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Antibody Response of Monkeys to Invasion Plasmid Antigen D after Infection with *Shigella* spp.

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The antigen preparation most often used for determining the levels of antibodies to virulence-associated proteins of *Shigella* spp. consists of a mixture of proteins (including IpaB, IpaC, IpaD, and VirG*) extracted from virulent shigellae with water (water extract). To overcome the lack of specificity for individual antigens in the water-extract enzyme-linked immunosorbent assay (ELISA), the *ipaD* gene from *S. flexneri* has been cloned, expressed to a high level, and purified for use in a new ELISA for the determination of the levels of antibody against IpaD in monkeys and humans challenged with shigellae. The IpaD ELISA for serum immunoglobulins G and A correlated well with the water-extract ELISA in that monkeys infected with *S. flexneri* or *S. sonnei* responded with high serum antibody titers in both assays. The IpaD assay required less antigen per well, had much lower background levels, and did not require correction with antigens from an avirulent organism. In conjunction with the water-extract ELISA, it was possible to identify infected animals that did not respond to IpaD but did produce antibodies that reacted in the water-extract ELISA. This indicates that even though IpaB, IpaC, and IpaD are essential for the invasiveness phenotype, the infected host does not always produce antibodies against all components of the invasiveness apparatus.

Bacillary dysentery is a severe diarrheal disease caused by *Shigella* species. Several essential virulence factors of *Shigella* spp., including the invasion plasmid antigen (Ipa) proteins (IpaB, IpaC, and IpaD), VirG (IcsA), and lipopolysaccharide (LPS) elicit substantial antibody responses in the serum as well as in secretions after infection (1, 5, 6, 10, 11, 13). The most frequently used serological assays for measuring the antibody response against *Shigella* protein antigens are Western blots (immunoblots) and enzyme-linked immunosorbent assays (ELISAs). Western blots provide qualitative and semiquantitative information about antibodies against specific protein antigens, but they must be interpreted carefully. For example, in the IpaB region of blots, three serologically recognized antigens, including IpaB, IpaH, and HSP60, exist. The titers of antibodies are more readily determined by ELISAs. Two antigens routinely used in ELISAs for *Shigella* serodiagnosis are purified LPS (which must be prepared for each *Shigella* serotype to be analyzed) and the water-extractable antigens (1, 5, 10, 13). The latter antigen preparation is a heterogenous product which includes both invasiveness plasmid antigens (IpaB, IpaC, IpaD, and VirG*) and chromosomally encoded proteins and LPS (9, 10, 15). This antigen preparation is frequently used for measuring antibody levels in serum and secretions as well as for the detection of circulating antibody-secreting cells producing antibodies against the virulence-specific antigen complex (3, 4). It can be used for any species of *Shigella* or enteroinvasive *Escherichia coli* because the *ipa* genes are conserved in all serotypes. Unfortunately, it does not measure antibody levels against specific Ipa proteins or VirG, and it requires a correction for antibodies against chromosomally encoded proteins. This is accomplished by preparing a water extract from a plasmid-free *Shigella* strain that is the same serotype as the virulent preparation. To overcome the shortcomings of the water-extract ELISA, we have developed a new ELISA using purified, recombinant IpaD protein. IpaD is present in the water extract from virulent organisms and is a crucial factor in the invasiveness of shigellae (7, 9, 10). It is located in the outer membrane of shigellae, is exposed on the surface, and is possibly involved in the modulation of secretion of IpaB and IpaC (7, 9, 10). The IpaD ELISA, described here, will provide specificity and sensitivity in serological assays for monitoring the immune response to virulence-associated antigens after infection with shigellae. This will be useful in determining infection with *Shigella* spp. in nonimmune humans and higher primates in outbreaks of diarrheal disease as well as in seroprevalence surveys.

The PCR was used to produce a 1,000-bp *ipaD* fragment for insertion into the plasmid expression vector pET-15b. Primers, based on the published nucleotide sequence of *ipaD* (14), were designed to give an *ipaD*-containing DNA fragment that could be inserted in frame into pET-15b after each was *Nde*I-*Bam*HI digested. The resulting PCR product was treated with *Nde*I and *Bam*HI and ligated into *Nde*I-*Bam*HI-digested pET-15b. *E. coli* BL21(DE3)pLysS was transformed with the ligation mixture, and transformants were selected on Luria-Bertani agar containing 0.03 mg of ampicillin per ml. Transformants were screened for inserts by the appearance of a 1,000-bp PCR product by using the T7 RNA polymerase promoter and terminator sequences as primers. Insertion of the *ipaD* fragment was confirmed by double-stranded DNA sequencing.

Overexpression of the resulting *ipaD* fusion gene in *E. coli* BL21 (DE3)pLysS was possible with IPTG (isopropyl-β-D-thiogalactopyranoside) induction (Fig. 1). The fusion protein product with a size of about 42 kDa (Fig. 1) consisted of full-length IpaD fused to a 3-kDa leader peptide containing six histidine residues. This leader peptide enabled the fusion protein to be affinity purified by passing a cytosolic extract, containing the recombinant protein, over a Ni^{2+} column followed by elution of the IpaD fusion protein with 1 M imidazole. The IpaD fusion protein was approximately 95% pure as determined with Coomassie-stained gels, and it reacted with three different rabbit anti-IpaD serum samples made against three different IpaD peptides (9).

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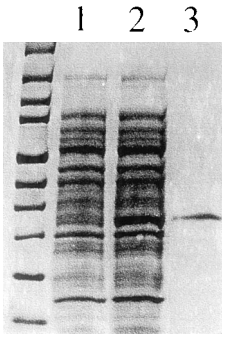

FIG. 1. Affinity purification of the IpaD-fusion protein. *E. coli* BL21(DE3)pLysS harboring the recombinant plasmid pWPD10 was induced with IPTG, resulting in the appearance of a protein observed on Coomassie-stained sodium dodecyl sulfate–10% polyacrylamide gels with an apparent molecular mass of 42 kDa (lane 2). No such protein species was observed in uninduced cells (lane 1). The crude cytosolic extract was passed over an immobilized support to which Ni^{2+} had been reversibly chelated. The column was washed extensively, and the IpaD fusion protein eluted with the same buffer containing 1 M imidazole. The purified IpaD fusion protein (lane 3), prepared in this way, was routinely greater than 95% pure as judged with Coomassie-stained sodium dodecyl sulfate-polyacrylamide gels. Molecular mass standards (in the unmarked lane) are 212, 158, 116, 97, 66, 56, 43, 37, 26, and 20 kDa, respectively, from top to bottom.

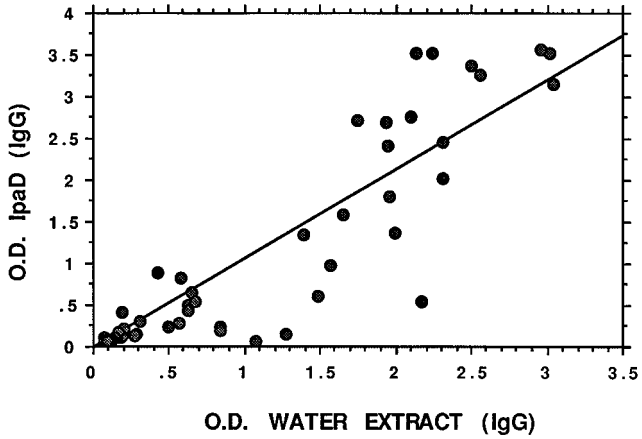

FIG. 2. Correlation between the IpaD ELISA and the water extract ELISA. A total of 47 serum samples were collected from monkeys 2 weeks after oral challenge with *S. flexneri* 2a. All serum samples were diluted 1/800 for this analysis and were probed with an anti-IgG conjugate. A comparison of the optical density values obtained for the IpaD ELISA versus those for the water-extract ELISA is shown (r of 0.942, P of <0.0001). The solid line represents the best fit obtained by regression analysis.

To date it has not been possible to measure antibody levels against individual Ipa proteins in an ELISA because of the lack of pure, stable Ipa proteins. To establish the usefulness of the IpaD ELISA, we compared the serum antibody levels against IpaD, water-extracted antigens, and LPS in monkeys infected with *S. flexneri* 2a.

Purified IpaD, diluted in phosphate-buffered saline (PBS; pH 7.4), was used to coat polystyrene 96-well plates (Corning/Costar, Cambridge, Mass.) at a final concentration of 0.05 μg per well. The antigen was incubated on the plates overnight at 4°C. The wells were subsequently blocked with 2% casein in a Tris-buffered saline solution. Immune sera, diluted in casein, were incubated with the antigen for 2 h. After the wells were washed with PBS containing 0.05% Tween 20, alkaline phosphatase-conjugated anti-human immunoglobulin G (IgG) or IgA (Kirkegaard & Perry, Gaithersburg, Md.) was added for 1 h, the wells were washed, and finally, the substrate (para-nitrophenylphosphate, 1 mg/ml in diethanolamine buffer) was added to each well and allowed to develop for 30 min. All incubations with antisera and washes were performed at room temperature. The optical density (OD) was determined with a Molecular Dynamics microplate reader at a wavelength of 405 nm. The levels of serum antibodies against LPS and virulence-associated antigens extracted with water were measured with an ELISA, as previously described (10).

Antibody levels to IpaD, water extract, and LPS were determined for sera collected from 47 monkeys from four different treatment groups, 2 weeks after infection with *S. flexneri* 2a (Table 1). The quantity of shigellae administered orally ranged from 2×10^3 CFU per monkey to 2×10^9 CFU per monkey, with each successive group of 12 monkeys receiving 100-fold-more shigellae. The various doses of shigellae gave a range of immune responses and clinical disease. The baseline anti-IpaD levels were uniformly low for all monkeys. Infection with lower doses of shigellae (2×10^3 and 2×10^5 CFU) did not produce disease and resulted in a minimal serum antibody response. At the highest dose (2×10^9 CFU per monkey), 10 of 11 monkeys had diarrhea, 10 of 11 produced serum IgG antibodies against IpaD, and all 11 were positive for IgG antibodies against the water extract (Table 1). Similar responses for IgA against IpaD and IgG for LPS were found. The level of response against LPS appeared somewhat lower in the group of animals receiving 2×10^7 CFU. The data in Fig. 2 indicate that a correlation

TABLE 1. Serological response to IpaD, water extract, and LPS in monkeys challenged with various doses of *S. flexneri* 2a

Dose (CFU)	No. of animals infected/no. tested[a]	Mean OD ± SD[b]				No. of animals that seroconverted against antigen indicated/no. tested[c]			
		IpaD/IgG		IpaD/IgA					
		Preinfection	Postinfection	Preinfection	Postinfection	IpaD (IgG)[d]	IpaD (IgA)[d]	Water extract	LPS[d]
2.0×10^3	0/12	0.26 ± 0.21	0.23 ± 0.17	0.44 ± 0.67	0.37 ± 0.60	0/12	0/12	0/12	0/12
2.0×10^5	0/12	0.33 ± 0.48	0.34 ± 0.37	0.46 ± 1.01	0.49 ± 0.97	1/12	1/12	2/12	0/12
2.0×10^7	2/12	0.62 ± 0.73	1.67 ± 1.01[d]	0.25 ± 0.18	0.82 ± 0.63[d]	8/12	8/12	7/12	4/12
2.0×10^9	10/11	0.32 ± 0.18	2.52 ± 1.27[d]	0.19 ± 0.19	0.93 ± 0.80[d]	10/11	8/11	11/11	10/11

[a] Monkeys exhibiting diarrhea within 72 h of infection were considered positive for disease (infected) due to the *Shigella* challenge.

[b] The mean OD values at a 1/1,000 dilution of the preinfection and postinfection serum samples are given. The postinfection serum samples were collected 14 days after challenge.

[c] The various antigens used to determine the serological response included purified recombinant IpaD, water-extractable virulence-associated antigens (water extract), and LPS from *S. flexneri* 2a. IgA and IgG levels were determined against IpaD. Only the IgG level was determined against water extract and LPS. Positive samples represent those serum samples which had a twofold-higher OD value in the postinfection serum sample than in the preinfection serum sample.

[d] The difference between the mean OD values was statistically significant, as determined by the Wilcoxon signed rank test ($P < 0.005$).

TABLE 2. Serum IgG response to IpaD in monkeys challenged with either *S. flexneri* 2a or *S. sonnei*

Challenge species[a]	No. of animals with clinical signs of disease/ no. tested	Mean OD ± SD[b]		No. of monkeys with IpaD seroconversion[c]
		Preinfection	Postinfection[d]	
S. flexneri 2a	8/12	0.06 ± 0.03	1.10 ± 0.77	10
S. sonnei	7/12	0.19 ± 0.18	1.01 ± 0.62	12

[a] Monkeys were challenged orally with 2×10^{10} CFU of either *S. flexneri* 2a or *S. sonnei*. The details of this challenge study have been previously published (2).

[b] The mean OD values at a 1/1,000 dilution of the pre- and postinfection samples are given. The postinfection serum samples were collected 9 days after challenge.

[c] Those animals which had a twofold-higher OD value (at a 1/1,000 dilution) in the postinfection serum sample than in the preinfection sample were considered positive (to have seroconverted).

[d] The difference between the pre- and postinfection mean OD value had a *P* value of <0.005 by the Wilcoxon signed rank test.

between the IpaD and water-extract ELISA (*r* of 0.942, *P* of <0.0001) existed when IgG levels were determined. Even though the relationship between the IpaD (IgG) ELISA and the water-extract (IgG) ELISA was very strong, there were two monkeys (of 47) that had serum IgG antibodies to the water-extracted antigens but not to IpaD. Sera from these monkeys did not react with IpaD on Western blots but did react with IpaB and IpaC (data not shown). Regression analysis between the LPS and IpaD ELISA and the LPS and water-extract ELISA gave *r* values of 0.814 (*P* of <0.0001) and 0.809 (*P* of <0.0001), respectively.

The water-extract ELISA is useful in determining antibody responses against all members of the genus *Shigella* in large part because the *ipa* genes, which encode the dominant antigens in the water extract, are conserved in all invasive *Shigella* spp. and enteroinvasive *E. coli*. The data in Table 2 indicate that, as expected, monkeys infected with either *S. sonnei* or *S. flexneri* produced serum antibodies against IpaD. Interestingly, 2 of the 12 *S. flexneri*-infected monkeys did not show increased levels of IgG against IpaD in serum collected 9 days postinfection. Similar to the single animal in the group inoculated with 2×10^9 CFU, these two animals did produce antibodies against the water extract, and specifically against IpaB and IpaC (as determined by Western blot analysis) (9).

In this study, we demonstrated that purified, recombinant IpaD can be used to monitor the specific immune response in monkeys infected with a *Shigella* sp. The assay requires small quantities of antigen, has low background levels, and is specific for IpaD. Unlike the water-extract ELISA, it does not require correction with an antigen from an avirulent source because it uses a pure antigen. In addition, the recombinant IpaD is expressed to high levels in the pET15b vector, is relatively stable (compared with the easily degraded IpaB and IpaC), and is easy to purify via affinity chromatography on a nickel column.

The IpaD ELISA correlated very well with the water-extract ELISA, which was expected since IpaD is present in the water extract. It was noted that a few monkeys infected with shigellae did not produce detectable antibodies in the IpaD ELISA but were positive in the water-extract ELISA. These serum samples, which were all evaluated by Western blot, were negative for IpaD and positive for IpaB and IpaC antibodies (9). The ability of the water-extract ELISA to pick up more positives than the IpaD ELISA is very likely related to a higher frequency of antibody responses against IpaB and IpaC in infected animals. Therefore, it is unlikely that the IpaD ELISA can replace the water-extract ELISA but that it will be useful because of its specificity for IpaD and the lack of background problems. Sensitivity approaching that of the water-extract ELISA will likely occur when purified IpaB or IpaC becomes available. Even so, by using the water-extract ELISA along with the IpaD ELISA, it is possible to identify subgroups of infected individuals that have unique immunological responses to the Ipa proteins. The ability to distinguish subgroups of infected individuals will enable a better understanding of the host immune response to *Shigella* infection. In addition, these results indicate that all components of the invasiveness protein complex of *Shigella* spp. (7, 8) are not recognized equally by all infected animals.

The IpaD ELISA may also be useful for seroepidemiological studies designed to identify populations with a high or low frequency of exposure to *Shigella* spp. without the need to consider multiple serotypes of the genus *Shigella*. For example, in a group of healthy, adult U.S. citizens, the incidence of individuals with IpaD antibodies was approximately 15% (9). A similar frequency was noted for antibodies against the water extract. In areas of endemicity, the frequency of IpaD antibodies in an adult population would probably be much higher (11, 13).

As other plasmid-encoded antigens, such as IpaB, IpaC, and VirG, are purified, it will be possible to evaluate the specific immune response both at the humoral and the totally uncharacterized cellular level for each of these components. This type of information, in conjunction with more-detailed analysis at the peptide level (12), may be necessary for determining immune response patterns that are correlated with disease outcome or protection against future disease.

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