-PAGE and Western blotting experiments. The avirulent strains do produce alpha-toxin; however, the amount produced was not quantified.

Of these six proteins, perfringolysin O is a known virulence factor of *C. perfringens* (20) and three are enzymes involved in energy production. There is growing evidence that such “housekeeping” enzymes have a role in the pathogenesis of, or immunity to, other infections (Table 2). Interestingly, a 190-kDa CP4-secreted protein that reacted strongly to both serum and intestinal IgY was identified as a hypothetical protein of *C. perfringens* strain 13. Our data demonstrate for the first time
that CPE1281 is a genuine protein. Searching for structural motifs in the amino acid sequence of this protein using a Web-based tool (ExPASy ScanProsite, http://www.expasy.org/tools/scanprosite/) suggested that this protein is a protease. The strong immune response suggests that it may have a role in NE pathogenesis or immunity. Elongation factor G, another antigenic protein identified in the current study (Table 1), is an essential factor in the protein synthesis machinery of bacteria, but there is no indication of a role in the pathogenesis of, or in immunity to, any other infection.

To assess whether the serum and intestinal washings from immune chickens had neutralizing antibodies to alpha-toxin, purified *C. perfringens* alpha-toxin (Sigma Laboratories, St. Louis, MO) reconstituted to 0.5 μg/μl of protein was diluted in phosphate-buffered saline, pH 7.2, to different concentrations, and 100 μl of each dilution was used to check the lecithinase activity of alpha-toxin on 5% egg yolk agar (Difco) plates. The dilution of alpha-toxin that showed the least detectable lecithinase activity (1:4,000) was the reference control. The dilution of test sera or intestinal washings required to neutralize this activity was the titer. The neutralizing antibody titer of pooled serum was 5,000, and that of intestinal antibodies was 10,000. Since the alpha-toxin reacted in Western blots to serum antibodies only under nonreducing conditions (data not shown).

FIG. 2. Immunoreactivities of secreted and whole-cell proteins of virulent, protective (CP4) and avirulent, nonprotective (CP5) *C. perfringens* strains to immune serum. (A) Secreted proteins of CP4 (lane 2) and CP5 (lane 3). The numbers indicated by arrows correspond to the identities of the proteins as shown in Table 1. (B) Whole-cell proteins of CP4 (lane 2) and CP5 (lane 3). Molecular mass standards (in kDa) are in lane 1 of each panel.

| Band no. | Protein | GenBank accession no. | Mol mass (Da) | Homology | Method |
|----------|---------|-----------------------|---------------|----------|--------|
| 1        | Hypothetical protein of *C. perfringens* strain 13 | 18144943 | 189,835 | 80% of sequence coverage | MS/MS and de novo sequencing |
| 2        | Pyruvate:ferredoxin oxidoreductase of *C. perfringens* strain 13 | 18311043 | 127,863 | 77% of masses matched | MALDI- peptide fingerprinting |
| 3        | EF-G of *C. perfringens* strain 13 | 18311390 | 75,988 | 80% of masses matched | MALDI- peptide fingerprinting |
| 4        | Perfringolysin O of *C. perfringens* strain 13 | 18143820 | 55,910 | 77% sequence coverage | nLC ESI-MS/MS |
| 5        | GAPDH of *C. perfringens* strain 13 | 18144966 | 37,000 | 55% sequence coverage | nLC ESI-MS/MS |
| 6        | FBA of *C. perfringens* strain 13 | 18310332 | 34,000 | 42% sequence coverage | nLC ESI-MS/MS |

*a* Band number corresponds to the bands indicated with arrows in Fig. 2A.

*b* nLC, nanospray liquid chromatography.
shown), it is apparent that the specificity of serum antibodies is to conformational neutralizing epitopes of alpha-toxin. No other differences between the immune serum reactivities of reduced and nonreduced virulent *C. perfringens*-secreted proteins were observed (data not shown).

The assumption behind the approach of identifying highly immunogenic proteins of *C. perfringens* in immune chickens is that some or all of these proteins are likely to be involved in the pathogenesis of, and immunity to, NE. An increased antibody response to a bacterial protein suggests the importance of that protein in some aspect of the host-pathogen interaction of the disease. However, in view of the difficulty apparent (5, 6, 18) in concluding that these immunoreactive proteins are involved in protective immunity and the fact that a recent work demonstrated that alpha-toxin is not essential in the pathogenesis of NE (10), further work is required to determine the role of proteins identified in this study in immunity to, or the pathogenesis of, NE.

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