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Antibodies to Shiga Holotoxin and to Two Synthetic Peptides of the B Subunit in Sera of Patients with *Shigella dysenteriae* 1 Dysentery

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Acute- and convalescent-phase sera from 18 Thai patients and convalescent-phase sera from two Israeli patients and one Bangladeshi patient with *Shigella dysenteriae* 1 (Shiga) dysentery were tested by enzyme-linked immunosorbent assay to detect antibodies that bind *S. dysenteriae* lipopolysaccharide (LPS), Shiga holotoxin, or two synthetic peptides representing epitopes from the B subunit of Shiga toxin. Paired sera from 24 Maryland adults with *Shigella flexneri* 2a or *Shigella sonnei* diarrhea served as negative controls. Of the 16 paired Thai serum samples tested for immunoglobulin G LPS antibody, 10 had ≥4-fold rises (the two subjects with the highest convalescent-phase titers exhibited toxin-neutralizing activity); acute-phase specimens from four of the remaining six individuals already had elevated Shiga LPS titers in their acute specimens ranging from 1:800 to 1:12,800. Similarly, convalescent-phase sera from the two Israeli patients and the Bangladeshi patient revealed LPS titers of 1:800 to 1:3,200. In contrast, none of the Maryland volunteers with *S. flexneri* or *S. sonnei* diarrhea manifested rises in Shiga anti-LPS ($P < 0.00001$ versus 10 of 16 Thai patients). Only 4 of the 18 Thai patients had significant rises in antibody to purified Shiga toxin, while one of the two Israeli patients and the one Bangladeshi patient had elevated convalescent-phase titers. None of the sera that reacted with Shiga holotoxin had antibody that bound to the peptides. This report, which describes a search for serum antibodies that bind Shiga toxin in patients with Shiga dysentery, demonstrates such antibodies in only a minority of patients with bacteriologically confirmed disease. During Shiga dysentery, Shiga toxin may be elaborated in such small quantities in vivo that it fails to elicit an immune response in most patients even though it may exert biological effects. In this behavior Shiga toxin resembles tetanus toxin, another potent exotoxin that fails to elicit antitoxic responses in people who recover from clinical tetanus.

Shiga toxin, the potent toxin elaborated by *Shigella dysenteriae* 1 (Shiga bacillus), has been intensively studied with respect to its sequence, tertiary structure, and molecular mode of action and the receptors that bind the toxin (24, 30, 38). This toxin, which was first discovered in 1903 (10), can be shown to be lethal for mice (neurotoxic) (14, 39), cytotoxic (14, 47), and enterotoxic (14, 29). A category of *Escherichia coli*, enterohemorrhagic *E. coli* (EHEC), that causes hemorrhagic colitis and the hemolytic-uremic syndrome elaborates a virtually identical toxin, Shiga-like toxin I (also referred to as verotoxin 1) (39, 43, 44); some EHEC strains express a related but distinct toxin, Shiga-like toxin II (43, 44). The precise role played by Shiga toxin in the pathogenesis of Shiga dysentery remains unclear. Mutants of *S. dysenteriae* 1 that lack Shiga toxin nevertheless cause diarrheal illness, as well as dysentery, in monkeys (16) and humans (32), although the clinical syndrome is marked by somewhat diminished colonic tissue damage and perhaps by less gross blood in the loose stools. Much is known of the structure and action of Shiga toxin (24, 30, 38), and there are a few reports of the immunological response to Shiga toxins in patients with hemolytic-uremic syndrome due to EHEC infection (4, 8, 25), but there are surprisingly few publications on the human immune response to Shiga toxin in patients with Shiga dysentery (28). Accordingly, we undertook to measure serum antibodies to purified Shiga toxin and two Shiga toxin B-subunit peptides in acute- and convalescent-phase sera of patients with bacteriologically confirmed Shiga dysentery.

MATERIALS AND METHODS

Sera. Paired acute- and convalescent-phase sera were obtained from 18 patients in Thailand (45), including 8 <5 years old, 2 5 to 9 years old, 4 10 to 19 years old, and 4 >20 years old. In addition, convalescent-phase sera from two Israeli adults and from one Bangladeshi subject with culture-confirmed Shiga dysentery were available. All the patients manifested overt dysentery with loose stools containing gross blood and mucus. For negative control sera, we used specimens obtained prechallenge and 10, 21, and 28 days

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after experimental challenge of adult volunteers with pathogenic *Shigella flexneri* 2a (9 subjects) or *Shigella sonnei* (15 subjects), serotypes that do not elaborate Shiga toxin. These volunteers served as unimmunized controls in experimental challenge studies to assess the efficacy of *Shigella* vaccines (23, 31a). The sera were stored at −20°C until tested.

Antigens. The antigens utilized in enzyme-linked immunosorbent assays (ELISAs) to detect antibodies in human serum include purified Shiga toxin and two synthetic peptides. Shiga toxin was purified by affinity chromatography as previously described (11).

An earlier analysis of the primary amino acid sequence of Shiga toxin revealed two highly hydrophilic peaks within the B subunit, at the amino terminus of the molecule. A computerized plot pattern confirmed that these two peptide segments, as well as another region at the carboxy terminus, contained high contents of surface residues. Two regions of the molecule, comprising residues 7 to 26 (peptide 7–26) and peptide 54–67 of the amino acid sequence, were predicted to be B-lymphocyte epitopes. This was confirmed in previous studies in which we showed that synthetic peptides corresponding to these sequences, when conjugated to a carrier protein and administered to mice as a parenteral vaccine, elicited antibodies that recognize Shiga toxin (21). These two synthetic peptides were used in the present study to coat microtiter plates for ELISAs.

Purified lipopolysaccharide (LPS) of *S. dysenteriae* 1 was prepared by the hot-water–phenol method (48).

ELISAs. The ELISAs to measure Shiga toxin and peptide antibodies were similar to other antibody detection ELISAs previously developed at the Center for Vaccine Development (University of Maryland School of Medicine) (33–35). Every other well of plastic 96-well microtiter plates (Cooke) was coated with 100 μl of a 1.0-μg/ml concentration of purified Shiga toxin in carbonate buffer (pH 9.6) and left overnight at 4°C. After being washed with phosphate-buffered saline (PBS) (pH 7.2)-Tween, 100-μl samples of sera (twofold dilutions at 1:10 to 1:320) were added to the wells and the plates were incubated for 60 min at 37°C. After a standard washing, goat anti-human immunoglobulin G (IgG) conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added at a dilution of 1:500. After appropriate incubation and washing, the color reaction was developed in a standard manner and recorded in an automated Titertek ELISA reader. The optical density (OD) in the control well without antigen was subtracted from the OD in the corresponding well with antigen and expressed as the net OD. A net OD of ≥0.1 was deemed to signify the presence of antibody, and the endpoint titer was the highest dilution that had a net OD equal to or greater than that value. When paired sera were tested, fourfold rises were considered significant.

To measure antibody to Shiga toxin peptides, Nunc flat-bottom plates (Nunc, Roskilde, Denmark) were first treated with 0.1 ml of PBS (pH 5.0) with 0.2% glutaraldehyde for 60 min at room temperature, after which the plates were washed with PBS (pH 7.2). The synthetic peptides, diluted to a final concentration of 10 μg/ml in 0.1 M PBS (pH 7.2) with 0.2% glutaraldehyde, were added to alternating wells of the plates, which were then incubated overnight at 4°C. After being washed with PBS, the plates were blocked to cover uncoated sites on the plastic and the rest of the assay was performed as described above. The net OD cutoff signifying the presence of specific antibody to peptide 7–26 was ≥0.20; for peptide 54–67 it was 0.10.

To measure antibody to LPS, purified *S. dysenteriae* LPS (100 μl of a 1.0-μg/ml concentration) in carbonate coating buffer (as described above) was applied to every other well of microtiter plates overnight at 4°C. After appropriate washing, twofold dilutions of sera (starting at 1:100) were added and the assay proceeded as described above. A net OD cutoff criterion of ≥0.30 was selected as signifying the presence of IgG antibodies to *S. dysenteriae* 1 LPS.

To determine the net OD cutoff mentioned above that signify the presence of antibody to Shiga toxin, peptide 54–67, peptide 7–26, and LPS, each of these antigens was tested against a battery of 24 putative negative human serum samples (24 Maryland volunteers who had never traveled to less-developed countries and with no history of shigellosis, hemolytic-uremic syndrome, or hemorrhagic colitis) at the starting dilution used in each assay (1:10 for the peptide ELISAs and 1:100 for the LPS ELISA). For each antigen tested, the mean net OD of the 24 serum samples was calculated, as was the standard deviation. The cutoff to signify the presence of antibody in any serum specimen subsequently tested was arbitrarily set as a net OD value greater than the mean plus 3 standard deviations. This proved to be ≥0.1 for two Shiga holotoxins and peptide 54–67, ≥0.2 for peptide 7–26, and ≥0.3 for LPS. The higher cutoff for LPS is not surprising, since O antigens of many Gram-negative bacteria share cross-reactivities in addition to the shared core sugar and lipid A specificities. Thus, even individuals who are unlikely to have ever been exposed to *S. dysenteriae* 1 may have antibodies to *E. coli* LPS, other *Shigella* serotypes, or other members of the family *Enterobacteriaceae* that result in some level of cross-reaction with *S. dysenteriae* 1 LPS.

Shiga toxin neutralization assay. Toxin neutralization assays were performed with the HeLa clone 229 cell line, purified Shiga toxin, and hyperimmune specific rabbit antisera in a modification of a previously described method (18). HeLa cell monolayers were established in 96-well microtiter plates (Nunc) with Eagle minimum essential medium (Whittaker Bioproducts, Walkersville, Md.) supplemented with 2% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml with 18 to 20 h of incubation at 35°C in 5% CO_2. Fifty-microliter samples of serial dilutions of toxin, in duplicate, starting at 2.5 ng/ml, were added to microtiter wells and incubated for 18 h to establish the toxin dilution resulting in 50% detachment of HeLa cells (50% cytotoxic dose). After incubation, plates were shaken and gently washed in PBS (pH 7.2), and detached cells were removed. The remaining cells were fixed in 2% Formalin in 0.067 M PBS for 1 min, the fixative was removed, and the cells were then stained with 0.13% crystal violet in 5% ethanol–2% Formalin–PBS for 20 min. Wells were examined by direct visualization and by microscopy. For the serum neutralization studies, the method was the same as that for determining the 50% cytotoxic dose of the toxin, except that equal volumes of 20 times the concentration of the 50% cytotoxic dose and serial twofold dilutions of heat-inactivated sera (starting at 1:10) were incubated for 1 h at 37°C before being added to the cell monolayers. A ≥50% inhibition of cytopathic effect was used as a cutoff for determining titer. Hyperimmune rabbit antisera and convalescent-phase sera obtained from subjects with *S. sonnei* diarrhea were used as positive and negative controls, respectively.

TABLE 1. Reciprocal titers of specific antibody to *S. dysenteriae* 1 antigens in paired acute- and convalescent-phase sera of confirmed Shiga dysentery patients[a]

Subject	Source of sera	Titer of antibody to:						Titer for Shiga toxin neutralization	
		LPS		Holotoxin		Peptides			
		Acute	Conv.	Acute	Conv.	Acute	Conv.	Acute	Conv.
UVS-11	Thailand	<100	800	<10	160	NT[b]	NT	<10	20
UVS-277	Thailand	NT	NT	<10	40	<10	<10	<10	40
UVS-266	Thailand	NT	NT	<10	20	<10	<10	<10	<10
UVS-572	Thailand	800	3,200	<10	20	<10	<10	<10	<10
UVS-222	Thailand	<100	800	<10	10	<10	<10	NT	NT
UVS-179	Thailand	<100	<100	<10	<10	<10	<10	NT	NT
UVS-214	Thailand	<100	<100	10	<10	<10	<10	NT	NT
UVS-445	Thailand	<100	1,600	<10	<10	<10	<10	NT	NT
UVS-514	Thailand	<100	800	<10	<10	<10	<10	NT	NT
UVS-518	Thailand	<100	3,200	<10	<10	<10	<10	NT	NT
UVS-522	Thailand	<100	400	<10	<10	<10	<10	NT	NT
UVS-530	Thailand	<100	800	10	<10	<10	<10	NT	NT
UVS-651	Thailand	<100	1,600	<10	<10	<10	<10	NT	NT
UVS-169	Thailand	12,800	12,800	<10	<10	<10	<10	NT	NT
UVS-172	Thailand	800	800	<10	<10	<10	<10	NT	NT
UVS-281	Thailand	6,400	25,600	10	<10	<10	<10	NT	NT
UVS-529	Thailand	6,400	6,400	10	<10	<10	<10	NT	NT
UVS-904	Thailand	6,400	3,200	<10	<10	<10	<10	NT	NT
DP1	Israel	NA[c]	800	NA	20	NA	<10	NA	<10
TM	Israel	NA	3,200	NA	<10	NA	<10	NA	NT
O16	Bangladesh	NA	3,200	NA	20	NA	<10	NA	NT

[a] Acute and conv., acute- and convalescent-phase sera, respectively.
[b] NT, insufficient serum available for testing.
[c] NA, specimen not available.

RESULTS

LPS antibodies. Paired serum samples from 16 of the 18 Thai patients with bacteriologically confirmed Shiga dysentery were tested for anti-LPS antibody. Ten of these 16 patients manifested at least fourfold rises in IgG antibody to purified Shiga LPS (Table 1). Among the six individuals who did not show significant rises, four had very high titers in their acute-phase specimens (one had a titer of ≥1:12,800, two had titers of 1:6,400, and one had a titer of 1:800). Acute-phase serum specimens from four Thai patients were collected after each patient had been ill for more than 7 days; these included patients 266 (8 days), 277 (11 days), 169 (13 days), and 527 (14 days). The convalescent-phase sera from the two Israeli patients and from the Bangladeshi patient with Shiga dysentery all had elevated titers of IgG antibody to *S. dysenteriae* 1 LPS (Table 1). In contrast, none of the 24 serum samples from Maryland adult volunteers who experienced *S. flexneri* 2a or *S. sonnei* diarrheal illness showed significant rises in IgG antibody to *S. dysenteriae* 1 LPS ($P < 0.00001$ versus the seroconversion rate [10 of 16] among Thai patients with Shiga dysentery). Notably, one Maryland volunteer had a baseline titer of 1:3,200 against Shiga LPS, presumably because of prior contact with a cross-reactive antigen.

Antibody to purified Shiga holotoxin. As summarized in Table 1, 4 (22%) of the 18 Thai patients with Shiga dysentery showed rises in serum IgG antibody to purified Shiga holotoxin. In one instance the convalescent-phase titer was high (1:160), while in the others the titers were low (1:20) or moderate (1:40). Elevated titers of IgG antibody to Shiga holotoxin (i.e., ≥1:20) in convalescent-phase sera from one of the two confirmed Israeli Shiga dysentery cases and from the Bangladeshi case were also recorded.

None of the 24 Maryland volunteers challenged with *S. flexneri* 2a or with *S. sonnei* had significant rises in levels of serum antibody to Shiga holotoxin, and their titers at all time points were <1:10.

Antibodies to synthetic peptides. None of the sera, including the six convalescent-phase serum samples that manifested elevated titers of IgG antibody to purified Shiga toxin, showed antibodies to the synthetic peptides.

Toxin neutralization assay. Paired serum samples from four Thai patients who showed seroconversions to purified Shiga toxin, as determined by ELISA, and from one patient who did not were tested for neutralizing activity. Single convalescent-phase serum samples from one Bangladeshi patient and one Israeli patient were also tested. None of the acute-phase sera showed neutralizing activity. However, in the convalescent-phase sera of the two patients who exhibited the most prominent rises in titer of anti-Shiga toxin antibody, as determined by ELISA, clear-cut evidence of neutralizing antibody was demonstrated (Table 1). Neutralizing antitoxin was limited to sera with ELISA titers of ≥1:40.

DISCUSSION

S. dysenteriae 1 is unique among *Shigella* serotypes because of the severity of the clinical illness that it causes, its epidemiologic behavior, which results in pandemics, and its ability to elaborate Shiga toxin. This potent toxin is believed to play a role in pathogenesis of the hemolytic-uremic syndrome (which occurs as a well-recognized, albeit uncommon, complication of Shiga dysentery) (31, 41) and may exacerbate local mucosal injuries in the colon (16, 32). The immune response to this powerful toxin has not been well studied in patients with Shiga dysentery.

In the early 1970s, Keusch et al. (28) reported that volunteers who were challenged with pathogenic *S. dysen-*

teriae 1 (in the course of vaccine development studies) developed modest increases in serum antibodies that could neutralize the HeLa cell cytotoxicity of crude Shiga toxin. This neutralizing anticytotoxin was confined to the IgM class and was present in such low concentrations that even a single twofold dilution resulted in loss of activity (28). In these early studies, Keusch et al. (26, 27) also reported that convalescent-phase sera from patients with *S. sonnei* and *S. flexneri* infections could similarly neutralize the cytotoxic effects of Shiga toxin. Finally, when they tested sera from volunteers who developed diarrheal illness after ingesting a genetically modified *S. dysenteriae* 1 strain in which the genes for Shiga toxin were inactivated (32), Keusch et al. (29) again reported rises in levels of antibodies that could neutralize the HeLa cell cytotoxic effects of Shiga toxin. Reviewing the data from those pioneering studies in the light of present knowledge leaves doubt that antibody to Shiga toxin was in fact being measured, because it is now known that *Shigella* serotypes other than *S. dysenteriae* 1 do not produce Shiga toxin, although they do elaborate minute quantities of other distinct cytotoxins that are active on HeLa cells (3, 5, 40). These other cytotoxins appear to be genetically unrelated to Shiga toxin but bear immunologic similarities such that Shiga antitoxin can neutralize their cytotoxicity and vice versa. In light of current knowledge, it would appear that in those early studies Keusch et al. (26–28), unbeknownst to them, were in large part measuring antibodies stimulated by the non-Shiga cytotoxins. Ultimately, elucidation of the primary structure of these other *Shigella* cytotoxins will lead to a rational classification and terminology for them.

It is against this background that the observations reported herein hold particular relevance, for they represent the first attempt to specifically measure antibodies that bind both to Shiga toxin and to peptide epitopes of Shiga toxin in patients with Shiga dysentery. To accomplish this, highly purified Shiga toxin and two synthetic peptides were used as antigen. Only 4 of 18 Thai patients manifested significant rises in antibody to the purified Shiga toxin. Similarly, elevated IgG titers were detected in convalescent-phase sera of only two of three Israeli and Bangladeshi patients with Shiga dysentery. Why was there such a poor serologic response? Possible explanations include the following. (i) Secretory IgA intestinal antibodies rather than serum antibodies should be measured. (ii) The antibody response to Shiga toxin may be muted, because this toxin is such a powerful suppressant of protein synthesis (6). (iii) Because of its potency, Shiga toxin may destroy the antigen-processing cells that pinocytose it and, in this way, the immune response may be muted. (iv) Since human B lymphocytes undergoing isotype switching transiently express receptors on their surface that bind Shiga toxin (9), such cells may be selectively targeted by the toxin, which would thereby interfere with development of an IgG antitoxin response. We consider these factors unlikely to have greatly influenced the rate of seroconversion, since patients with severe *Shigella* dysentery are known to mount brisk serum antibody responses to other antigens such as LPS and surface protein antigens such as invasion plasmid-associated antigens (7, 12, 13, 32, 37). Indeed, most of the patients in the present study had elevated convalescent-phase titers of serum antibody against *S. dysenteriae* 1 LPS (Table 1).

Two other explanations are more plausible. One is that during Shiga dysentery, Shiga toxin is produced in vivo in only minute quantities, which are sufficient to induce a biological effect (because of the potency of the toxin) but are insufficient to reliably generate antibody responses. If this is true, in this property Shiga toxin would resemble tetanus toxin. It is of note that Shiga toxin and tetanus toxin are comparably potent in lethality for mice (20). Alternatively, the immune response to Shiga toxin may be genetically restricted, and the few responders observed may represent particular major histocompatibility complex class II genotypes disposed to respond.

Synthetic peptides 7–26 and 54–67 were originally selected because a computer analysis predicted that they would be B-cell epitopes. When conjugated to carrier proteins and thereupon used as conjugate vaccines in mice, these peptides readily stimulated binding antibodies that showed neutralizing properties against the enterotoxic effect of Shiga toxin (22). However, the data summarized in Table 1, from the testing of human sera suggest that in vivo these amino acid sequences are not recognized by the immune system as immunodominant B-cell epitopes. Increasingly, we are coming to recognize that B-cell epitopes are often conformational (2, 19, 42) and difficult to predict. While short hydrophilic peptide sequences may readily stimulate antibodies when used as haptens in conjugate vaccines, this does not necessarily imply that antibodies to these putative epitopes are stimulated in the course of natural infection. Under natural conditions, such linear amino acid sequences may be masked by the tertiary folding of the protein, or for other reasons they may not function in vivo to stimulate immune responses.

Patients with hemolytic-uremic syndrome caused by EHEC typically show rises in titers of serum toxin-neutralizing antibody (25). It is possible that in such EHEC infections, either an enhanced amount of toxin, which better elicits an antitoxin response, is elaborated or more toxin enters the bloodstream.

The fact that only a minority of patients with bacteriologically confirmed natural Shiga dysentery infection manifested significant rises in antibody to Shiga toxin does not necessarily preclude a potentially important role for a Shiga toxoid as an important component of a vaccine against *S. dysenteriae* 1 disease. For example, fewer than 20% of patients with acute typhoid fever develop serum antibodies to the Vi antigen of *Salmonella typhi*, yet inducement of serum Vi antibodies by parenteral inoculation with purified Vi polysaccharide vaccine confers significant protection (1). Similarly, tetanus toxoid is an excellent immunogen, while antitoxin is usually not detectable following clinical tetanus. Thus, a properly designed and formulated toxoid, whether consisting of a parenteral vaccine (36), a microsphere-encapsulated oral vaccine (15), a modified protein expressed by an attenuated *S. dysenteriae* 1 strain (46), or another live vector (such as *Salmonella typhi*) (17), may yet prove useful.

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