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Serum Immune Response to *Shigella* Protein Antigens in Rhesus Monkeys and Humans Infected with *Shigella* spp.

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The serum antibody response to proteins encoded by the virulence-associated plasmid of *Shigella flexneri* was determined in monkeys challenged with virulent *S. flexneri* serotype 2a. With water-extractable antigen in an enzyme-linked immunosorbent assay, a significant increase in antibody titer against proteins from a plasmid-carrying, virulent strain of *S. flexneri* serotype 5 could be demonstrated in convalescent sera. There were minimal antibody titers against proteins from an avirulent (plasmid-free) organism. Previously identified plasmid-coded polypeptides a, b, c, and d were predominant antigens recognized by a majority of the convalescent sera in immunoblots. An additional 140-megadalton plasmid-coded polypeptide was also recognized by half of the sera. Convalescent serum from an infected monkey recognized antigens on the bacterial surface in several different plasmid-containing *Shigella* species and in an enteroinvasive *Escherichia coli* strain. A survey of sera obtained from children 5 to 10 years of age who had been infected with *S. flexneri* or *S. sonnei* revealed high enzyme-linked immunosorbent assay titers in both acute and convalescent sera against a water extract from a virulent *Shigella* strain. In contrast, children under 3 years of age had no antibody titer in either acute or convalescent sera against the virulence-associated shigella proteins, while 3- to 4-year-old children mounted an immune response against these proteins only in convalescence.

Bacteria of the genus *Shigella*, the etiological agents of bacillary dysentery, require both chromosomal and extrachromosomal loci for expression of the virulent phenotype (4, 7, 8, 23). The extrachromosomal genes, which are necessary for invasion of the colonic epithelium, are carried on 120- to 140-megadalton (MDa) plasmids (17, 24). Although these plasmids are not identical, there are highly conserved genes present in plasmids from all virulent *Shigella* strains (22). At least seven polypeptides (designated a through g) have been identified as unique products of the virulence plasmids of *Shigella* spp. and enteroinvasive *Escherichia coli* (9, 10). Four of these polypeptides (a, b, c, and d) are synthesized from a 37-kilobase fragment of cloned plasmid DNA that has the capacity to restore HeLa cell invasiveness in a shigella recipient which has lost the 140-MDa Plasmid (17). Recently, we have shown that plasmid proteins b, c, and d are antigenically cross-reactive in different shigella serotypes and in an enteroinvasive strain of *E. coli* (9).

The association of the plasmid-coded polypeptide antigens with the invasive phenotype implies that they might be key antigens eliciting a protective immune response in primates and humans. Indeed, immunoblot analysis of convalescent serum from a rhesus monkey which had been challenged with shigellae indicates that antibody recognizing polypeptides a, b, c, and d is present after infection (9). Therefore, we have further characterized the immune response against plasmid-coded antigens by enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis of sera from a large group of monkeys challenged with *S. flexneri* serotype 2a. In addition, acute and convalescent sera from humans infected with either *S. flexneri* or *S. sonnei* have been analyzed. These studies indicate that plasmid-coded polypeptides a through d, along with a newly identified plasmid-associated polypeptide of 140 kDa, are the principal proteinaceous antigens inducing a serum immune response during shigella infection in the natural host.

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MATERIALS AND METHODS

Bacterial strains. The thirteen bacterial cultures used in this study are listed in Table 1. Cultures were routinely grown in Penassay broth (Difco Laboratories, Detroit, Mich.) at 37°C.

Extraction of plasmid proteins b and c. Overnight cultures (5 liters) of *S. flexneri* 5 strain M90T or WR6078 were grown at 37°C in Penassay broth. The bacteria were collected by centrifugation and suspended in 500 ml of distilled water containing 5 μM phenylmethylsulfonyl fluoride. This mixture was placed on a rotary shaker at 37°C for 2 h. The preparation was then centrifuged at 16,000 × *g* for 20 min, and the supernatant was collected. This supernatant was centrifuged at 100,000 × *g* for 2 h. The supernatant from this procedure was concentrated 10-fold in an ultrafiltration apparatus (Amicon Corp., Danvers, Mass.) with a PM-10 membrane. The concentrated extract was assayed for protein content by the method of Lowry et al. (15) and then stored at −20°C. Before use in the ELISA, the water extract was assessed for the presence of polypeptides b and c by immunoblot procedures with rabbit antisera recognizing these polypeptides. The same lot of water extract from M90T or WR6078 was used throughout this study.

ELISA. An ELISA was used to quantitate serum antibody recognizing shigella antigen preparations and to determine the cross-reactivity of the antibody with several invasive and noninvasive strains of *Shigella* and *E. coli*.

To measure serum antibody levels in monkeys and humans, the water extract from either invasive or noninvasive strains of *S. flexneri* 5 was diluted in phosphate-buffered saline (PBS) (pH 7.4). This diluted extract was used to coat 96-well polystyrene plates (Costar 3590; Costar, Cambridge, Mass.) at a concentration of 1 μg of protein per well. The

TABLE 1. Bacterial strains and plasmid content

Strain	Serotype	Plasmid content	Virulence	Origin (reference) and comments
S. flexneri				
2457T	2a	Two large plasmids and small, cryptic plasmids	+	WRAIR[a]
2457O	2a	Same as 2457T	−	LaBrec et al. (12); spontaneous colonial mutant of 2457T; plasmid proteins are not synthesized (9)
WR6070 (M4243A1)	2a	One large plasmid and cryptic plasmids	−	Sansonetti et al. (24); spontaneous colonial mutant of M4243
J17B	3a	One large plasmid and cryptic plasmids	+	WRAIR
M76-49	4b	One large plasmid and small, cryptic plasmids	+	WRAIR
M90T	5	One large plasmid and cryptic plasmids	+	WRAIR
WR6078 (M90TA2)	5	Small, cryptic plasmids	−	Sansonetti et al. (24); spontaneous colonial variant of M90T
CCH060	6	Two large plasmids and small, cryptic plasmids	+	WRAIR
S. sonnei				
53G	Form I	One large plasmid and small cryptic plasmids	+	WRAIR
53GII	Form II	Small, cryptic plasmids	−	Kopecko et al. (11); spontaneous colonial variant of 53G
S. dysenteriae				
JVA70	1	One large plasmid and small, cryptic plasmids	+	WRAIR
E. coli				
395-1			−	WRAIR; Nal^r of K-12 1133
4608-58	0143	One large plasmid	+	WRAIR

[a] WRAIR, Walter Reed Army Institute of Research.

antigen was allowed to attach overnight at 25°C, and the supernatant was aspirated. Filler consisting of 100 μl of 5% fetal calf serum (FCS) in PBS was added to the wells for 1 h at 25°C. The antigen-coated plates were then rinsed twice with washing buffer (PBS containing 0.1% FCS and 0.05% Tween 20). Monkey sera, serially diluted in PBS containing 1% FCS, was added to the antigen-coated wells for 2 h at 25°C, followed by four rinses in washing buffer. Alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) in a 1/500 dilution (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added for 1 h, followed by another wash. The substrate (*p*-nitrophenylphosphate, 1 mg/ml, 100 μl per well) was added and developed as previously described (5) with the optical densities at 405 nm determined on a Microelisa Auto-Reader (MR 500; Dynatech Laboratories, Inc., Alexandria, Va.). Endpoints for titer determination were the last dilution having an optical density of 0.200 or greater.

The reactivity of the monkey antiserum with *Shigella* strains of various serotypes and enteroinvasive *E. coli* was also determined by an ELISA. Bacterial cultures were grown overnight in Penassay broth at 37°C. The cells were collected by centrifugation and suspended in carbonate buffer (27) to approximately 10^9 cells per ml, and 100 μl was added per well to a 96-well polystyrene microtiter plate. The cells were allowed to attach overnight at 25°C, and then filler was added the next morning, followed by washing as described above. The monkey serum used in this assay was diluted in PBS with 1% FCS from 1:200 to 1:10,000. This diluted serum was added to microtiter plates (100 μl per well) for 2 h at 25°C. The antiserum was removed, and the plates were washed and processed for the ELISA as described above.

Oral challenge of monkeys. Rhesus monkeys were challenged with virulent *S. flexneri* 2a (2457T) during a vaccine trial. Over a span of 7 days, 20 monkeys in the placebo group were fed three doses of approximately 10^{10} cells of *E. coli* 395-1, while the vaccine group (also 20 monkeys) was fed three doses of an *E. coli* vaccine strain expressing the *S. flexneri* 2a somatic antigen. This vaccine also harbored the 140-MDa plasmid from *S. flexneri* 5 (5). The animals were challenged 2 weeks later with 10^{10} cells of virulent *S. flexneri* 2a (2457T). Blood was collected before vaccination, 10 days after the regimen of *E. coli*, and 2 weeks after challenge with the virulent shigellae. A more detailed account of the vaccine study has been reported previously (5).

Human sera. The human sera used in this study were kindly provided by Peter Echeverria of the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Blood samples were obtained from culture-positive children during the acute phase of gastrointestinal illness (within 24 h of presentation with symptoms) and during convalescence (four weeks after the acute phase).

Electrophoresis. Gels consisting of 14% (wt/vol) acrylamide cross-linked with N_1N^1-diallytartardiamide in a discontinuous polyacrylamide gel electrophoresis (PAGE) system with Laemmli buffers (13) were employed for separation and analysis of shigella polypeptides.

Immunoblotting (Western blotting) procedure. Whole-cell sodium dodecyl sulfate (SDS) lysates of bacteria were prepared as previously described (9), and the bacterial components were separated by SDS-PAGE. These components

were then electroeluted onto nitrocellulose (Bio-Rad Laboratories, Richmond, Calif.) in a Tris-glycine-methanol buffer (26) at 1 A for 90 min. The nitrocellulose sheets were blocked with a Tris-buffered saline solution containing 2% (wt/vol) casein (Sigma Chemical Co., St. Louis, Mo.). Human or monkey sera, diluted 1/200 in casein filler, were incubated for 2 h at 25°C with the nitrocellulose strips. The strips were then washed as previously described (2) and incubated with staphylococcal protein A (Pharmacia, Inc., Piscataway, N.J.) labeled with ^{125}I. After 1 h, the strips were washed again, air dried, and exposed to X-ray film (Kodak Blue Brand; Eastman Kodak Co., Rochester, N.Y.) overnight. In all immunoblot experiments, control strips were reacted with rabbit antisera containing antibody against plasmid proteins b, c, or d to identify these polypeptides. These rabbit antisera have been described (9).

RESULTS

Water extraction of shigella proteins. Several plasmid-coded proteins of invasive shigellae are associated with the outer membrane (10), and extraction of such outer membrane proteins is often possible by shaking bacteria in deionized water or low-ionic-strength buffers (3, 25). An immunoblot with monkey antiserum containing antibody against plasmid-coded polypeptides (9) showed that water extracts from a virulent strain of *S. flexneri* 5 contained plasmid proteins b, c, and d, with proteins b and c being present in relatively high concentration (Fig. 1).

ELISA with water-extractable antigen. Retrospective studies were performed on monkey sera collected during a vaccine trial (5) and on human sera collected from patients presenting with naturally acquired shigella infections. The antibody response of 40 monkeys as measured by ELISA is shown in Table 2. The animal sera did not contain significant levels of antibody against a water extract from virulent or avirulent shigellae (strains M90T and WR6078, respectively) either before or after administration of the vaccine or placebo. Two weeks after oral challenge with virulent *S. flexneri* 2a (2457T), most of the animals exhibited significantly increased levels of serum antibody (average titer of 1,855 and 1,128 in the placebo and vaccine groups, respectively) against a water extract from virulent *S. flexneri* 5. Only background levels of antibody reacted with an extract from the avirulent strain. Since the antigens used in the ELISA were extracted from a serotype 5 strain and the monkeys were challenged with a serotype 2a strain, detection of cross-reacting serum antibody against the heterologous lipopolysaccharide was also minimized.

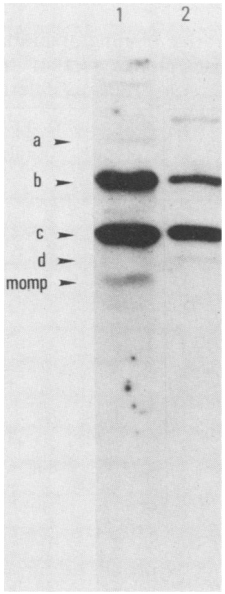

FIG. 1. Immunoblot of whole-cell extracts and water extracts from virulent *S. flexneri* 5 (M90T) separated by SDS-PAGE. Lanes: 1, whole-cell extract; 2, water extract. Convalescent monkey serum containing antibody against plasmid proteins a, b, c, and d was incubated with the antigen as described in the text.

TABLE 2. Serum IgG responses measured by ELISA in pre- and postchallenge sera from monkeys fed shigella vaccine or an *E. coli* placebo[a]

Serum	Placebo group titers[b]		Vaccine group titers[b]	
	Vir$^+$	Vir$^-$	Vir$^+$	Vir$^-$
Prechallenge	70 ± 10	50	55 ± 3	50
Postplacebo (or postvaccine)	72 ± 18	53 ± 2	72 ± 11	50
Postchallenge	1855 ± 493	75 ± 19	1,128 ± 436	57 ± 4

[a] Pertinent details of the vaccine study are given in materials and methods. The placebo group was fed an *E. coli* K-12 strain, and the vaccine group was fed a similar *E. coli* strain carrying the 140-MDa plasmid of *S. flexneri*, in addition to other *Shigella* spp. chromosomal loci. All animals were challenged orally with virulent *S. flexneri* 2a.

[b] Expressed as the mean ± the standard error of the mean. Antigens in the ELISA were water extracts obtained from either invasive, virulent, (Vir$^+$) *S. flexneri* 5 (M90T) or noninvasive, avirulent (Vir$^-$) *S. flexneri* 5 (WR6078).

The serum antibody response in several confirmed cases of shigellosis in patients living in Thailand is summarized in Table 3. Although the ELISA data from this field study were not as consistent as the data from the monkey challenge study, it appears that humans also produce high levels of antibody against the shigella plasmid-coded protein antigens after naturally acquired infection. Paired sera from four of five children less than 5 years of age had minimal antibody in acute sera recognizing shigella antigens and increased antibody titers in convalescent sera against plasmid-coded antigens. Children older than 4 years had antibody in acute sera which recognized virulence-associated antigens as measured by the ELISA. These antibody levels were increased fourfold in only one of four of the convalescent sera from older children.

ELISA with whole bacterial cells as antigens. It has been shown recently that rabbit antisera raised against plasmid-coded polypeptides b, c, or d from *S. flexneri* 5 (M90T) cross-reacts with similar polypeptides in immunoblots of various *S. flexneri* serotypes, *S. sonnei*, and enteroinvasive *E. coli* (9). To determine whether the antibody in convalescent monkey serum also cross-reacts with plasmid-coded antigens in enteroinvasive strains of *Shigella* and *E. coli*, an ELISA was performed with these bacteria as antigens. Whole bacterial cells were used to coat the ELISA plates. The serum was absorbed with plasmid-free, avirulent *S. flexneri* 2a (WR6070) to minimize recognition of chromosomal products. The reactivity of sera collected from a monkey before and after challenge with virulent *S. flexneri* 2a is presented in Table 4. The convalescent sera exhibited high antibody titers against undisrupted cells of enteroinvasive strains of *S. flexneri*, *S. dysenteriae* 1, *S. sonnei*, and *E. coli*. *Shigella* strains that did not contain a virulence-associated plasmid (WR6078 and 53GII) or which contained a plasmid with an expression defect (2457O) (9) were not recognized by the monkey sera.

TABLE 3. Human serum antibody response to *Shigella* spp. plasmid polypeptides[a]

Patient	Patient age (yr)	Infecting organism	Acute serum			Convalescent serum		
			ELISA titer[b]		Plasmid antigens recognized[c]	ELISA titer[b]		Plasmid antigens recognized[c]
			Vir+	Vir−		Vir+	Vir−	
AE-31	1	*S. flexneri* 2	<50	<50	ND[d]	400	<50	ND
AE-30	2	*S. flexneri* 2	50	<50	ND	100	100	ND
AE-16	3	*S. sonnei* I	50	<50	ND	1,600	<50	a, b, c, d
AE-36	4	*S. flexneri* 2	<50	<50	ND	1,600	200	ND
AE-39	4	*S. flexneri* 2	200	<50	b[e]	≥6,400	100	a[e], b, c, d
AE-8	5	*S. flexneri* 3	6,400	<200	a, b, c, d[f]	3,200	<200	140K, a, b, c, d[f]
AE-15	6	*S. flexneri* 2	3,200	<200	a, b, c, d	1,600	<200	140K, a, b, c, d[f]
AE-13	7	*S. flexneri* 2	1,600	<200	a, b, c, d[f]	12,800	<200	140K, a, b, c, d[f]
AE-7	10	*S. flexneri* 2	1,600	<200	a, b, c, d[f]	3,200	<200	140K, a, b, c, d[f]

[a] Shigellae were isolated from patients after 6 to 72 h of illness. Acute sera were obtained at this time. Convalescent sera were collected 20 to 28 days after collection of the acute sera. Antigen was obtained from virulent (Vir+) or avirulent, plasmid-negative (Vir−) *S. flexneri* 5 strains M90T and WR6078, respectively.
[b] The reciprocal of the last dilution having an optical density at 405 nm of 0.200 or greater.
[c] Determined by immunoblot procedures.
[d] ND, None detected.
[e] Weak reactivity on immunoblots.
[f] Other protein antigens were also recognized by these sera.

Immunoblot analysis of monkey sera. The ELISA results indicated that a significant portion of the serum antibody response in shigella-infected monkeys was directed against plasmid-associated antigens. To determine whether the antibody recognized any of the plasmid-coded proteins previously identified (9), immunoblot analysis was performed on serum taken pretreatment, posttreatment, and post-challenge. The sera obtained pretreatment and either postplacebo or postvaccine did not contain demonstrable antibodies against either the plasmid-containing or the plasmid-negative strain (data not shown). After challenge with *S. flexneri* 2a, all 40 animals mounted an antibody response that was measurable by immunoblot analysis. The immunodominant antigens in the vaccine and placebo groups were the plasmid-coded polypeptide antigens 140K, a, b, c, and d (Fig. 2). The immunoblot results obtained from the placebo and vaccine groups are summarized in Table 5. Plasmid protein antigens a, b, and c were recognized by a high percentage of sera in both groups, while plasmid proteins 140K and d were recognized by 50 and 62% of the animal sera, respectively. Antibody to the major outer membrane protein (MOMP) of *S. flexneri*, which is present in both invasive and noninvasive strains, was present in 35 of 40 monkeys tested. These results conclusively demonstrate that the plasmid-coded shigella proteins are dominant immunogens recognized by a majority of animals that have been orally challenged with virulent *S. flexneri*.

Immunoblot analysis of human sera. Immunoblots were also used to determine which shigella proteins are immunodominant antigens in humans infected with shigellae. Figure 3 shows the antigens recognized by the sera of a 6-year-old male infected with *S. flexneri* 2a (lanes 1 through 4) and a 3-year-old male infected with *S. sonnei* (lanes 5 through 8). In this gel the separated polypeptides from virulent *S. flexneri* 5 (M90T) were in the odd-numbered lanes, and in the even-numbered lanes were polypeptides of the plasmid-free mutant (WR6078). The acute serum (obtained 7 h after the onset of illness) from the 6-year-old (lanes 1 and 2) contained antibody against plasmid-coded polypeptides 140K, a, b, c, and d (lane 1), in addition to several other polypeptides that are in both plasmid-containing (lane 1) and plasmid-free strains (lane 2). Convalescent serum (obtained 30 days after infection) from the same patient contained increased quantities of antibody to plasmid-coded polypeptides a, c, and d, as indicated by the heavier bands on the immunoblot (lane 3). This antibody specificity was typical of children over 4 years of age (Table 3). Two children under 3 years of age did not exhibit antibody against plasmid-coded polypeptides in either acute or convalescent sera (data not shown), but one 3-year-old who had been infected with *S. sonnei* did mount an immune response against these polypeptides in convalescence (Fig. 3, lane 7).

DISCUSSION

Recent immunoblot analysis of serum from a rhesus monkey which had been orally challenged with a virulent strain of *S. flexneri* established that four virulence-associated, plasmid-coded polypeptides were recognized by serum IgG in this animal (9). Since only one monkey was

TABLE 4. Comparative binding of antibody from prechallenge and convalescent rhesus monkey serum[a] to whole cells of various *Shigella* and *E. coli* strains as determined by ELISA

Strain	Serotype	Virulence	ELISA titer[b]	
			Pre-challenge	Post-challenge
S. flexneri				
2457T	2a	+	<200	≥10,000
2457O	2a	−	<200	≤200
J17B	3	+	<200	≥10,000
M76-49	4b	+	<200	≥10,000
M90T	5	+	<200	≥10,000
WR6078	5	−	<200	<200
CCH060	6	+	<200	≥10,000
S. dysenteriae JVA70	1	+	<200	≥10,000
S. sonnei				
53G	Form I	+	<200	≥10,000
53GII	Form II	−	<200	400
E. coli 4608-58	0143	+	<200	≥10,000

[a] Convalescent serum was from a monkey which has been infected with *S. flexneri* 2a strain 2457T. The serotype-specific antibody was absorbed from this serum with *S. flexneri* 2a strain WR6070, an avirulent strain which did not carry a 140-MDa plasmid.
[b] Reciprocal of the last dilution of antiserum showing an optical density of 0.2 or more.

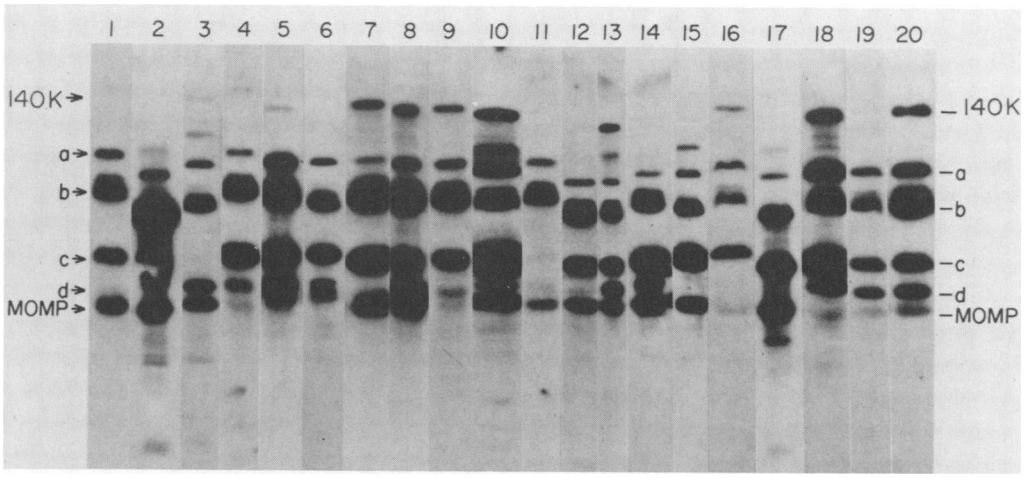

FIG. 2. Recognition of plasmid protein antigens by monkeys infected with *S. flexneri* 2a. Sera collected 2 weeks postchallenge from 20 monkeys (lanes 1 through 20) were reacted with virulent *S. flexneri* 5 (M90T) whole-cell lysates that had been separated by SDS-PAGE and electroblotted to nitrocellulose. The plasmid protein antigens 140K, a, b, c, and d are noted. One MOMP was also antigenic. This figure is a composite of several blots.

used in the initial study, additional experiments were undertaken to determine the reproducibility of this immune response in monkeys and humans. In addition to qualitative immunoblot analysis of antibodies against individual polypeptides, the ELISA was adapted for rapid quantitative analysis of the immune response to shigella antigens. The ELISA was also used to demonstrate that antibody against plasmid-coded proteins recognized outer membrane constituents on the surface of virulent but not avirulent strains of *Shigella* and enteroinvasive *E. coli* regardless of serotype. This indicates that the antibody was not directed against chromosomal products, such as porins, which would be expressed by both the virulent parent and the avirulent mutant. Pal et al. (20) have recently demonstrated cross-reactive antigens with an ELISA using invasive, plasmid-containing strains of *Shigella* and enteroinvasive *E. coli*. These investigators used hyperimmune rabbit antiserum which was raised against the latter organisms and absorbed with a mutant *E. coli* strain which could not evoke keratoconjunctivitis. Preliminary experiments done in collaboration with T. Pal have indicated that this rabbit antiserum recognizes plasmid-coded polypeptides b and c (unpublished data). Therefore, both the ELISA which we developed with convalescent monkey serum and the previously developed ELISA with hyperimmune rabbit serum are probably assessing similar bacterial constituents.

The results of these studies indicate that each monkey in a group of 40 animals mounted a significant serum antibody response against at least five (140K, a, b, c, and d) plasmid-coded protein antigens after oral challenge with virulent *S. flexneri* 2a. Seven of nine children with naturally acquired shigella infection also exhibited high titers of serum antibody against these antigens. Immunoblots indicated that many of these sera had antibody only against plasmid-coded proteins, with polypeptides b and c being particularly antigenic. Some sera (human and monkey) also contained antibody against a MOMP which is encoded by the *S. flexneri* chromosome. The selective immune response against plasmid-coded antigens was rather striking, since these proteins are relatively minor components of the outer membrane. A possible explanation of these results is that primates may be tolerant to most *S. flexneri* proteins, which are similar to those expressed by *E. coli* in the normal flora, so the predominant immune response is directed against unique antigens expressed only by transient enteric pathogens.

Young children (less than 5 years old) did not have high levels of antibody in acute sera against the plasmid protein antigens. Four out of five of these children did show an increase in titer in convalescent sera against these antigens as determined by the ELISA, but only two children had sufficient levels of IgG to be positive in immunoblots. The various antibody responses to the plasmid-coded proteins could be due to several factors, including the age of the patient, previous exposure to shigellae, severity of infection, or the possibility that the antibody response was predominantly IgM and therefore not measured in the ELISA or immunoblot procedures. It has been reported that shigella infection tends to cause chronic enterocolitis with numerous acute phases in children 1 to 3 years of age (18), and this chronicity could be related to the virtual absence of an immune response against plasmid-coded antigens in children under 3 years of age.

All children 5 years old or older had antibody titers of 1,600 or more in acute sera, and only one of these four children had increased levels of antibody against plasmid-coded protein antigens in convalescent serum. The presence of acute-serum antibody is not surprising since these patients live in an area of endemic shigellosis. This antibody may have been a preexisting residual response from a previous infection, or it may have been produced as a rapid secondary response to the current infection. Similar studies on sera from healthy adults in the United States indicate that detectable levels of antibody to the plasmid-coded polypeptides are not present in a majority of individuals (unpublished

TABLE 5. Summary of protein antigens recognized by immune sera of monkeys challenged with virulent *S. flexneri* 2a[a]

Monkey group	No. of monkeys responding to protein:					
	140K	a	b	c	d	MOMP
Vaccine	10	19	20	20	11	18
Placebo	10	17	20	18	14	17

[a] Proteins 140K, a, b, c, and d are found only in virulent, plasmid-containing shigellae. The MOMP is present in both virulent and avirulent organisms. There were 20 animals in each group. The antigen specificity was determined by immunoblot procedures.

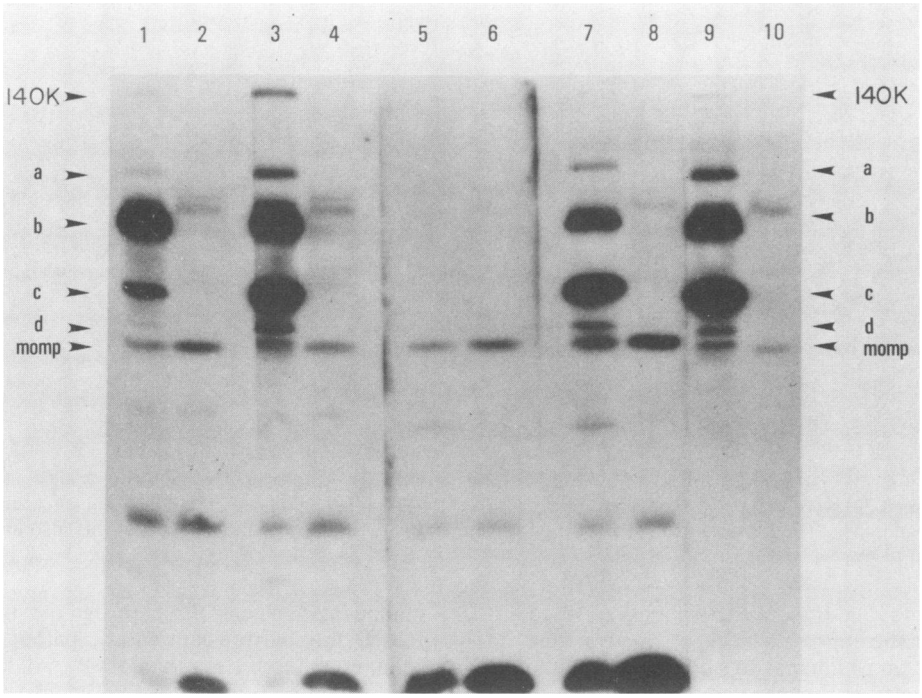

FIG. 3. Immunoblot analysis of *S. flexneri* antigens recognized by sera obtained from humans infected with shigellae. Separated polypeptides of virulent *S. flexneri* 5 (M90T; odd-numbered lanes) and avirulent *S. flexneri* 5 (WR6078; even-numbered lanes) were incubated with the antisera and then with radiolabeled protein A. Lanes: 1 through 4, antigens recognized by sera from a 6-year-old male infected with *S. flexneri* 2; 1 and 2, acute sera obtained after 7 h of illness; 3 and 4, reaction with sera obtained 4 weeks later; 5 through 8, antigens recognized by a 3-year-old male infected with *S. sonnei*; 5 and 6, reaction with acute sera; 7 and 8, reaction with convalescent sera; 9 and 10, incubation with monkey sera containing antibody to plasmid proteins 140K, a, b, c, and d, as well as the MOMP.

observations). The amount of secretory antibody present during the acute phase of disease against the plasmid-coded proteins is unknown. These children showed minimal increases in serum antibody titers during convalescence, so it is possible that only a localized immune response to shigella infection occurs in previously exposed individuals.

The biological function of IgG recognizing plasmid-coded proteins is a matter of conjecture. Antibody against somatic antigen mediates opsonization and complement-mediated killing, in addition to the antibody-dependent, cell-mediated antibacterial activity of lymphocytes (14, 16, 21). Since antibody against plasmid-coded antigens was readily bound to the surface of bacterial cells in an ELISA, it is possible that this antibody against proteinaceous antigens has antibacterial activity similar to that associated with antibody against lipopolysaccharide antigens. Studies in progress suggest that IgG and IgA antibody recognizing plasmid-coded proteins is secreted by cultured colonic biopsies taken from challenged monkeys 2 to 3 weeks postinfection (G. Dinari, personal communication). Since expression of the 140-MDa plasmid is associated with the invasive phenotype, it is conceivable that antibody secreted onto the colonic mucosa could inhibit the first stage of infection, i.e., the invasion of the colonic epithelium, by neutralizing the plasmid products which may be responsible for this function.

Even though the biological implications of antibody recognizing plasmid-coded polypeptides are not yet clear, the discovery of such antibody in convalescent sera allows some speculation on previously reported observations. For example, *S. flexneri* 2a vaccine (T_{32}-Istrati) evoked protection against both homologous and heterologous serotypes of *Shigella* strains in large-scale field trials in Romania (18). It has also been reported that an outer membrane protein preparation from *S. flexneri* 2a induces protection against development of keratoconjunctivitis for 48 h after homologous or heterologous challenge in guinea pigs and rabbits. This protection was passively transferred by serum (1). The antigens evoking heterologous protection in these studies were not identified, but the plasmid-coded polypeptides are obvious candidates.

Nonetheless, it should be noted that earlier vaccine trials have indicated that protection against shigellosis is associated with serotype-specific somatic antigens (6, 19). In addition, the occurrence of major epidemics of *S. dysenteriae* 1 in areas such as Central America, Bangladesh, West Bengal, and Africa, which are endemic for other *Shigella* species, indicates that the immunity of adults in these areas is at least somewhat species specific. In the final analysis, the discovery of antibody in convalescent sera which recognizes the products of the 140-MDa virulence-associated plasmids of *Shigella* species and enteroinvasive *E. coli* may have only diagnostic and epidemiological relevance, but the existence of commonly expressed, virulence-associated antigens in most enteroinvasive pathogens also presents intriguing possibilities for the development of enteric vaccines. The protective potential of such plasmid-coded antigens should be investigated.

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