Characterization of Virulence Marker Antigen of *Shigella* spp. and Enteroinvasive *Escherichia coli*

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Antisera produced in rabbits immunized with an enteroinvasive O143 strain of *Escherichia coli* were absorbed with an avirulent derivative of the same strain. The resulting sera have been previously shown to recognize enteroinvasive pathogens when used in an enzyme-linked immunosorbent assay. In the current study, Western blots (immunoblots) showed that such an absorbed rabbit antiserum recognized two proteins (IpaB and IpaC) which are encoded by a large, virulence-associated plasmid. These proteins are the apparent constituents of the virulence marker antigen which is expressed by shigellae and enteroinvasive *E. coli*.

Shigella species and enteroinvasive strains of *Escherichia coli* (EIEC) cause disease by invading and multiplying within the colonic epithelial cells. While isolation and identification of shigellae from clinical isolates is a routine matter, this is not the case for EIEC. Identification of these organisms by biochemical tests is not possible, and many isolates belonging to recognized EIEC serogroups are avirulent (11, 12, 14). Therefore, diagnostic tests employing DNA probes or enzyme-linked immunosorbent assay (ELISA) have been designed to allow identification of enteroinvasive pathogens. The latter assay uses hyperimmune rabbit antiserum raised against an EIEC strain of serogroup O143 and absorbed with an isogenic avirulent mutant of the same serogroup (11, 12). The cross-reacting antigenic moiety recognized by this serum was designated the virulence marker antigen (VMA) (11). The VMA ELISA successfully identified 83 virulent strains of *Shigella* species and EIEC (12), and the assay differentiated EIEC that were isolated from children with dysentery in Thailand (14). Avirulent strains of *Shigella* species and of EIEC serogroups were negative in the VMA ELISA (9).

Although the VMA ELISA proved to be a useful diagnostic tool, the nature of VMA was unclear (11). DNA probes recognizing portions of the 140-megadalton (MDa) plasmids which are necessary for virulence in EIEC and in *Shigella flexneri* (5, 13) have successfully identified enteroinvasive organisms (1, 14, 15), so it seemed likely that the virulence plasmids might encode cross-reacting antigen(s) such as VMA (12). Arguing against this hypothesis was the fact that the avirulent EIEC mutant (strain 2/33), which was used to absorb the VMA rabbit serum, retains the 140-MDa plasmid (11). However, it has recently been shown that VMA reactivity is transferred with the 140-MDa plasmid (10), and this finding strongly suggests that the VMA is plasmid encoded.

Since antibody recognizing four plasmid-encoded proteins of *S. flexneri* has been shown to cross-react with similar proteins in Western blots (immunoblots) using other *Shigella* species and EIEC O143 (4), we considered it likely that one or more of these proteins might constitute the VMA. Western blot analysis of convalescent-stage serum from monkeys or humans has shown that these plasmid-encoded proteins

are the immunodominant antigens in the event of a *Shigella* infection (8). Therefore, these proteins have been designated "invasion plasmid antigens," and the plasmid locus encoding them has been designated the *ipa* locus (2).

Figure 1 shows the results of a Western blot of the *E. coli* O143 strain which was used in the manufacture of VMA serum (lane A) and the avirulent derivative of this strain which was used to absorb the VMA serum (lane B). This immunoblot was developed with serum from a monkey which had been infected with *S. flexneri* 2a for 2 weeks before being bled. As expected, the convalescent-stage monkey serum recognized four proteins in the virulent EIEC strain which were not present in the avirulent mutant. These proteins are *ipa* gene products which have been designated IpaA (78 kDa), IpaB (62 kDa), IpaC (43 kDa), and IpaD (38 kDa) (7). In addition to these constituents, a 140-kDa plasmid-encoded protein (3, 8) was recognized in both strains. In contrast to its expression of the *ipa* gene products, the avirulent mutant apparently expresses wild-type levels of this protein.

Since Fig. 1 indicates that virulent EIEC O143 strain 2 expresses *ipa* gene products and avirulent strain 2/33 does not, it is possible that rabbit antiserum raised against the former strain would contain anti-*ipa* antibody which could not be absorbed by the latter strain. For Fig. 2, such a hyperimmune rabbit serum was used in the Western blot procedure. Strip I was incubated with unabsorbed serum raised against EIEC 2, strip II was incubated with the same antiserum after absorption with avirulent strain 2/33, and strip III was incubated with antiserum absorbed with the virulent immunizing strain. The rabbit antiserum recognized IpaB (62 kDa) and IpaC (43 kDa) from EIEC 2 (lane 1). Antibody recognized lipopolysaccharide O antigen and a protein of approximately 80 kDa, in addition to these plasmid-encoded proteins. The latter constituents were also recognized in the 2/33 avirulent mutant (lane 2). Absorption of the hyperimmune rabbit serum with this mutant removed antibody that recognized these shared antigens (strip II). This absorbed serum, which is similar to the VMA serum previously used in ELISA (9, 11, 12, 14), recognized only IpaB and IpaC proteins from virulent strain 2 (lane 3), and this serum did not react with strain 2/33 (lane 4). When the hyperimmune rabbit serum used on strip I was absorbed with whole cells of strain 2, the antibody recognizing *ipa* proteins was removed (strip III). The VMA ELISA uses

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FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis of *E. coli* O143 strain 2 (lane A) and strain 2/33 (lane B). Whole-cell lysates were prepared by heating bacteria at 100°C for 3 min in sodium dodecyl sulfate sample buffer, and bacterial components were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (3). An electroblot of the separated components was incubated overnight at 25°C with monkey serum diluted 1:50 in 2% casein filler. Two weeks before being bled, this monkey had been orally challenged with *S. flexneri* 2a. Serum immunoglobulin G binding to the immunoblot was stained with protein A-alkaline phosphatase (Polskies, Warrington, Pa.). The polypeptides labeled a through d are the previously reported IpaA, IpaB, IpaC, and IpaD plasmid-encoded products, respectively (1, 6, 7).

whole bacterial cells as antigen, and the latter absorption experiment suggests that the VMA serum recognizes *ipa* proteins which are located on the bacterial surface.

To confirm that VMA serum recognizes *ipa* proteins in many species of *Shigella* as well as in EIEC, a Western blot was performed with *Shigella* strains of different serogroups. The VMA serum recognized IpaB and IpaC in virulent strains of *Shigella sonnei* (Fig. 3, lane 2), *S. flexneri* (lanes 4, 5, and 10), and *Shigella dysenteriae* (lane 11). In contrast, plasmid-negative strains of *S. sonnei* (lane 1) and *S. flexneri* (lane 7 and 9) were not recognized by the VMA serum. An avirulent colonial variant of *S. flexneri* 2a (strain 2457-O, lane 6) also did not express the *ipa* proteins. Like the 2/33 EIEC strain, this strain carries the 140-MDa plasmid (6). This indicates that occasional avirulent strains of *Shigella* or EIEC may fail to express the *ipa* gene products even though they retain the invasion plasmid. Nonetheless, previous VMA ELISA results indicate that such VMA-negative, plasmid-positive strains are not usually found among clinical isolates (14).

In summary, Western blot analysis indicates that the VMA ELISA specifically recognizes proteins which are encoded by invasion plasmids of EIEC and *Shigella* species. Although the role of these proteins in the pathogenesis of enteroinvasive disease is unknown, there are presently no examples of enteroinvasive strains which do not express these products. Therefore, the VMA ELISA should be more reliable than serological testing as a diagnostic tool for

FIG. 2. Western blot analysis of *E. coli* O143 strain 2 (lanes 1, 3, and 5) and strain 2/33 (lanes 2, 4, and 6). Panel I was incubated with a 1:50 dilution of hyperimmune rabbit antiserum raised against live O143 strain 2, panel II was incubated with the same serum after absorption with avirulent strain 2/33, and panel III was incubated with hyperimmune antiserum which had been absorbed with O143 strain 2.

epidemiological studies of EIEC incidence. It should be pointed out, however, that animal-to-animal variations can be expected in absorbed rabbit sera such as the VMA antiserum. This quantitative variability can be overcome somewhat by pooling sera, but monoclonal antibodies recognizing *ipa* gene products, such as those which have recently been reported (7; J. A. Mills, J. M. Buysse, and E. V. Oaks, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D78, p. 84), should allow the development of more standardized ELISA reagents for identification of all enteroinvasive pathogens.

FIG. 3. Western blot analysis of virulent and avirulent strains of *Shigella* and EIEC (3, 4, 6) with anti-VMA serum. Lane 1, *S. sonnei* 53G form II (spontaneous avirulent mutant which has lost a 120-MDa plasmid); lane 2, *S. sonnei* 53G form I; lane 3, *S. flexneri* M90TA-2 serotype 5 (spontaneous avirulent mutant which has lost a 140-MDa plasmid); lane 4, *S. flexneri* M90T serotype 5; lane 5, *S. flexneri* J17B serotype 3; lane 6, *S. flexneri* 2457-O serotype 2a (spontaneous avirulent mutant retaining a 140-mDa plasmid); lane 7, *S. flexneri* 2457A₁ serotype 2a (spontaneous avirulent mutant which has lost a 140-MDa plasmid); lane 8, *S. flexneri* 2457T serotype 2a; lane 9, *S. flexneri* M25-8A serotype 1 (spontaneous avirulent mutant which has lost a 140-MDa plasmid); lane 10, *S. flexneri* M25-8 serotype 1; lane 11, *S. dysenteriae* JVA70 serotype 1; lane 12, *E. coli* 4608-58 serotype O143.

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