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Molecular Characterization of Proteins from Porcine Spirochetes†

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Sonicated preparations of *Treponema hyodysenteriae* and *Treponema innocens* were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Treponemal proteins were electrophoresed on a 10% polyacrylamide slab gel in a discontinuous Tris-glycine system and either stained with Coomassie blue dye or transferred electrophoretically at 20 mA for 16 h and 30 mA for 3 h to nitrocellulose paper. Staining of the gels revealed at least 42 distinct *T. hyodysenteriae* and *T. innocens* proteins, with molecular sizes ranging from >100 to 14 kilodaltons (kDa). Each species contained 12 to 16 major protein bands; five of the proteins were common to both species. Fourteen major antigens were identified in *T. hyodysenteriae* isolate B204 by using serum specimens from pigs in the acute stage of swine dysentery. Twelve additional antigens were detected in isolate B204 when convalescent-phase serum specimens were reacted to the blot. A wide band at 16 kDa was identified with convalescent-phase serum specimens in *T. hyodysenteriae* but not in *T. innocens*. This 16-kDa antigen was also identified in *T. hyodysenteriae* with colonic secretions from convalescent pigs.

It has been shown by a number of investigators that pigs which recover from swine dysentery (SD) are immune to rechallenge with *Treponema hyodysenteriae* (8, 12; I. T. Egan, Ph.D. thesis, Iowa State University, Ames, 1983). The immune response generated by pigs to the pathogenic spirochete is a humoral and mucosal antibody response consisting of immunoglobulin G (IgG) or IgA or both (5, 8). The humoral response can be detected in serum as early as 12 days postinfection with *T. hyodysenteriae*. The serum antibody titer develops during the acute phase of the disease and reaches a plateau at the time of cessation of clinical signs (8, 11). The antibody response of the colon to *T. hyodysenteriae* has been assayed only in convalescent pigs. The colonic response generated against this spirochete is at moderate levels, and reaction in the enzyme-linked immunosorbent assay occurs only to specific glycoproteins of *T. hyodysenteriae* (5). The role of antibody in protecting pigs against rechallenge is not known, except that it has been shown that colonic loops are protected against challenge after passive transfer of convalescent-phase serum antibody (2, 6). The antigens involved in this immune response have not been identified.

Treponema innocens is a spirochete which is considered normal flora of swine (3, 7, 13, 17). *T. innocens* is morphologically identical to *T. hyodysenteriae* and can be differentiated from the pathogenic species by hemolysin production or by serotyping (1, 2, 16). Numerous serological tests have been developed which demonstrate the presence of cross-reactive antibodies to both *T. hyodysenteriae* and *T. innocens* in sera from convalescent swine (4, 9, 10). It is assumed that these cross-reactions are due to protein antigens since lipopolysaccharide antigens of the two species are serologically different (11). To better define this cross-reaction and to identify the proteins of *T. hyodysenteriae* involved in the initiation of the immune response of pigs to SD, proteins of *T. hyodysenteriae* and *T. innocens* were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blot analysis (14, 18).

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MATERIALS AND METHODS

Bacteria. *T. hyodysenteriae* isolate B204 was originally isolated from a pig with SD and is pathogenic when inoculated orally into mice and pigs. *T. innocens* isolate B256 was isolated from a pig showing signs of diarrhea and is nonpathogenic when inoculated orally into mice and pigs (7, 13). Both of these isolates were originally obtained from J. M. Kinyon, Iowa State University, Ames. The isolates have been maintained in our laboratory and are stored frozen at −70°C.

Cultivation. The *T. hyodysenteriae* and *T. innocens* isolates were grown in Trypticase soy broth (BBL Microbiology Systems) supplemented with 5% fetal bovine serum and incubated for 24 h at 38°C under an atmosphere of H_2-CO_2 (1:1). The isolates used in the study had undergone <20 in vitro passages.

Immunization of animals. Six-week-old pigs were orally infected with 10^9 viable cells of *T. hyodysenteriae* isolate B204. Pigs which demonstrated clinical signs of SD were allowed to convalesce without drug therapy. Serum specimens were obtained from the pigs before and during infection and after recovery from the disease. The serum specimens were filter sterilized and stored at −20°C.

Colonic secretions were obtained from normal pigs and pigs which had convalesced from SD. A 30-cm portion of the colon of each pig was flushed with a phosphate-buffered saline solution through exterior tubing every other day for 30 days. The flushings were heat inactivated (56°C for 30 min), clarified, filtered through a membrane (pore size, 0.8 μm), and concentrated 10-fold. The colonic secretions were stored at −20°C.

Preparation of antigen. *T. hyodysenteriae* and *T. innocens* cells in the log phase were harvested by centrifugation and adjusted to 10^9 cells per ml. The cells were washed with a phosphate-buffered saline solution (pH 7.2; 0.01 M) and suspended in a final volume of 10 ml. The cells were disrupted by sonication (30 min), and the soluble portion of the mixture was harvested by centrifugation (10,000 × *g*) and assayed for protein by the method of Lowry et al. (15).

Gel electrophoresis. Discontinuous SDS-slab gel electrophoresis was performed with stacking and separating gels of

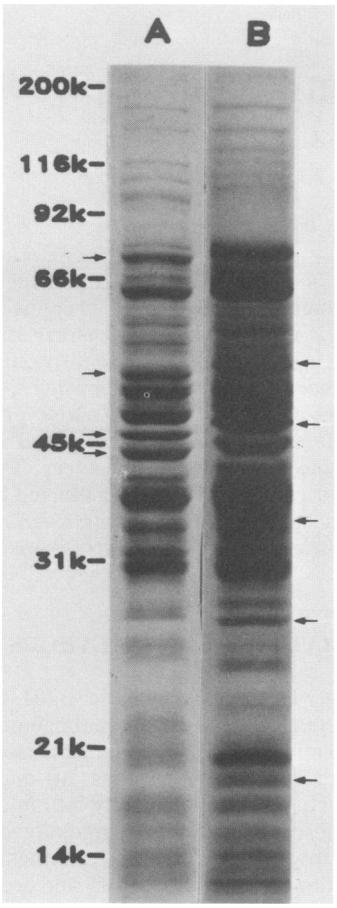

FIG. 1. Protein profiles of sonicated *T. hyodysenteriae*, isolate B204 (lane A), and *T. innocens*, isolate B256 (lane B). The proteins were separated by SDS-polyacrylamide gel electrophoresis with 4% stacking and 10% separating gels, and the gel was stained with Coomassie blue. Molecular size markers in kilodaltons are shown on the left. The arrows indicate unshared proteins in the two isolates.

4 and 10% acrylamide, respectively. Antigen (~2.3 mg of protein per ml) and marker proteins were diluted 1:4 and 1:20, respectively, in sample buffer containing 0.0625 M Tris (pH 6.8), 0.1% SDS, 2% 2-mercaptoethanol, 10% glycerol, and bromphenol blue. Each sample was clarified by centrifugation (10,000 × *g*), boiled for 4 min, and added (50 μl) to wells in the stacking gel.

Mixing experiments were conducted to determine whether proteins which migrated similarly were actually of the same molecular weight. Isolates B204 and B256 were combined in equal amounts (25 μl each) and electrophoresed next to B204 and B256 alone (50 μl each). The profiles of the paired isolates were compared with those of the individual isolates.

Protein transfer. Electrophoretic transfer of proteins from the gel to nitrocellulose paper was performed by the method of Towbin et al. (18). The gel and nitrocellulose paper were sandwiched between Whatman no. 1 filter paper, Scotch-Brite pads, and a plastic holder. With the paper toward the anode of the blotting device, proteins from the gel were transferred electrophoretically at 20 mA for 16 h and 30 mA for 3 h in a 25 mM Tris–192 mM glycine–20% methanol buffer (pH 8.3) at 4°C. The nitrocellulose paper was cut into 10-mm-wide strips and assayed. The transfer of proteins to the nitrocellulose paper was confirmed by amido black staining and destaining in distilled water.

Blotting. The nitrocellulose strips were placed in a 5% skim milk solution for 30 min at room temperature for blocking of nonspecific reactions. The strips were probed with either swine antisera (1:200) or swine colonic secretions (1:2) diluted in 5% skim milk for 1.5 h at room temperature. The strips were washed for 30 min with six changes of a phosphate-buffered saline–0.5% Tween 20 buffer (washing buffer). Blots were then probed with either anti-swine biotinylated IgG (1:100) or anti-mouse biotinylated IgG (1:100) for 30 min with five changes of washing buffer. The blots were reacted to an avidin-peroxidase conjugate (1:100) for 30 min at room temperature. The strips were washed for 20 min with five changes of washing buffer. The blots were then reacted to a 4-chloro-1-naphthol-H_2O_2 substrate solution and photographed.

Absorption. Convalescent-phase swine sera (67 days postinfection) were absorbed for 3 h at 37°C and for 21 h at 4°C with 5×10^{10} cells of *T. innocens*. The sera were harvested by centrifugation and stored at −20°C until used in the blotting assay.

RESULTS

Protein profiles. Proteins of *T. hyodysenteriae* and *T. innocens* were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. At least 42 distinct proteins were detected in each of the two species

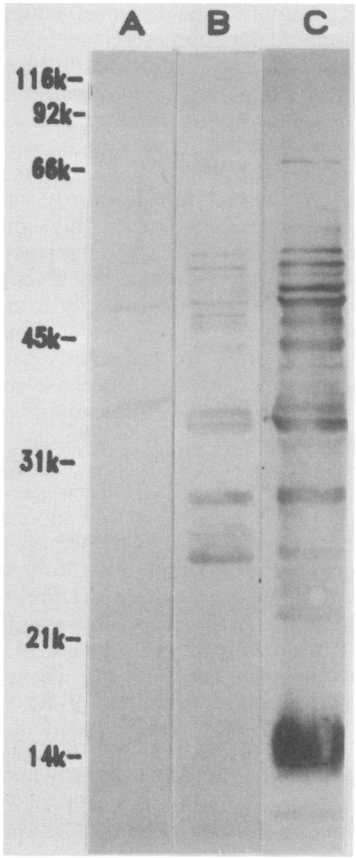

FIG. 2. Antigens of *T. hyodysenteriae*, isolate B204, identified by probing with porcine sera at preinfection (lane A) and at acute (lane B) and convalescent (lane C) stages of SD and with biotinylated anti-swine IgG. Molecular size markers in kilodaltons are shown on the left.

after staining (Fig. 1). These proteins ranged in molecular size from >200 to <14 kilodaltons (kDa).

Common proteins. Each species contained 12 to 16 major protein bands; five of the proteins were common to both species (Fig. 1). Unshared major protein bands were also detected. Peptides at 72, 53, 46, and 44 kDa were present in isolate B204 (*T. hyodysenteriae*) but absent in isolate B256 (*T. innocens*). Also, major protein bands at 74, 55, 47, 45, 37, 27, 19, and 10 kDa were present in the *T. innocens* isolate but absent in *T. hyodysenteriae*.

Mixing experiments. Differences in protein staining were resolved by performing mixing experiments. These experiments confirmed that the 72-, 53-, 46-, and 44-kDa peptides in isolate B204 and the 55-, 47-, 37-, 27-, and 19-kDa major proteins in isolate B256 were actually unique to each isolate. The differences originally noted between peptides were due to differences in staining intensities.

Swine antisera. Proteins were transferred from gels to nitrocellulose strips and probed with pooled swine serum specimens. Reactions of swine antibody to *T. hyodysenteriae* blots are shown in Fig. 2. Fourteen antigens from isolate B204 were identified reacted to antisera from pigs with acute SD. Twelve additional antigens were detected when antisera from recovered pigs were reacted to B204 proteins. Two of these antigens were also recognized by normal swine sera. The molecular sizes of the antigens ranged between 66 and 14 kDa (Fig. 2).

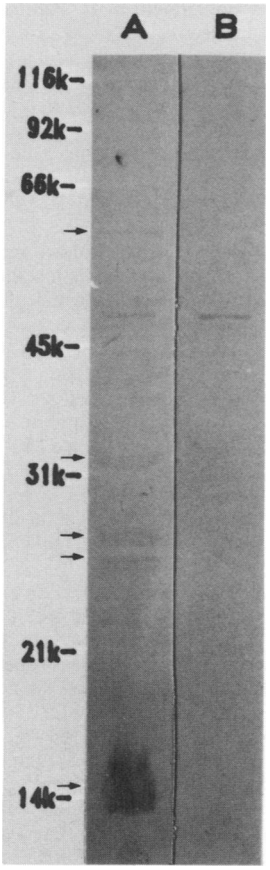

FIG. 3. Antigens of *T. hyodysenteriae*, isolate B204, identified by probing with porcine convalescent-phase (lane A) and normal (lane B) colonic secretions and with biotinylated anti-swine IgG. The arrows indicate antigens in B204 identified by the convalescent-phase secretions only. The bottom arrow indicates a 16-kDa antigen also recognized by convalescent-phase sera. Molecular size markers in kilodaltons are shown on the left.

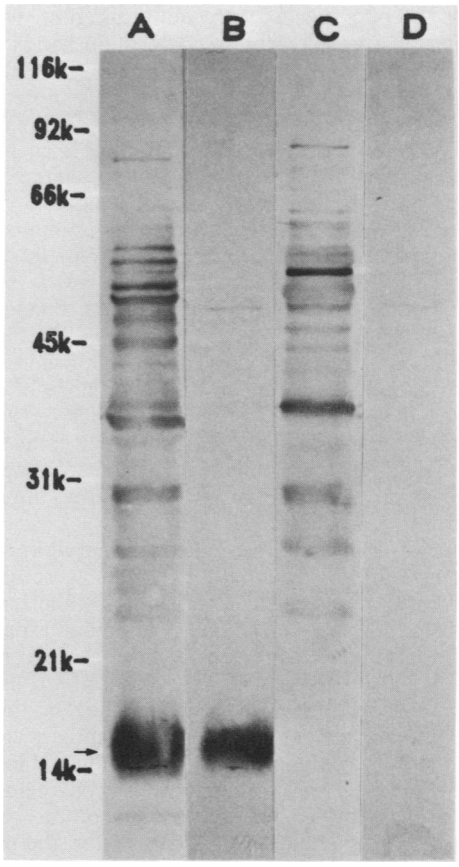

FIG. 4. Antigens of *T. hyodysenteriae*, isolate B204 (lanes A and B), and *T. innocens*, isolate B256 (lanes C and D), identified by probing with porcine convalescent-phase sera before (lanes A and C) and after (lanes B and D) absorption with isolate B256 cells and biotinylated anti-swine IgG. The arrow indicates a 16-kDa antigen in lane B unique to *T. hyodysenteriae*. Molecular size markers in kilodaltons are shown on the left.

Colonic secretions. Antigens from isolate B204 were reacted to colonic secretions from normal and convalescent pigs and to anti-swine biotinylated IgG (Fig. 3). One antigen at 49 kDa was identified with secretions from both the normal and convalescent pigs, whereas additional antigens were detected by antibody from convalescent-phase secretions at 59, 32, 27, 26, and 16 kDa. The 16-kDa antigen recognized by the convalescent-phase swine secretions was the same diffusely staining antigen recognized by convalescent-phase sera.

Absorbed swine sera. Antigens from *T. hyodysenteriae* and *T. innocens* B256 were reacted to antisera from convalescent pigs before and after absorption with 5×10^{10} cells of isolate B256. The unabsorbed sera recognized numerous antigens common to the two species of spirochetes (Fig. 4). The results with the absorbed sera confirmed the observation of shared antigens, with the sera recognizing a 49-kDa antigen in both isolates and a 16-kDa antigen only in *T. hyodysenteriae* isolate B204.

DISCUSSION

This is the first report of the separation of proteins from an isolate of *T. hyodysenteriae* or *T. innocens*. The protein

profiles for the two species were quite different, with only some similarities between the major protein bands. Mixing experiments confirmed these differences. The differences were quite surprising given the previously noted cross-reactions of each species to various sera (4, 9, 10). Perhaps the shared proteins are the dominant antigens in each species and are more immunogenic or perhaps the shared antigens are on proteins with different molecular weights.

The ontogeny of the immune system was examined in pigs infected with isolate B204. This demonstrated a quantitative increase in antigens detected with pooled swine sera at 0 days (preinfection) as compared with 27 (acute stage) and 67 (convalescent stage) days postinfection (Fig. 2). Pigs at 67 days postinfection had recovered from SD, whereas at 27 days postinfection, they were in the acute stage of disease, with clinical signs of SD. However, pigs which recover from SD are immune to reexposure (8, 12), which indicates that the additional antigens identified with serum specimens from convalescent pigs may be involved in initiating the immune response to the spirochete.

A number of antigens appeared to be shared between *T. hyodysenteriae* and *T. innocens*, but there were also antigenic differences between the two isolates when reacted to antisera from convalescent pigs. To actually determine whether these antigens were the same, convalescent-phase swine sera were absorbed with cells of *T. innocens* isolate B256. After absorption with the nonpathogen, the swine antisera identified two antigens, with one antigen at 16 kDa unique to the pathogenic species. This antigen stained as a broad band and may be two separate glycoproteins which migrate closely together. These results contrast with the results shown in the stained gels. Apparently, the unshared proteins found in isolate B204 at 72, 53, 46, and 44 kDa are not antigenic or the epitopes shared between the isolates are on different-molecular-weight proteins of *T. hyodysenteriae* and *T. innocens*. Other than differences in the lipopolysaccharide antigens of the two species, this is the first indication that *T. hyodysenteriae* contains an antigen at 16 kDa which is not found in nonpathogenic *T. innocens*. Because of this finding and the fact that the 16-kDa antigen was identified only with sera from pigs which had recovered from the disease, it is possible that this antigen is responsible for protecting pigs against SD.

As expected, fewer antigens were detected in *T. hyodysenteriae* isolates after probing with colonic secretions from convalescent pigs, mainly because the secretions had a lower antibody titer than did the sera. However, it was a surprise that only five antigens were identified with colonic secretions from convalescent pigs. Five of these antigens, at 58, 32, 27, 26, and 16 kDa, were not detected with secretions from normal pigs. The 16-kDa antigen is the same antigen which was prominent in blots from convalescent antisera. This further confirms that this antigen is involved in the immune response of pigs to SD.

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