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Shiga and Shiga-Like Toxins

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INTRODUCTION

Although *Shigella dysenteriae* serotype 1 (Shiga) toxin was discovered more than 80 years ago (18) and has long been recognized as one of the most potent bacterial toxins (18, 116), early efforts to characterize its structure, biologic activity, genetics, role in pathogenesis, and other properties were hindered by difficulties in preparing pure toxin, obtaining appropriate immunologic reagents, and conducting genetic studies with *S. dysenteriae* 1. The enterotoxicity of Shiga toxin was first reported in 1972 (59). Other important advances that have been made during the past decade include the following: (i) purification of Shiga toxin to homogeneity (7, 21, 81, 87, 120); (ii) identification of cell surface receptors for toxin (8, 45; A. A. Lindberg, J. E. Brown, N. Strömberg, M. Westling-Ryd, J. E. Schultz, and K. A. Karlson, J. Biol. Chem., in press; A. A. Lindberg, J. E. Schultz, M. Westling, J. E. Brown, S. W. Rothman, K. A. Karlsson, and N. Strömberg, Proc. FEMS Meet. Mol. Biol. Pathogenic Microorganisms, in press); (iii) demonstration that the intracellular action of toxin causes an inhibition of protein synthesis (7, 8a, 97, 111; T. G. Obrig, T. P. Moran, and J. E. Brown, J. Biol. Chem., in press); (iv) recognition that *Escherichia coli* and other enteric bacteria produce Shiga-like toxins (82; A. D. O'Brien, M. E. Chen, R. K. Holmes, J. Kaper, and M. M. Levine, Letter, Lancet i:702, 1983); (v) appreciation that Vero cytotoxins and Shiga-like toxins are the same (A. D. O'Brien, T. A. Lively, M. E. Chen, S. W. Rothman, and S. B. Formal, Letter, Lancet i:702, 1983); (vi) purification of Shiga-like toxin to homogeneity (80); (vii) demonstration that production of Shiga-like toxins can be controlled by phage conversion (84, 108; S. M. Scotland, H. R. Smith, G. A. Willshaw, and B. Rowe, Letter, Lancet ii:216, 1983); (viii) cloning and characterization of the structural genes for Shiga-like toxin from toxinogenic coliphages (44, 78, 119); (ix) demonstration of antigenic heterogeneity among Shiga-like toxins (110; S. M. Scotland, H. R. Smith, and B. Rowe, Letter, Lancet ii:85–886, 1985; M. A. Karmali, M. Petric, S. Louie, and R. Cheung, Letter, Lancet i:164–165, 1986); and (x) association of Shiga-like toxin-producing strains of *E. coli* with hemorrhagic colitis (W. M. Johnson, H. Lior, and G. S. Bezanson, Letter, Lancet i:76, 1983; A. D. O'Brien, T. A. Lively, M. E. Chen, S. W. Rothman, and S. B. Formal, Letter, Lancet i:702, 1983), and hemolytic uremic syndrome (HUS) (52; M. A. Karmali, M. Petric, B. T. Steele, and C. Lim, Letter, Lancet i:619–620, 1983). The primary goal of this review is to provide an overview of the current status of the rapidly evolving research on Shiga and Shiga-like toxins.

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SHIGA TOXIN IN *SHIGELLA* SPP.

History

Shigellosis, the severest form of which was historically called bacillary dysentery, is a disease limited to humans and certain other primates (24). The bacteria that cause shigellosis belong to the genus *Shigella* and are classified into four species and over 40 serotypes on the basis of the characteristics of their O antigens. The species of *Shigella* are *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D). *Shigella* spp. colonize the large bowel and must penetrate and multiply within colonic epithelial cells to cause disease in volunteers (25, 70). The pathophysiology of shigellosis is beyond the scope of this review and is discussed in detail elsewhere (57, 58). Typical symptoms of shigellosis include cramps, painful defecation, fever, diarrhea, dysentery (blood and mucus in stools), or diarrhea and dysentery (24). All four species of *Shigella* have been shown to contain large plasmids (120 to 140 megadaltons) that are required for the organism to invade epithelial cells (101–104). At least three chromosomally encoded determinants must also be expressed for shigellae to be fully virulent in animal models (102), but the specific gene products made by them have not been established.

Shigella spp. also elaborate a toxin, designated as Shiga toxin, that may contribute to the development of necrotic lesions of the colon. Several detailed reviews on Shiga toxin have been published over the last 50 years (12, 30, 57, 58, 113, 116). The Shiga toxin was first described in 1903 by Conradi, who reported that intravenous inoculation of autolysates of Shiga's bacillus (*Shigella dysenteriae* 1) paralyzed and killed rabbits (18). It was subsequently shown that various animal species exhibit differential susceptibilities to the lethal effects of Shiga toxin, but only the rabbit and mouse display neurological symptoms (14). Studies in the 1950s suggested that Shiga toxin does not act directly on neurons (i.e., is not actually a neurotoxin) but that it can cause secondary neurological disorders by its action on the vascular system of the brain and spinal cord (6, 43). More recent data concerning the interaction of Shiga toxin with neurons are controversial. Wiley et al. (118) reported that purified Shiga toxin can be axonally transported to rat vagal sensory neurons and can kill those neurons. In contrast, Brown et al. failed to demonstrate axonal transport of purified Shiga toxin, nor were they able to show cytotoxicity or specific binding of toxin to neuroblastoma hybrid cells (J. E. Brown, W. H. Habig, J. G. Kenimer, and M. C. Hardegree, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B109, p. 36). The reason for the diverse results of Wiley et al. and Brown et al. is not apparent.

Although the paralytic-lethal effects of crude Shiga toxin were originally demonstrated in the early part of this century, it was not until the 1940s that such preparations were reported to be cytotoxic for selected mammalian cells (69, 115). Subsequently, Keusch et al. (59) showed that partially purified preparations of Shiga toxin could elicit fluid accumulation, i.e., were enterotoxic, in ligated segments of rabbit ileum. When purified Shiga toxin became available in the 1980s, several groups independently demonstrated that the paralytic-lethal, cytotoxic, and enterotoxic activities described for extracts or culture filtrates of Shiga's bacillus could be ascribed to a single toxin (7, 21, 28, 81). These varied biological properties appear to reflect the molecular action of Shiga toxin, i.e., inhibition of protein synthesis in susceptible target cells (9, 10, 97, 111), as discussed in a subsequent section of this review.

Purification and Structure

The following two points relevant to all purification schemes should be emphasized: (i) Shiga toxin is a cell-associated toxin that may be located in the periplasmic space (20, 37) and released into the culture medium after cell death (23, 114); and (ii) the yield of Shiga toxin is increased if the bacteria are cultured in a medium with a reduced iron content or a medium that has been adjusted to a high pH (23, 79, 114). Purification of the Shiga toxin to homogeneity was first reported in 1980 (81, 87), and several new and modified purification procedures have subsequently been published (7, 21, 80, 88, 120). The protocol for toxin purification now used in our laboratory (80) is representative of the various schemes. The shigellae are grown in an iron-depleted, modified glucose syncase broth (79, 82). The organisms are harvested by centrifugation and lysed in a French pressure cell, and the lysates are then clarified by ultracentrifugation and passed over an Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) column. Toxin which binds to the Affi-Gel Blue is eluted from the column and subsequently purified by chromatofocusing and antitoxin affinity chromatography. About 60% of the cytotoxin activity present in the starting cell lysates is recovered, but the amount of purified protein is low (100 to 200 μg of toxin per 8 liters of culture). The yield of purified toxin per 3 liters of culture as reported by Olsnes et al. (88) was 150 ng with a recovery of 5%. By contrast, Donohue-Rolfe et al. (21) reported yields of up to 800 μg of purified protein toxin from 4 liters of culture (recovery, 47%). In addition to the different yields of purified Shiga toxin obtained by various investigators, a range of values has been reported for the specific activity of purified Shiga toxin as measured in 50% cytotoxic doses (CD_{50}) per milligram of toxin or 50% protein synthesis-inhibitory doses (ID_{50}) per milligram of toxin. The specific activities of purified Shiga toxin published to date are 4×10^7 CD_{50}/mg (21), 3×10^8 CD_{50}/mg (7), 5×10^8 to 1×10^9 CD_{50}/mg (79, 80), and 1×10^9 ID_{50}/mg (88). These differences in specific activity could reflect differences in sensitivity of the HeLa cell sublines used to detect cytotoxin, variations among laboratories in the method used to measure cytotoxicity, or differences in the purity or extent of denaturation of the various toxin preparations.

Past estimates of the molecular weight (MW) of purified Shiga holotoxin have ranged from 58,000 to 70,000 (7, 21, 80, 88). The 70,000 value appears to be correct as calculated from recent data on subunit MW and subunit stoichiometry. The newest fidings indicated that Shiga toxin comprises one A subunit of MW ca. 32,000 (21) and five copies (21) of a B subunit of MW 7,700 (106). The mature B subunit consists of 69 amino acids (106) which agree exactly in composition and sequence with the 69 amino acids inferred from the nucleotide sequence (47a) of the gene for the B subunit of *E. coli* Shiga-like toxin type I (SLT-I). The A subunit must be nicked by a proteolytic enzyme and its disulfide bonds must be reduced for it to form the enzymatically active A_1 fragment of MW 27,000 (88). The subunit structure of purified Shiga toxin is reminiscent of those of cholera toxin and *E. coli* heat-labile toxin, but no antigenic cross-reactivity is demonstrable between Shiga toxin and those toxins (81). Nonetheless, Seidah et al. (106) have proposed on the basis of amino acid sequence data that the B subunits of *E. coli* heat-labile toxin and cholera toxin are distantly related to the B subunit of Shiga toxin.

Although some strains of *S. flexneri* and *S. sonnei* (61, 85) produce very low levels of Shiga-like toxin that is neutralizable by anti-Shiga toxin, this toxin has not yet been obtained in pure form. O'Brien and LaVeck used a solid-phase radioimmunoassay to measure the amounts of toxin antigen produced by *Shigella* spp. other than *S. dysenteriae* 1 (79). Their results indicated that the variations in levels of cytotoxin produced by the different shigellae reflect differences in toxin yields rather than variations in cytotoxicity per unit of toxin antigen. The question remains whether the Shiga-like toxins of some *S. flexneri* and *S. sonnei* isolates and the Shiga toxin of *S. dysenteriae* 1 are identical toxins or different but closely related toxins. Until amino acid or nucleotide sequence data are available for comparison of the proteins or structural genes of the *Shigella* toxins, it is appropriate to continue to use the term Shiga-like to describe toxins neutralized by anti-Shiga toxin that are produced by *Shigella* serotypes other than *S. dysenteriae* 1.

Mode of Action

Each subunit of Shiga toxin plays an essential role in the intoxication of sensitive mammalian cells. The B subunit is responsible for the binding of toxin to the mammalian cell receptor, whereas the A subunit, after it is proteolytically nicked and reduced to the A_1 fragment, is the component responsible for inhibiting protein synthesis in the target cell. With respect to the localization on different subunits or domains of the functions that mediate toxic activity and cell binding, Shiga toxin is similar to many other bifunctional bacterial toxins such as cholera toxin, *E. coli* heat-labile toxin, diphtheria toxin, *Pseudomonas* exotoxin A, and pertussis toxin (75). Details of the mode of action of Shiga toxin are given below and are represented diagramatically in Fig. 1.

Receptor binding and internalization. Several reviews have been published in which the receptor-mediated binding and internalization of Shiga toxin by mammalian cells is discussed (26, 56, 75, 89). Keusch and Jacewicz (62) first demonstrated the specific binding of toxin to rat liver membranes and to HeLa cells by means of an indirect assay which measures the reduction of cytotoxicity in cell culture medium. In the same study (62), the chemical nature of the Shiga toxin receptor was analyzed. The receptor was destroyed by proteolytic enzymes, phospholipases, and lyzozyme but not by neuraminidase or galactose oxidase. Chitin-containing compounds competitively inhibited Shiga toxin binding to HeLa cells, and wheat germ agglutinin, which is known to possess specific binding affinity for *N,N',N''*-triacyl chitotriose, blocked toxin uptake as assessed by an indirect toxin consumption assay. Keusch and Jacewicz (62) surmised from these data that the receptor for Shiga toxin on mammalian cells may be a glycoprotein. It should be noted that Lindberg et al. (J. Biol. Chem., in press) were unable to confirm that chiotriose derivatives inhibit the binding of Shiga toxin to HeLa cells when they used a direct binding assay. Recently, Keusch et al. (63) reexamined the chemical nature of the Shiga toxin receptor with purified ^{125}I-labeled toxin as a probe to measure toxin binding to HeLa cells. They concluded that the functional receptor (defined as the receptor that mediates the cytotoxic effect of Shiga toxin) is an N-linked glycoprotein on the basis of a series of experiments in which they examined the effects of trypsin, tunicamycin, β-galactosidase, β-*N*-acetyl glucosaminidase, and various sugars and lectins on toxin binding and cytotoxicity. In that report (63), Keusch et al. proposed that two other binding sites for Shiga toxin exist on HeLa cells (one tunicamycin sensitive and one tunicamycin resistant), but neither is a functional receptor. A subsequent publication (45) from that laboratory reported that a nonfunctional, glycolipid receptor (presumably the tunicamycin-resistant receptor) for HeLa cells had been isolated and identified as a globotriaosylceramide. Lindberg

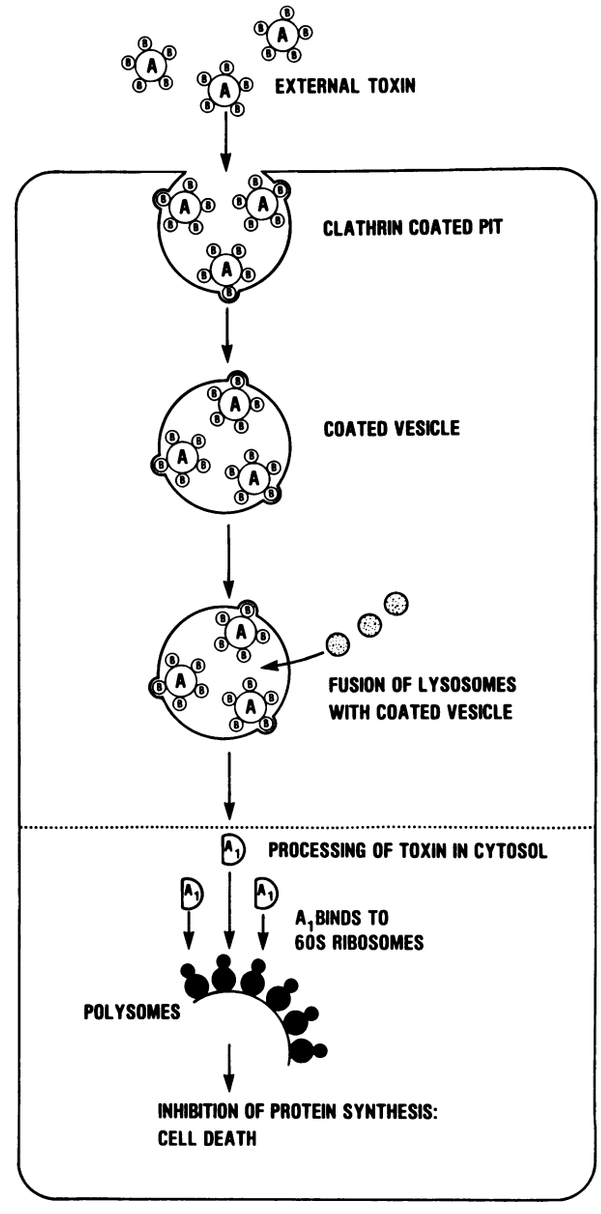

FIG. 1. Model for the receptor-mediated endocytic entry of Shiga toxin and processing of Shiga toxin in a mammalian cell (adapted from reference 57, Fig. 6). Shiga toxin enters the cell by receptor-mediated endocytosis. The B subunit of the toxin binds to the mammalian cell receptor. The clathrin-coated pit is pinched off, and the coated vesicle is formed. The vesicle is acidified, and it may fuse with lysosomes. The mechanism by which the enzymatically active A_1 fragment of Shiga toxin is generated and reaches the cytosol is not known but is presumed to involve proteolytic nicking and reduction of disulfide bonds of the A subunit. The A_1 fragment within the cytosol binds to the 60S ribosome, leading to inhibition of protein synthesis and cell death.

and co-workers (8; Lindberg et al., J. Biol. Chem., in press) have also isolated and characterized Shiga toxin receptors from HeLa cells. Lindberg et al. (J. Biol. Chem., in press) studied the binding of purified Shiga toxin to a series of glycolipids and to cells by using inhibition with synthetic receptor analogues. These investigators proposed that the functional HeLa cell receptor is a galactose $\alpha 1 \rightarrow 4$ galactose (i.e., galabiose)-containing glycolipid but that glycoproteins containing galabiose may mediate toxin binding without toxin internalization (i.e., are nonfunctional receptors). Thus, both Keusch and co-workers and Lindberg and co-workers agree that Shiga toxin can specifically bind to galabiose-containing glycolipids and galabiose-containing glycoproteins on HeLa cells. The two groups disagree on which of the receptors are the functional receptors for toxin. Whatever the exact chemical nature of the Shiga toxin receptors on HeLa cells, indirect evidence suggests that the B subunit of Shiga toxin binds to those receptors; monoclonal antibodies to the B subunit of Shiga toxin block the binding of ^{125}I-labeled native toxin to the HeLa cell surface (21). The isolated B subunit does not bind to intact HeLa cells (88) but does bind to the glycolipid receptor extracted from those cells (45). A glycolipid identified as a globotriaosylceramide (45) that binds ^{125}I-labeled Shiga toxin has been isolated from rabbit jejunum and may be the same as the microvillus membrane-binding site described by Fuchs et al. (32). Expression of this glycolipid receptor on rabbit intestinal microvillous membranes increases with the age of the rabbit (M. Mobassaleh, A. Donohue-Rolfe, R. K. Montgomery, G. T. Keusch, and R. J. Grand, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 323, 1985). This observation on the developmental expression of Shiga toxin intestinal receptors may explain why ligated segments of newborn-rabbit ileum are resistant to the secretory effects of Shiga toxin (M. Mobassaleh, A. Donohue-Rolfe, R. K. Montgomery, R. J. Grand, and G. T. Keusch, Gastroenterol. Abstr., in press).

Only certain sublines of HeLa and Vero cells are highly sensitive to Shiga toxin (35, 87); Hep-2 cells are slightly sensitive to the toxin, whereas WI-38 cells, baby hamster kidney cells, Chinese hamster ovary cells, Y-1 adrenal cells, L cells, and a human melanoma cell line are refractory to Shiga toxin (35, 87). Eiklid and Olsnes (27) observed that ^{125}I-labeled Shiga toxin bound to toxin-sensitive cell lines and to some, but not all, toxin-insensitive lines. These findings indicate that the presence of toxin receptor on a eucaryotic cell is necessary but not sufficient for Shiga toxin to kill that cell; toxin must be internalized and must reach its ribosome target. Shiga toxin appears to enter toxin-sensitive HeLa cells by an endocytotic mechanism (29, 46, 89).

Inhibition of protein synthesis. Several groups of investigators have demonstrated that Shiga toxin inhibits protein synthesis in eucaryotic cells (8a, 9, 10, 86, 97, 111). Analysis of the mechanism by which Shiga toxin inhibits protein synthesis has been modeled on previous studies of the mode of action of other bacterial toxins. The prototype protein synthesis-inhibiting bacterial toxins are diphtheria toxin and *Pseudomonas* exotoxin A (75). These toxins inactivate elongation factor 2 (EF-2) by NAD-dependent adenosine diphosphate (ADP) ribosylation of a modified histidine residue called diphthamide (75); inactivation of EF-2 inhibits peptide chain elongation. Although Shiga toxin also inhibits peptide chain elongation (10, 97), Obrig et al. (in press) found that Shiga toxin does not directly affect EF-2 but does affect EF-1-dependent reactions. Thus, Obrig et al. (in press) and Brown et al. (8a) concluded that EF-1-dependent aminoacyl transfer ribonucleic acid (tRNA) binding is the primary site of Shiga toxin action. How Shiga toxin alters EF-1-dependent steps in protein synthesis remains to be determined. One possible explanation proposed by Obrig et al. (in press) is that Shiga toxin structurally modifies 60S ribosomal subunits, which results in reduced affinity of EF-1 (and subsequently EF-2) for the ribosome. Obrig et al., in a separate report (86), found that Shiga toxin exhibits ribonuclease (RNase) activity with naked 5S or 5.8S ribosomal RNA (rRNA) of 60S ribosomal subunits acting as substrates; 28S rRNA was not tested. In the same report, Obrig et al. presented evidence that two other ribosome-inactivating toxins, ricin and phytolaccin, also exhibit RNase activity specific for 5S or 5.8S rRNA as substrates (86). However, the three toxins generated distinct patterns of RNA fragments, which suggests different cleavage sites on the RNAs. As discussed by Obrig et al. (86), one possible explanation for the findings with Shiga toxin is that the toxin preparation contained a contaminating RNase. The argument presented by Obrig et al. (86) against such a possibility was that the heat inactivation profiles for the RNase and protein synthesis-inhibitory activities of Shiga toxin were similar. It should be noted that despite the apparent RNase activity of Shiga toxin against naked rRNA, no changes in any of the three rRNAs present in intact ribosomes after exposure to Shiga toxin have been observed (Obrig et al., in press).

Immunology and Immunochemistry

Evidence that Shiga and Shiga-like toxins are produced in vivo in sufficient quantities to elicit an immune response was first presented by Keusch and Jacewicz (60). These investigators reported that the sera of patients with shigellosis due to either *S. flexneri* or *S. sonnei* contained antibody that could neutralize the cytotoxic effects of Shiga toxin on HeLa cells. In the same study, Shiga toxin-neutralizing antibody responses in patients with previous *S. dysenteriae* 1 and *S. dysenteriae* 2 infections were also demonstrated. The anti-Shiga toxin response in serum for both natural and experimental infections of humans was reported to be restricted to the immunoglobulin M heavy-chain class (64). The neutralizing antitoxin in hyperimmune rabbit sera has been reported as being restricted to the immunoglobulin M fraction (64) and the immunoglobulin G fraction (81). The basis for these discrepant results has not been found.

The question of whether secretory immunoglobulin A anti-Shiga toxin antibodies in the gastrointestinal tract will protect against diarrhea or dysentery caused by *Shigella* spp. has not been resolved. However, it is clear from studies with rhesus monkeys that high levels of circulating anti-Shiga toxin antibodies will not protect against oral *Shigella* infection (74). Monkeys that were vaccinated parenterally with Formalin-inactivated Shiga toxin developed levels of antitoxin in serum which protected them against injection with 1,000 50% mouse lethal doses, but they had diarrhea or dysentery as severe as (or more severe than) unimmunized controls did when they were fed *S. dysenteriae* 1.

Two types of antigenic variants could potentially exist among the family of Shiga and Shiga-like toxins: (i) toxins that are not cross-neutralized by a single reference antiserum, and (ii) partially cross-reacting toxins that are cross-neutralized but are not antigenically identical. No cross-reacting variants have been detected among the Shiga toxins produced by different strains of *S. dysenteriae* 1 (A. D. O'Brien, L. R. M. Marques, and S. B. Formal, unpublished observations). Prado et al. (95) examined sonic lysates of 35

strains of shigellae and found that 86% of the lysates (other than *S. dysenteriae* 1) could not be neutralized by anti-Shiga toxin. Whether the cytotoxin(s) present in these strains are related to Shiga toxin but are not cross-neutralizable remains to be determined.

Genetics

Little historical information is available on the genetics of Shiga toxin production. The only mutations reported to affect the yield of shiga toxin are chlorate-resistant mutations in *S. dysenteriae* 1 (34) that reduce the level of cytotoxin produced (64, 79). The mutations in those strains were not mapped, nor was the mechanism by which they reduced the yield of cytotoxin determined. In fact, the chlorate-resistant mutants were originally (34) classified as nontoxigenic rather than as low-level toxin producers, because the cytotoxicity assay used in the early 1970s was less sensitive than that now available.

The genes for the Shiga toxin of *S. dysenteriae* 1 and the Shiga-like toxin of *S. flexneri* appear to be located on the chromosome; no evidence for toxin genes on plasmids or bacteriophages of *Shigella* spp. has been found. Timmis et al. (112) reported the first in vivo cloning of the Shiga toxin determinant. These investigators generated a series of R′ plasmids in *E. coli* K-12 that carry sections of the *S. dysenteriae* 1 chromosome. The plasmids were constructed to carry portions of the *S. dysenteriae* 1 chromosome previously demonstrated (102) for *S. flexneri* to correlate with the ability of the organism to provoke fluid secretion in rabbit ileal segments. The results of the study by Timmis et al. (112) localized the Shiga toxin genes (designated *sht*) near the "*metB* distal side of *argE*". The close linkage of *sht* to *argE* is in some disagreement with the report of Sansonetti et al. (102), who tentatively localized the region on the *S. flexneri* chromosome for fluid production in rabbits as being near the lysine decarboxylase (LDS)-negative locus and in the *rha-mtl* region. Timmis et al. (112) offered two quite plausible explanations for these differences in the localization of the toxin genes: the maps of *S. dysenteriae* 1 and *S. flexneri* may not be exactly the same, and *S. dysenteriae* 1 may carry a second copy of *sht*. The latter possibility is supported by the mention (112) of an unpublished finding that a second determinant for cytotoxin production is carried on an R′-*asn* plasmid that also carries the LDS-negative locus of *Shigella* spp. Timmis et al. (112) stated that they had successfully cloned the toxin determinants of the R′-*argE* plasmid in vitro. It will be of considerable interest to compare the operon structure and nucleotide sequence of *sht* with the structural genes of *E. coli* SLT-I and *E. coli* SLT-II (see below). Toward that end, the structural genes for Shiga toxin have recently been cloned in our laboratory (N. A. Strockbine, R. K. Holmes, and A. D. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B-39, p. 78).

Role of Toxin in Disease

Dysentery. The subject of the possible role of Shiga and Shiga-like toxins in the pathogenesis of several *Shigella*- and *E. coli*-associated diseases was recently reviewed by Cantey (12). There is no direct proof that Shiga and Shiga-like toxins function as important virulence factors, but there are circumstantial data to support that hypothesis. Dysentery in volunteers fed an invasive, low-toxin-producing (64, 79), chlorate-resistant mutant of *S. dysenteriae* 1 designated strain 725 was milder than the disease caused by the invasive, highly toxinogenic parent strain M131. The duration and height of the fever and severity of the dysentery were less in volunteers fed strain 725 than in those fed strain M131 (70). These clinical observations are in keeping with the idea that the higher level of toxin produced by *S. dysenteriae* 1 than other *Shigella* spp. correlates with the more severe disease caused by Shiga's bacillus; dysentery occurs more commonly in patients infected with *S. dysenteriae* 1 than in those infected with any other serotype of *Shigella* (57). One explanation for how Shiga toxin promotes dysentery is that toxin present free in the colonic lumen binds to colonic epithelia and ultimately kills those cells by inhibiting protein synthesis. Two lines of evidence support such a theory. First, measurable toxin is found in human fecal specimens from patients with *S. dysenteriae* 1 infection (19). Second, Shiga toxin binds to and is cytotoxic for primary cultures of human colonic epithelial cells (M. P. Moyer, S. W. Rothman, P. S. Dixon, and J. E. Brown, submitted for publication; M. P. Moyer, S. W. Rothman, and J. E. Brown, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B108, p. 36).

Diarrhea. The results of gut perfusion studies (100) done with monkeys also provide evidence for a role of Shiga or Shiga-like toxins in causing the watery diarrhea sometimes seen during shigellosis (24). All *S. flexneri*-infected animals showed decreased colonic absorption or net colonic secretion. However, animals with diarrhea also showed net secretion in the jejunum, whereas no jejunal secretion was evident in monkeys with dysentery alone. No invasion of jejunal tissues by *Shigella* spp. was found in any of the animals, although invasion of the colonic epithelium occurred regularly. Subsequently, Kinsey et al. (65) showed that monkeys inoculated intracecally with *S. flexneri* had dysentery but not diarrhea. Thus, in monkeys, the diarrhea of shigellosis appears to require a secretory response of the small bowel superimposed on the colonic dysfunction. More recent findings with humans (11) suggest that shigella diarrhea results from colonic dysfunction without evidence for an increased small-bowel flow rate. Wherever the intestinal site for the secretion responsible for shigella diarrhea, one may postulate that the secretion is a consequence of toxin produced by the *Shigella* spp.

If Shiga toxin is responsible for the diarrhea of shigellosis, a key question is how can a protein synthesis-inhibiting toxin elicit fluid secretion in the intestine? Shiga toxin does not appear to act like cholera toxin or *E. coli* heat-labile toxin. Although Shiga toxin can elevate the cyclic adenosine monophosphate (cAMP) content of rabbit intestinal mucosa (22), as can cholera toxin and *E. coli* heat-labile toxin, Shiga-toxin-induced accumulation of cAMP does not occur until long after fluid secretion begins (H. T. Paulk, A. O. Cardamone, G. S. Gotterer, and T. R. Hendrix, Gastroenterology **72**:1164, 1977). Shiga toxin also does not appear to act like *E. coli* heat-stable enterotoxin. Unpublished observations from collaborative studies with R. Guerrant indicate that Shiga toxin does not increase the cyclic guanosine monophosphate (cGMP) content of the rabbit ileum. An alternative mechanism by which Shiga toxin could cause net fluid secretion is to block (or inhibit) fluid absorption. Indeed, Keenan et al. (55) recently reported that purified Shiga toxin and purified *E. coli* Shiga-like toxin selectively destroyed the mature absorptive epithelial cells of the rabbit ileum. One can therefore propose that Shiga toxin binds to mature absorptive epithelial cells through glycolipid receptors, inactivates protein synthesis in the host cells, and ultimately causes the death of those cells. In this model,

TABLE 1. Characteristics of *E. coli* enterotoxins[a]

Entero-toxin[b]	Structure	MW	Mass of subunits A and B (kilodaltons)	No. of subunits A and B	Immuno-genic	Bioassays	Mode of action
LT-I	Protein	86,000	28; 11.8	1; 5	Yes	Y1 or CHO cells; rabbit ileal loops	Adenylate cyclase production
LT-II	Protein	ND[c]	28; 11.8	ND	Yes	Y1 or CHO cells	Adenylate cyclase production
SLT-I	Protein	70,000	32; 7.7	1; 5	Yes	HeLa or Vero cell cytotoxicity; rabbit ileal loops; lethality to mice	Protein synthesis inactivation
SLT-II	Protein	ND	ND	ND	Yes	HeLa or Vero cell cytoxicity; rabbit ileal loops; lethality to mice	ND
ST_a	Polypeptide	2,000			No	Suckling mouse	Guanylate cyclase production
ST_b	Polypeptide	5,000			No	Weaned pig jejunal loops	ND

[a] For recent reviews of the enterotoxins, see Guerrant et al. (39) and O'Brien and Nataro (83).
[b] LT, Heat-labile toxins; ST, heat-stable toxins. For details about the recently characterized toxin LT-II, see Pickett et al. (93), Holmes et al. (42), and Guth et al. (40, 41).
[c] ND, Not determined.

diarrhea would result from inhibition of absorption rather than from active secretion.

HUS. HUS was originally described by Gasser et al. (33) as the association of hemolytic anemia, thrombocytopenia, and acute renal failure. HUS can occur after infection with *S. dysenteriae* 1 (36, 68, 96) or after infection with *E. coli* serotypes, such as *E. coli* O157:H7, that produce high levels of Shiga-like (Vero) toxin (52, 91; Karmali et al., Letter, 1983). *S. dysenteriae* 1-associated HUS tends to be more severe than *E. coli*-associated HUS.

The signs of HUS after Shiga infection include disseminated intravascular coagulation, renal microangiopathy, and hemolytic anemia (68). In one study (68), 50% of the patients with *S. dysenteriae* 1-associated HUS had endotoxemia as assessed by the *Limulus* assay. These symptoms presage a poor prognosis. The clinical manifestations of *E. coli* O157:H7-associated HUS are the classic triad described by Gasser et al. (33). Karmali et al. (52) have suggested that the Shiga toxin of *S. dysenteriae* 1 or the Shiga-like toxin of *E. coli* O157:H7 may enter the bloodstream and cause damage to vascular endothelial cells such as those in the kidneys. This is an attractive hypothesis, because the damage to vascular epithelium seen on autopsy of patients who have died of HUS (68) resembles the histopathological damage to small vessels in the central nervous systems of animals inoculated with crude Shiga toxin (6, 14). An animal model of HUS has not yet been developed, and the limited availability of purified toxin has hindered studies on the effects of purified toxin in animals.

SHIGA-LIKE TOXINS IN *E. COLI*

History and Nomenclature

Several different toxins that act on the intestinal tract can be produced by *E. coli* (Table 1). *E. coli* organisms that cause diarrheal disease in humans and animals have been classified into four groups (71). Enterotoxigenic *E. coli* (ETEC) adhere to the mucosa of the small bowel and produce a heat-labile toxin or heat-stable toxin or both. Enteroinvasive *E. coli* (EIEC) do not produce heat-labile or heat-stable toxin, but, like shigellae, they penetrate and multiply within colonic epithelial cells. Enteropathogenic *E. coli* (EPEC) were originally incriminated epidemiologically as pathogens for infants and young children. EPEC belong to a limited number of serotypes (71), and some EPEC strains have been shown to adhere avidly to the mucosa of the small intestine and produce characteristic effacement of microvilli (1, 16, 77, 99). Enterohemorrhagic *E. coli* (EHEC) cause hemorrhagic colitis and are of the serotype O157:H7 (98). EPEC and EHEC are not enteroinvasive and do not produce the classical heat-stable and heat-labile enterotoxins (71, 98, 117). We and others (12, 52, 73) have proposed that some EPEC and all EHEC strains may cause intestinal disease by producing elevated levels of Shiga-like cytotoxins. In this section, the discovery and nomenclature of *E. coli* Shiga-like toxins, as well as the relationship of the toxins to Vero toxins, will be discussed.

Discovery that EPEC and EHEC make Shiga-like toxins. The initial search for a Shiga-like toxin of *E. coli* focused on EPEC that caused diarrhea in infant rabbits (13) or infant humans, because no virulence mechanism was established for EPEC, and, as mentioned above, they are neither enteroinvasive nor able to produce heat-labile or heat-stable enterotoxins. The results of a preliminary survey of selected *E. coli* strains for production of a Shiga-like toxin (defined at that time as a cytotoxin for HeLa cells that could be neutralized by rabbit antitoxin raised against crude Shiga toxin) indicated that certain EPEC can make such a toxin (A. D. O'Brien, M. R. Thompson, J. R. Cantey, and S. B. Formal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B103, p. 32). This hypothesis was confirmed in 1982 (82), when monospecific antibody to Shiga toxin became available. In the same investigation (82), it was also observed that the level of Shiga-like toxin produced by *E. coli* strains varied from trace amounts that were detectable only in bacterial lysates to levels equivalent to that produced by *S. dysenteriae* 1.

That EHEC also make Shiga-like toxins was first reported in 1983. O'Brien et al. (A. D. O'Brien, T. A. Lively, M. E. Chen, S. W. Rothman, and S. B. Formal, Letter, Lancet **i**:702, 1983) found that isolates of *E. coli* O157:H7 isolated during outbreaks that had occurred in the United States made high levels of a cell-associated cytotoxin for HeLa and Vero cells that could be neutralized by rabbit anti-Shiga toxin. In the same study, it was reported that culture supernatants of *E. coli* O157:H7 strains 931, 932, and 933 also contained a cytotoxin for HeLa and Vero cells that could be completely neutralized by rabbit anti-Shiga toxin. On further analysis of the 933 strain, it is now clear that this organism makes two kinds of cytotoxins, one of which can be neutralized by anti-Shiga toxin (110; Scotland et al., Letter, 1985). We have designated (110) the anti-Shiga toxin neutralizable cytotoxin as Shiga-like toxin I (SLT-I), and we have named the other cytotoxin Shiga-like toxin II (SLT-II).

It is important to note that SLT-I predominates in cell lysates and SLT-II is the more active toxin in culture filtrate (110) when both toxins are produced by the same strain.

Relationship of Shiga-like toxins to Vero toxins. The first description of an EPEC Vero toxin was made in 1977 by Konowalchuk et al. (67). The Vero toxin produced by the strain used as a prototype in that classical study, EPEC O26:H11 strain H-30, appears to be the same toxin as SLT-I of *E. coli* (A. D. O'Brien, T. A. Lively, M. E. Chen, S. W. Rothman, and S. B. Formal, Letter, Lancet **i**:702, 1983). Konowalchuk et al. identified nine strains in addition to *E. coli* H-30 that contained a Vero toxin in culture filtrates of the organisms (67). Of these 10 strains, 7 were associated with diarrhea in human infants, 1 was associated with diarrhea in a weanling pig, and 2 were isolated from cheese. None of the *E. coli* strains that made Vero toxin also made *E. coli* heat-labile or heat-stable enterotoxin. Vero toxin from 8 of the 10 strains was neutralized by antiserum against the Vero toxin from *E. coli* H30; Vero toxins from an EPEC O128:B12 and from the pig isolate O138:K81 were not neutralized by the antiserum. This was the earliest indication that *E. coli* strains produce more than one toxin active against Vero cells.

The first studies on *E. coli* isolated from animals Vero toxin concentrated on EPEC and EPEC-like strains (see above) (15, 53, 54, 66, 105; W. G. Wade, B. T. Thom, and N. Evans, Letter, Lancet **ii**:1235–1236, 1979; S. M. Scotland, N. P. Day, G. A. Willshaw, and B. Rowe, Letter, Lancet **i**:90, 1980; M. W. Wilson and K. A. Bettelheim, Letter, Lancet **i**:201, 1980). Subsequently, Johnson et al. (Letter, 1983) found that Canadian isolates of EHEC which had been incriminated as the agents of hemorrhagic colitis made Vero toxin. As was the case with *E. coli* O157:H7 strains isolated during two outbreaks of hemorrhagic colitis in the United States (98, 117), the Canadian strains were not enteroinvasive and did not make *E. coli* heat-labile or heat-stable enterotoxin. With the observation that EHEC make a Shiga-like toxin active on Vero cells (A. D. O'Brien, T. A. Lively, M. E. Chen, S. W. Rothman and S. B. Formal, Letter, Lancet **i**:702, 1983), O'Brien et al. concuded that Vero toxin and Shiga-like toxin were the same toxin. When it was subsequently realized that EHEC can make more than one antigenically distinct Vero toxin (110; Scotland et al., Letter, 1985), Scotland et al. adopted the nomenclature Vero toxin 1 (VT1) and Vero toxin 2 (VT2). VT1 is equivalent to SLT-I, and VT2 is synonymous with SLT-II.

Purification and Structure

SLT-Is from cell lysates of EPEC strain H-30 and from cell lysates of EHEC strain 933 have been purified to homogeneity in our laboratory (80; A. D. O'Brien, T. A. Lively, T. W. Chang, and S. L. Gorbach, Letter, Lancet **ii**:573, 1983) by the same series of steps used to purify toxin from *S. dysenteriae* 1: i.e., French pressure cell disruption of bacteria grown in iron-depleted medium, Affi-Gel Blue chromatography, chromatofocusing, and anti-Shiga toxin affinity chromatography. The SLT-I from EHEC has also been purified by a method that includes ammonium sulfate fractionation, DEAE-cellulose column chromatography, and high-pressure liquid chromatography (M. Noda, N. Nakabayaski, T. Yutsudo, T. Hirayama, and Y. Takeda, Abstr. Toxicon **23**:600, 1985). Each SLT-I purified from these EPEC and EHEC strains was comprised of an A subunit and multiple copies of a B subunit.

The relative mobilities on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the A and B subunits of the SLT-Is purified from EPEC strain H30 and EHEC strain 933 were indistinguishable from those of the purified Shiga toxin of *S. dysenteriae* 1 strain 60R when all three samples were run in parallel (O'Brien, et al., Letter, Lancet **ii**:573, 1983). This observation, together with the data now available on the subunit structure of Shiga toxin (see above) and the translated nucleotide sequence of SLT-I from EHEC strain 933 (see Genetics and Regulation), indicate that the SLT-I A subunit has a MW of 32,200 and the SLT-I B subunit has a MW of 7,700. The value for the A subunit MW is close to that of the original estimate obtained with purified toxin of EPEC strain H30 (31,500 [80]), but the value for the B subunit is almost twice that estimated from cross-linking studies (80). By analogy with Shiga toxin, the most likely A-to-B subunit ratio for SLT-I is 1:5. If that is indeed the case, then the MW of holotoxin is ca. 70,000. Previous estimates of the MW of SLT-I have ranged from a low of 10,000 to 30,000 (67; W. G. Wade, B. T. Thom, and N. Evans, Letter, Lancet **ii**:1235–1236, 1979) to a high of 48,000 (80). The reason for this range in the reported MW of SLT-I is not known.

SLT-II may have been purified to homogeneity by Padhye et al., who recently reported (90) that they had purified a Vero cell cytotoxin from culture filtrates of *E. coli* O157:H7 strain 932 by concentration of filtrates followed by molecular sieve and anion-exchange chromatography. Since the Vero cell cytotoxin prepared by Padhye et al. (90) was not neutralized by anti-Shiga toxin, and since strain 932 produces both SLT-I and SLT-II (73), it seems reasonable to presume that the material isolated by Padhye et al. is SLT-II. Neutralization studies with anti-SLT-II must be done to prove or disprove that hypothesis. One of the problems with isolating one type of SLT from *E. coli* strains that make both SLT-I and SLT-II is that a toxin preparation could be contaminated with the variant toxin. Indeed, with the discovery by Scotland et al. (Letter, 1985) that *E. coli* 933 makes not only VT1 (SLT-I) but also VT2 (SLT-II), we reexamined our SLT-I preparation from *E. coli* 933 for SLT-II. We found no evidence that our preparation of SLT-I was contaminated with SLT-II.

Mode of Action

Purified SLT-Is from *E. coli* H30 (80) and *E. coli* 933 (O'Brien et al., Letter, Lancet **ii**:573, 1983) have the same biological activities as and comparable specific activities to purified Shiga toxin: cytotoxicity for HeLa and Vero cells, enterotoxicity for ligated rabbit ileal segments, and lethality at doses between 100 ng and 2 μg for mice. By analogy with Shiga toxin, the molecular basis for these three seemingly diverse biological activities is catalytic inactivation of 60S ribosomes in toxin-sensitive (receptor-expressing) cells. To our knowledge, the experiments to test the validity of this analogy have not been done, although crude preparations of *E. coli* SLT-I do inhibit protein synthesis in HeLa cells (81). The receptor for SLT-I has been identified (C. A. Lingwood, H. Law, S. Richardson, M. Petric, J. L. Brunton, and M. Karmali, J. Biol. Chem., in press) and is the same as for Shiga toxin. Cell lysates of the lysogenic strain *E. coli* C600 (933W) were recently used to assess the biological properties of SLT-II (110). The results of that study indicate that SLT-II has the same three biological properties as SLT-I, but, per unit of protein in cell lysates, it is more lethal for mice and less cytotoxic than SLT-I (110).

Immunology and Immunochemistry

Recent studies have established that SLT-I and VT1 are alternative names for the same toxin (110; Scotland et al., Letter, 1985; Karmali et al., Letter, 1986). SLT-I was purified to apparent homogeneity from both EPEC and EHEC strains (80; O'Brien et al., Letter, Lancet ii:573, 1983). In immunodiffusion tests with anti-Shiga toxin, both preparations of purified SLT-I gave lines of identity with Shiga toxin. Two monoclonal antibodies raised against *E. coli* SLT-I neutralized both homologous *E. coli* toxin and purified Shiga toxin (109). Monoclonal antibodies against Shiga toxin have also been isolated, but their reactivities against purified SLT-I were not determined (21, 38). Systematic studies of cross-reactivity between Shiga toxin and SLT-I from various strains of *E. coli* in immunobinding assays were not performed, primarily because sufficient quantities of the purified toxins were not available. Further studies are needed to determine whether Shiga toxin and SLT-I are immunochemically identical or whether there are unique epitopes on SLT-I that are not present on Shiga toxin, or vice versa.

Although the SLT-II of *E. coli* has cytotoxic, enterotoxic, and lethal activities similar to those of SLT-I and Shiga toxin (110), it is not neutralized by antiserum against purified Shiga toxin or monoclonal antibodies against SLT-I. In contrast, it is neutralized by antiserum prepared against a crude preparation of SLT-II. Antigenic diversity among Vero cytotoxins of *E. coli* has also been documented, and VT2 has been recognized that is not neutralized by anti-Shiga toxin but is neutralized by antiserum against crude VT2 (Scotland et al., Letter, 1985; Karmali et al., Letter, 1986). Reference SLT-II antiserum (110) neutralized the cytotoxicity of lysates from *E. coli* E32511 (O157:H$^-$), the prototype strain against which the reference VT2 antiserum was raised (Scotland et al., Letter, 1985), indicating that SLT-II and VT2 are probably alternate names for the same or closely related toxins. No toxin that has been identified immunologically as SLT-II or VT2 has been purified to homogeneity or characterized structurally, nor has the mode of action of this toxin been investigated (see preceding section). As noted above, Padhye et al. (90) have purified a toxin from *E. coli* 932 that is distinct from SLT-I, but the immunologic identity of this toxin with SLT-II has not yet been established. The possibility that SLT-I and SLT-II have shared epitopes that are not involved in neutralization of toxicity by antibody has not yet been investigated.

There is preliminary evidence that *E. coli* from a variety of sources may produce Vero cytotoxins of additional antigenic types. The Vero cytotoxins produced by strains of *E. coli* isolated from pigs with edema disease or neonatal pigs with diarrhea were not neutralized by antisera that were active against the cytotoxin(s) from *E. coli* strains from humans (67, 108). Karmali et al. (Letter, 1986) prepared a reference antiserum against a toxoid made from purified VT from *E. coli* H-30 (which is known to produce VT1) and compared its neutralizing activity with that of serum obtained from a patient convalescing from *HUS*. In tests with culture filtrates from a collection of *E. coli* strains from human and animal sources, all of the toxins that were completely neutralized by the antitoxoid (presumed to be VT1) were also neutralized by the convalescent serum. However, the toxins that were not neutralized by the antitoxoid could be divided into two groups on the basis of their reaction with the convalescent serum: some that were partially neutralized (and presumably contained VT2 exclusively or in highest titer) and others that were not neutralized at all. Karmali et al. interpreted the data as supporting the existence of antigenically distinct VT1 and VT2 toxins produced by *E. coli*, a conclusion with which we agree, but they did not address the nature of the toxin(s) that were not neutralized by either of their antisera. Unpublished observations from our laboratory (L. R. M. Marques, J. S. M. Peiris, S. J. Cryz, and A. D. O'Brien) suggest that at least one Vero toxin from *E. coli* strains isolated from pigs with edema disease is related to, but not identical with, the prototype SLT-II. Such strains contain an extracellular Vero toxin that, unlike SLT-II, is not active on HeLa cells but is neutralized by reference antisera against SLT-II. How this variant of SLT-II is related to the Vero toxins produced by the strains described by Karmali et al. (Letter, 1986) and Smith et al. (108) remains to be determined.

Genetics and Regulation

Phage conversion is responsible for controlling the production of several important bacterial toxins, including diphtheria toxin, streptococcal erythrogenic toxin, botulinum toxin, and staphylococcal enterotoxin A (2, 5, 72). Toxin-converting phages contain genes that are essential for toxinogenesis, and susceptible bacteria that become lysogenic for such *tox*$^+$ phages acquire the capacity to produce the corresponding toxin. Recently, production of Shiga-like toxins was also shown to be determined by specific phages in selected strains of both EPEC (O26, O128) and EHEC (O157) serotypes isolated from humans (84, 108; Scotland et al., Letter, 1983; H. R. Smith, N. P. Day, S. M. Scotland, R. J. Gross, and B. Rowe, Letter, Lancet i:1242–1243, 1984). In contrast, production of Shiga-like toxin in *E. coli* strains isolated from pigs was not found to be controlled by phages (108).

The phages that determine the production of Shiga-like toxins in *E. coli* are of several different types that were initially distinguished on the basis of differences in properties such as host range, immunity specificity, and susceptibility to thermal inactivation (84, 108). *E. coli* H19 (O26) and 933 (O157) are both lysogenic for two different toxin-converting phages. The phages, originally designated H19A (108) and H19J (108) are independent isolates of the same phage from strain H19 that are closely related to phage 933J from strain 933 (108) in terms of their morphology, virion polypeptides, DNA restriction fragments, and immunity specificity, but phage H19B has a different immunity specificity. Phages H19A/J, 933J, and H19B are all lambdoid phages that code for SLT-I. A 8.1-kilobase (kb) *Eco*RI fragment of the H19B genome was shown to be homologous with the region of the gene of phage lambda that encodes the genes *cI*, *ninR*, *O*, *P*, and the origin of replication (44); the regions of homology between phage lambda and phages H19A/J and 933J have not yet been mapped in detail. Phage 933W from *E. coli* 933 has quite different characteristics from the other phages described above and codes for SLT-II (84, 110). A converting phage was also isolated from an O157:H$^-$ *E. coli* strain, E32511 (119), which was subsequently shown to produce SLT-II (110; Scotland et al., Letter, 1985).

The organization and expression of the operon for SLT-I are shown schematically in Fig. 2. The structural genes that encode SLT-I were cloned on a 2.5-kb DNA fragment from a phage from strain H19 (not characterized as H19A or H19B) (119), on a 3.1-kb DNA fragment from phage 933J (78), and on a 1.7-kb DNA fragment from phage H19B (44). The restriction maps of phages 933J and H19B were similar

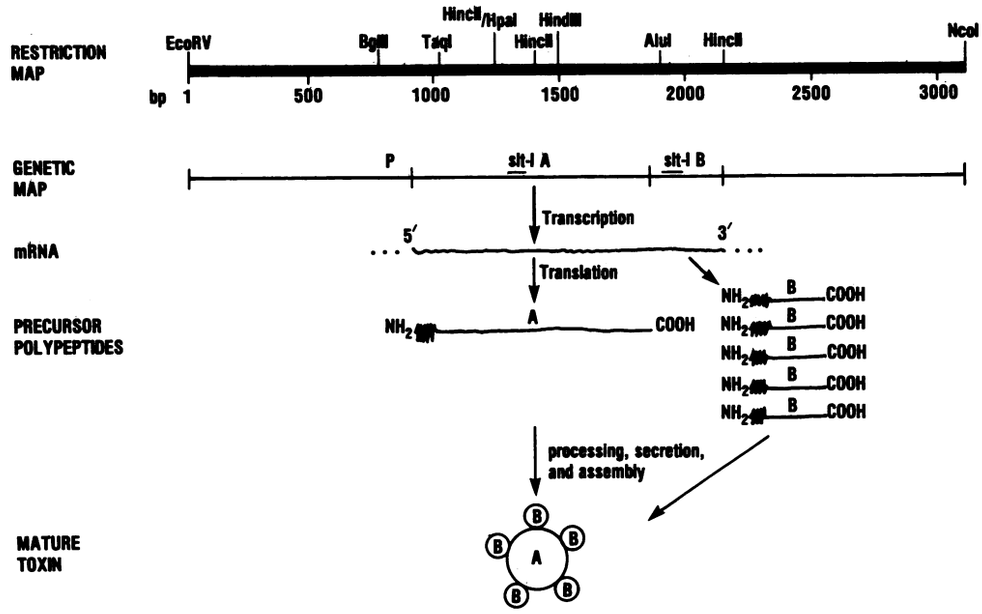

FIG. 2. Working model of the organization and expression of the SLT-I operon. The restriction map of the cloned 3.1-kb *Eco*RI-*Nco*I fragment of DNA from phage 933J is adapted from Jackson et al. (47a). The genetic map shows the location for the promotor (P) for the SLT-I operon and the structural genes *slt*-I A and *slt*-I B. The mRNA is presumed to be polycistronic. The primary translation products correspond to the unprocessed A and B polypeptides of SLT-I which have amino-terminal signal sequences represented by the wavy lines. The oligomeric holotoxin is presumed to be assembled from the A and B polypeptides after they are secreted and processed to remove the signal sequences.

but not identical. Analysis of appropriate DNA clones and subclones from phage 933J in minicells (78) and from phage H19B in an in vitro protein synthesis system (44) provided direct evidence that the phage genomes code for SLT-I, permitted assignment of the locations of the contiguous sequences coding for polypeptides A and B, and provided evidence for the orientation of transcription. Expression of the B gene by clones from which the promoter and the proximal part of the A gene was deleted indicated that the B gene can also be transcribed from a second promoter. This organization of the toxin operon was confirmed and extended by analysis of transposon insertions and mini-mu *lac* operon fusions within the genes for SLT-I (119; R. J. Neill, N. R. Serrano, S. W. Rothman, D. A. Foret, and D. H. Wells, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B112, p. 36; D. L. Weinstein and A. D. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B24, p. 28). The complete nucleotide sequence of the structural genes for the A and B polypeptides of SLT-I and the adjacent regulatory sequences from phage 933J have recently been determined in our laboratory (47a), and preliminary results from the sequencing of the A and B subunit genes from phage H19B have been reported by others (5). Analysis of the translated amino acid sequences indicates that the A and B polypeptides of SLT-I contain hydrophobic signal sequences at their amino termini and are probably synthesized as precursor forms of secreted periplasmic proteins. The deduced sequence for the mature form of the B polypeptide of SLT-I was identical with the 69-amino-aid sequence determined recently for the B polypeptide of Shiga toxin from *S. dysenteriae* (106). These studies therefore establish conclusively that SLT-I from *E. coli* and Shiga toxin from *S. dysenteriae* 1 are very closely related, and possibly identical, proteins.

The genetic determinants for a second Vero cytotoxin (VT2) were cloned on a 4.7-kb *Eco*RI fragment from a phage from *E. coli* E32511 into the plasmid vector pACYC184 (119). A 2-kb DNA probe prepared from the 4.7-kb insert of this hybrid plasmid was shown to hybridize with toxinogenic strains of *E. coli* O157 that failed to react with another 0.75-kb DNA probe for the SLT-I structural genes, providing direct evidence for two genetically distinct cytotoxins of *E. coli*. On the basis of evidence that *E. coli* E32511 produces SLT-II but not SLT-I (110; Scotland et al., Letter, 1985) and that *E. coli* K-12 lysogenized with the phage from strain E32511 also produces SLT-II only (Scotland et al., Letter, 1985), it seems almost certain that the second toxin identified by genetic criteria is the same as the second toxin identified by immunologic criteria. Under conditions of low stringency, the 2-kb DNA probe for SLT-II cloned from the E32511 phage hybridized weakly with the 0.75-kb DNA probe for SLT-I (119), and the 3.1-kb DNA fragment encoding SLT-I from phage 933J described above hybridized with specific restriction fragments of DNA from the SLT-II-converting phage 933W (110). These observations indicate that there is some homology between the structural genes for SLT-I and SLT-II. Very recently, the SLT-II genes were cloned and their polypeptide gene products were identified (J. W. Newland, N. A. Strockbine, and R. J. Neill, submitted for publication). Sequencing of the genes for SLT-II is in progress in our laboratory (M. P. Jackson, R. J. Neill, R. K. Holmes, A. D. O'Brien, and J. W. Newland, manuscript in preparation). Preliminary data based on the sequencing indicate that the genes for SLT-I and SLT-II have approximately 50 to 60% overall homology, with some regions having substantially greater homology. The molecular masses of the processed A and B polypeptides of SLT-II are predicted to be approximately 33.1 and 7.7 kilodaltons, respectively.

Little is known about the regulation of production of SLT-I or SLT-II in *E. coli*. For strains of *E. coli* that produce both SLT-I and SLT-II, SLT-I is often the predominant toxin present in cell lysates, whereas SLT-II may be present

in much higher titer than SLT-I in culture supernatants (16). This observation suggests that there may be significant differences in secretion or localization of SLT-I and SLT-II in *E. coli*. Wild-type strains of *E. coli* have been classified as high-, moderate-, or low-toxin-producing strains on the basis of quantitative titrations of toxin produced by strains grown under rigidly controlled conditions in deferrated medium (73). All strains of *E. coli* O157:H7 associated with hemorrhagic colitis have been characterized as highly toxinogenic, and a significant proportion of EPEC isolates are highly toxinogenic. Most highly toxinogenic strains produce SLT-I, and some also produce SLT-II. Production of SLT-II only is common among strains classified as moderately toxinogenic (73, 110). All strains of *E. coli* K-12 examined to date produce low levels of Shiga-like toxin that can be neutralized by polyclonal anti-Shiga toxin or monoclonal antibodies against SLT-I, but the DNA probe for SLT-I from phage 933J described above does not hybridize to any significant extent with DNA from *E. coli* K-12 strains. The reasons for this apparent discrepancy between the results of immunologic and genetic tests for the SLT of *E. coli* K-12 strains have not been resolved.

Role in Disease

Epidemiological evidence. As is the case with Shiga toxin, there is no direct proof that *E. coli* SLT-I or SLT-II plays a role in disease. Some of the strongest circumstantial evidence comes from epidemiological studies of *E. coli* strains isolated from humans (17, 48a, 52, 73, 105, 108) and animals (15, 53, 54, 73, 76, 107). Most of the epidemiological studies to date have exclusively examined culture filtrates of bacteria for Vero toxin. By contrast, Marques et al. (73) recently tested culture supernatants and sonic lysates of 418 strains of *E. coli* isolated from human or animal stools or from food for cytotoxic effects on HeLa cells. These investigators also asked whether cytotoxicity could be neutralized by anti-Shiga toxin. Marques et al. (73) classified strains according to the level of cell-associated toxin they produced. The categories were as follows: low, 2×10^2 to 6×10^2 CD_{50} per ml of sonic lysate; moderate, 1×10^3 to 1×10^4 CD_{50} per ml of sonic lysate; and high, 1×10^5 to 1×10^8 CD_{50} per ml of sonic lysate. Of the 418 strains analyzed, 107 had no detectable cell-associated cytotoxicity. A total of 262 strains made low levels of cytotoxin. The culture supernatants from the low-level cytotoxin producers had no detectable activity. Strains that fell in the low-level toxin producer category included *E. coli* isolated from healthy adults, *E. coli* K-12 substrains, EIEC, ETEC, *E. coli* strains of classical EPEC serogroups isolated from patients with diarrhea, other strains isolated from patients with diarrhea, and three strains (not of the O157:H7 serotype) isolated from patients with hemorrhagic colitis. Ten strains produced moderate levels of cell-associated cytotoxin, and cytotoxicity could also be detected in culture supernatants. All 10 strains were associated with diarrhea, hemorrhagic colitis, or HUS, and the 10 strains made only SLT-II (110). A total of 39 strains made high levels of cytotoxin, and 38 of the 39 were associated with diarrhea, hemorrhagic colitis, or HUS. All 39 strains made SLT-I, and 17 of the 39 strains also made SLT-II (110). The finding that moderate or high levels of cytotoxin were almost exclusively (48 of 49) found in strains isolated from people with diarrhea, hemorrhagic colitis, or HUS suggests that such elevated levels of cytotoxin may play a role in the pathogenesis of these diseases. However, the mechanism by which elevated levels of SLT-I or SLT-II or both might mediate these diseases has not been determined. It is also not clear whether one of the two Shiga-like toxins is more important than the other in the pathogenesis of each type of enteric disease. It should be emphasized that low levels of cytotoxin might also damage host cells if toxin were produced by an *E. coli* strain that could adhere avidly to or invade intestinal epithelial cells.

With the realization that moderate- and high-level Shiga-like toxin-producing strains are strongly associated with enteric disease and HUS (48a, 52, 73) comes the need for an efficient and sensitive means of detecting such strains in stools. Karmali et al. (51) have described a method for detecting low numbers of Vero toxin-producing *E. coli* in mixed cultures by a combination of colony "sweeps" (loopfuls of confluent bacterial growth) and polymyxin extraction of cell pellets. The advantages of this procedure are its sensitivity and its capacity to detect both SLT-I and SLT-II-producing strains of *E. coli*. The major disadvantage of the procedure is the need to have tissue culture facilities available in the clinical laboratory. This is a particular problem for small laboratories and for nearly all laboratories in developing countries. A colony enzyme-linked immunosorbent assay that permits direct detection of high-level SLT-I-producing *E. coli* on agar plates was recently described (109). In that assay, cultures are grown on iron-depleted agar supplemented with subinhibitory doses of trimethoprim-sulfamethoxazole, an antimicrobial mixture that stimulates Shiga and Shiga-like toxin synthesis (50). This observation was made when Karch et al. (48) tested several antimicrobial agents for their effects on toxin production. After samples are inoculated onto the agar containing trimethoprim-sulfamethoxazole, plates are incubated overnight, and bacterial colonies are then transferred to nitrocellulose by blotting. Toxin is released from the colonies on the nitrocellulose with polymyxin B, and the toxin bound to the membrane is detected by addition of monoclonal antibody to the B subunit of SLT-I, a horseradish peroxidase-coupled second antibody, and substrate. The advantages of the colony enzyme-linked immunosorbent assay are its direct application to stool cultures and its sensitivity and specificity (109). The disadvantage of the assay is that it will detect only *E. coli* that produce high levels of SLT-I (109). A monoclonal antibody specific for SLT-II will have to be developed and incorporated into the assay to detect *E. coli* that make only SLT-II.

Animal models. Several animal models have been developed for studying the virulence mechanisms of EHEC O157:H7 (3, 4, 31, 92, 94; J. J. Farmer, M. E. Potter, L. W. Riley, T. J. Barrett, P. A. Blake, M. L. Cohen, G. K. Morris, B. M. Thompson, C. A. Bopp, A. Kaufmann, R. S. Remis, and J. G. Wells, Letter, Lancet i:702–703, 1983) and other *E. coli* strains that produce elevated levels of Shiga-like toxins (92). The infant rabbit, the gnotobiotic pig, and the gnotobiotic calf (D. Francis, personal communication) seem to be appropriate models for studying colonization and induction of diarrhea after oral infection with *E. coli* O157:H7 strains. The mechanism of colonization of the gnotobiotic pig by *E. coli* O157:H7 resembles the attach-and-efface mode of classical EPEC (reviewed in reference 71), except that colonization by EHEC of the gnotobiotic pig is apparently not plasmid mediated (S. Tzipori and R. Robins-Browne, personal communication). However, a large plasmid of 60 to 70 megadaltons is present in most isolates of *E. coli* O157:H7 (117; Johnson et al., Letter, 1983), and that plasmid is associated with the expression of a unique fimbrial antigen (49).

In a very interesting study by Pai et al. (92), it was observed that 3-day-old rabbits inoculated intragastrically with 10^8 *E. coli* O157:H7 cells consistently developed diarrhea. The damage to the mucosal epithelium was seen mainly in the mid- and distal colon and was characterized by apoptosis (defined by Pai et al. [92] as "individual cell death") in the surface epithelium, increased mitotic activity in the crypts, mucin depletion, and a mild to moderate infiltration of neutrophils in the lamina propria and epithelium. Pai et al. (92) also found that infant rabbits developed diarrhea when given partially purified Vero toxin (isolated from culture filtrates of *E. coli* O157:H7 and therefore probably predominately SLT-II). Furthermore, these investigators noted that the mucosal abnormalities of rabbits inoculated with toxin alone mimicked those seen when the intact organism was used. It is of note that apoptosis was a striking feature of the histopathological damage to adult rabbit ileum after exposure to purified Shiga toxin or SLT-I (55).

SHIGA-LIKE TOXINS IN OTHER BACTERIA

Some strains of *Vibrio cholerae* and *Vibrio parahaemolyticus* make low levels of a cell-associated cytotoxin for HeLa cells that can be neutralized by polyclonal anti-Shiga toxin (A. D. O'Brien, M. E. Chen, R. K. Holmes, J. Kaper, and M. M. Levine, Letter, Lancet, i:77–78, 1984) and monoclonal antibody to the B subunit of SLT-I (109). We have previously speculated (O'Brien et al., Letter, 1984) that a Shiga-like toxin might be responsible for the diarrhea occasionally seen with volunteers fed *V. cholerae* strains that do not make cholera enterotoxin, such as JBK70 (47). One of the ways to test this hypothesis is to construct *V. cholerae* strains that no longer contain the Shiga-like toxin genes and to compare the diarrhea-evoking potential of the mutant with that of its parent. Experiments to produce such mutants are in progress (J. Kaper, personal communication). In addition to the *Vibrio* sp., a strain of *Salmonella typhimurium* (82, 109) and several isolates of *Campylobacter jejuni* (M. M. Moore, M. J. Blaser, G. I. Perez-Perez, and A. D. O'Brien) have been found to produce low levels of a cytotoxin neutralized by anti-Shiga toxin. The role, if any, that these toxins play in the pathogenesis of salmonellosis and campylobacteriosis remains to be determined. Moreover, the frequency of Shiga-like toxin synthesis by *S. typhimurium* and *C. jejuni* is unknown. To date, no bacteria other than *E. coli* have been shown to produce a cytotoxin neutralized by reference anti-SLT-II sera.

CONCLUSION

The main purpose of this review was to summarize the results of recent studies leading to the purification and characterization of *S. dysenteriae* 1 toxin and the immunologically related *E. coli* Shiga-like toxin, as well as the cloning and characterization of the structural genes for *E. coli* SLT-I. Determination of the nucleotide sequences of the structural genes and the corresponding amino acid sequences for the structural polypeptides for all known members of the Shiga toxin family in the near future is technically feasible. Such studies will provide unequivocal evidence about the extent of genetic and structural homology among these toxins, but additional studies are required for detailed knowledge concerning the three-dimensional structures of the toxins and the relationships between structure, receptor-binding activity, mechanism(s) for inactivation of protein synthesis, and biologically important antigenic determinants. The availability of the cloned toxin genes, highly specific monoclonal antitoxic antibodies, and purified toxins provides a powerful set of tools for molecular analysis of these and other aspects of Shiga and Shiga-like toxins.

At present, the role of Shiga toxin in the pathogenesis of infectious diseases caused by *Shigella* spp. remains highly suggestive but inconclusive. No strains of *S. dysenteriae* 1 have been identified that are virulent but fail to produce Shiga toxin. It should be feasible to produce genetically engineered, isogenic strains of *S. dysenteriae* 1 that differ only with respect to their toxinogenicity or nontoxinogenicity and to perform direct tests to determine whether the toxin is an essential or an accessory virulence factor for the pathogenesis of shigellosis. Analogous studies should also be done with other *Shigella* species. All efforts to date to prevent shigellosis by antitoxic immunity have been unsuccessful, but reliable methods for inducing secretory antitoxic antibodies in the gut are not yet available.

Epidemiological studies have established that production of high or moderate levels of Shiga-like toxins is associated with many EPEC and most EHEC strains that cause disease in humans. Furthermore, *E. coli* or *Shigella* strains that produce high or moderate levels of toxin are frequently isolated from patients with HUS. Most strains of *E. coli* that cause edema disease in pigs also produce elevated levels of Shiga-like toxin. Many of these studies do not differentiate between the antigenic variants of Shiga-like toxins; therefore, additional studies are needed to define more precisely the prevalence of SLT-I and SLT-II among such strains and to establish whether other serotypes or serovariants exist among the Shiga-like toxins of *E. coli*.

The observation that active immunization against Shiga toxin does not provide protection against shigellosis in experimental animals (74) does not provide definitive evidence against a pathogenic role for Shiga or Shiga-like toxins. The pathogenesis of such infections involves several different kinds of interaction between bacteria and host cells. *Shigella* spp. are able to invade and multiple within colonic epithelial cells, and EPEC become closely associated and efface the microvilli of enterocytes. Toxin could be produced in the intracellular environmnt or delivered to target cells by direct contact with bacterial cells, and such toxin might not be accessible to neutralization by antibodies in the extracellular milieu. The nature of the interaction between EHEC and target cells in the intestines of infected humans is not yet clearly defined. For HUS and edema disease, it has been postulated that toxin may be disseminated hematogenously from the intestinal tract and reach putative target cells such as the vascular endothelium in distant organs. If toxin released from bacterial cell has an essential or adjunctive role in pathogenesis, then specific antitoxic immunity might be effective for prevention or treatment of some of these diseases.

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LITERATURE CITED

1. **Baldini, M. M., J. B. Kaper, M. M. Levine, D. C. A. Candy, and H. W. Moon.** 1983. Plasmid-mediated adhesion in enteropathogenic *Escherichia coli*. J. Pediatr. Gastroenterol. Nutr. **2**:534–538.
2. **Barksdale, L., and S. B. Arden.** 1974. Persisting bacteriophage infections, lysogeny and phage conversions. Annu. Rev. Microbiol. **28**:265–279.
3. **Beery, J. T., M. P. Doyle, and N. A. Higley.** 1984. Cytotoxic activity of *Escherichia coli* O157:H7 culture filtrate on the mouse colon and kidney. Curr. Microbiol. **11**:335–342.
4. **Beery, J. T., M. P. Doyle, and J. L. Schoeni.** 1985. Colonization of chicken cecae by *Escherichia coli* associated with hemorrhagic colitis. Appl. Environ. Microbiol. **49**:310–315.
5. **Betley, M. J., V. L. Miller, and J. J. Mekalonos.** 1986. Genetics of bacterial enterotoxins. Annu. Rev. Microbiol. **40**:577–605.
6. **Bridgwater, F. A. J., R. S. Morgan, K. E. K. Rowson, and G. P. Wright.** 1955. The neurotoxin of *Shigella shigae*. Morphological and functional lesions produced in the central nervous system of rabbits. Br. J. Exp. Pathol. **36**:447–453.
7. **Brown, J. E., D. E. Griffin, S. W. Rothman, and B. P. Doctor.** 1982. Purification and biological characterization of Shiga toxin from *Shigella dysenteriae* 1. Infect. Immun. **36**:996–1005.
8. **Brown, J. E., K. A. Karlsson, A. Lindberg, N. Stromberg, and J. Thurin.** 1983. Identification of the receptor glycolipid for the toxin of *Shigella dysenteriae*, p. 678–679. *In* M. A. Chester, D. Heinegard, A. Lundblad, and S. Svensson (ed.), Glycoconjugates. Proceedings of the 7th International Symposium on Glycoconjugates. Lund-Ronneby, Sweden.

8a. **Brown, J. E., T. G. Obrig, M. A. Ussery, and T. P. Moran.** 1986. Shiga toxin from *Shigella dysenteriae* 1 inhibits protein synthesis in reticulocyte lysates by inactivation of aminoacyl-tRNA binding. Microb. Pathogenesis **1**:325–334.

9. **Brown, J. E., S. W. Rothman, and B. P. Doctor.** 1980. Inhibition of protein synthesis in intact HeLa cells by *Shigella dysenteriae* 1 toxin. Infect. Immun. **29**:98–107.
10. **Brown, J. E., M. A. Ussery, S. H. Leppla, and S. W. Rothman.** 1980. Inhibition of protein synthesis by Shiga toxin. Activation of the toxin and inhibition of peptide elongation. FEBS Lett. **117**:84–88.
11. **Butler, T., P. Speelman, I. Kabir, and J. Banwell.** 1986. Colonic dysfunction during shigellosis. J. Infect. Dis. **154**:817–823.
12. **Cantey, J. R.** 1985. Shiga toxin—an expanding role in the pathogenesis of infectious diseases. J. Infect. Dis. **151**:766–771.
13. **Cantey, J. R., and R. K. Blake.** 1977. Diarrhea due to *Escherichia coli* in the rabbit: a novel mechanism. J. Infect. Dis. **135**:454–462.
14. **Cavanagh, J. B., J. G. Howard, and J. L. Whitby.** 1956. The neurotoxin of *Shigella shigae*. A comparative study of the effects produced in various laboratory animals. Br. J. Exp. Med. **37**:272–278.
15. **Chen, C., T. Kume, T. Hohdatsu, and S. Tsubaki.** 1984. *Escherichia coli* originated from diarrhea of suckling piglets in Taiwan. III. Ability to produce enterotoxin and Vero cytotoxin. Kitasato Arch. Exp. Med. **57**:221–226.
16. **Clausen, C. R., and D. L. Christie.** 1982. Chronic diarrhea in infants caused by adherent enteropathogenic *Escherichia coli*. J. Pediatr. **100**:358–361.
17. **Cleary, T. G., J. J. Mathewson, E. Faris, and L. K. Pickering.** 1985. Shiga-like cyototoxin production by enteropathogenic *Escherichia coli* serogroups. Infect. Immun. **47**:335–337.
18. **Conradi, H.** 1903. Ueber löslishe, durch aseptische Autolyse, erhaltene Giftstoffe von Ruhr- und Typhus bazillen. Dtsch. Med. Wochenschr. **29**:26–28.
19. **Donohue-Rolfe, A., N. A. Kelley, M. Bennish, and G. T. Keusch.** 1986. Enzyme-linked immunosorbent assay for *Shigella* toxin. J. Clin. Microbiol. **24**:65–68.
20. **Donohue-Rolfe, A., and G. T. Keusch.** 1983. *Shigella dysenteriae* 1 cytotoxin: periplasmic protein releasable by polymyxin B and osmotic shock. Infect. Immun. **39**:270–274.
21. **Donohue-Rolfe, A., G. T. Keusch, C. Edson, D. Thorley-Lawson, and M. Jacewicz.** 1984. Pathogenesis of *Shigella* diarrhea. IX. Simplified high yield purification of *Shigella* toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies. J. Exp. Med. **160**:1767–1781.
22. **Donowitz, M., G. T. Keusch, and J. J. Binder.** 1975. Effect of *Shigella* enterotoxin on electrolyte transport in rabbit ileum. Gastroenterology **69**:1230–1237.
23. **Dubos, R. J., and J. W. Geiger.** 1946. Preparation and properties of Shiga toxin and toxoid. J. Exp. Med. **84**:143–156.
24. **Dupont, H. L.** 1985. *Shigella* species (bacillary dysentery), p. 1269–1274. *In* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), Principles and practice of infectious diseases, 2nd ed. John Wiley & Sons, Inc., New York.
25. **Dupont, H. L., R. B. Hornick, A. T. Dawkins, M. J. Snyder, and S. B. Formal.** 1969. The response of man to virulent *Shigella flexneri* 2a. J. Infect. Dis. **119**:296–299.
26. **Eidels, L., R. L. Proia, and D. A. Hart.** 1983. Membrane receptors for bacterial toxins. Microbiol. Rev. **47**:596–620.
27. **Eiklid, K., and S. Olsnes.** 1980. Interaction of *Shigella shigae* cytotoxin with receptors on sensitive and insensitive cells. J. Recept. Res. **1**:199–213.
28. **Eiklid, K., and S. Olsnes.** 1983. Animal toxicity of *Shigella dysenteriae* cytotoxin: evidence that the neurotoxic, enterotoxic, and cytotoxic activities are due to one toxin. J. Immunol. **130**:380–384.
29. **Eiklid, K., and S. Olsnes.** 1983. Entry of *Shigella dysenteriae* toxin into HeLa cells. Infect. Immun. **42**:771–777.
30. **Engley, F. B., Jr.** 1952. The neurotoxin of *Shigella dysenteriae* (Shiga). Bacteriol. Rev. **16**:153–178.
31. **Francis, D. H., J. E. Collins, and J. R. Duimstra.** 1986. Infection of gnotobiotic pigs with an *Escherichia coli* O157:H7 strain associated with an outbreak of hemorrhagic colitis. Infect. Immun. **51**:953–956.
32. **Fuchs, G., M. Mobassaleh, A. Donohue-Rolfe, R. K. Montgomery, R. J. Grand, and G. T. Keusch.** 1986. Pathogenesis of *Shigella* diarrhea: rabbit intestinal cell microvillus membrane binding site for *Shigella* toxin. Infect. Immun. **53**:372–377.
33. **Gasser, C., E. Gautier, A. Steck, R. E. Siebenmann, and R. Oechslin.** 1955. Hämolytisch-urämische Syndrome: bilaterale niereninden-nekrosen bei akuten erworbenen hämolytischen Anamien. Schweiz. Med. Wochenschr. **85**:905–909.
34. **Gemski, P., Jr., A. Takeuchi, O. Washington, and S. B. Formal.** 1972. Shigellosis due to *Shigella dysenteriae* 1: relative importance of mucosal invasion versus toxin production in pathogenesis. J. Infect. Dis. **126**:523–530.
35. **Gentry, M. K., and J. M. Dalrymple.** 1980. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J. Clin. Microbiol. **12**:361–366.
36. **Gianantonio, C., M. Vitacco, F. Mendilaharzu, A. Rutty, and J. Mendilaharzu.** 1964. The hemolytic-uremic syndrome. J. Pediatr. **64**:478–491.
37. **Griffin, D. E., and P. Gemski.** 1983. Release of shiga toxin from *Shigella dysenteriae* 1 by polymyxin B. Infect. Immun. **40**: 425–428.
38. **Griffin, D. E., M. K. Gentry, and J. E. Brown.** 1983. Isolation and characterization of monoclonal antibodies to Shiga toxin. Infect. Immun. **41**:430–433.
39. **Guerrant, R. L., R. K. Holmes, D. C. Robertson, and R. N. Greenberg.** 1985. Roles of enterotoxins in the pathogenesis of *Escherichia coli* diarrhea, p. 68–73. *In* L. Leive (ed.), Microbiology—1985. American Society for Microbiology, Washington, D.C.
40. **Guth, B. E., C. L. Pickett, E. M. Twiddy, R. K. Holmes, T. A. T. Gomes, A. A. M. Lima, R. L. Guerrant, B. D. G. M. Franco, and L. R. Trabulsi.** 1986. Production of type II heat-labile enterotoxin by *Escherichia coli* isolated from food and human feces. Infect. Immun. **54**:587–589.
41. **Guth, B. E. C., E. M. Twiddy, L. R. Trabulsi, and R. K. Holmes.** 1986. Variation in chemical properties and entigenic determinants among type II heat-labile enterotoxins of *Escherichia coli*. Infect. Immun. **54**:529–536.
42. **Holmes, R. K., E. M. Twiddy, and C. L. Pickett.** 1986. Purification and characterization of type II heat-labile

enterotoxin of *Escherichia coli*. Infect. Immun. **53**:464–473.
43. **Howard, J. G.** 1955. Observations on the intoxication produced in mice and rabbits by the neurotoxin of *Shigella shigae*. Br. J. Exp. Pathol. **36**:439–446.
44. **Huang, A., S. de Grandis, J. Friesen, M. Karmali, M. Petric, R. Congi, and J. L. Brunton.** 1986. Cloning and expression of the genes specifying Shiga-like toxin production an *Escherichia coli* H19. J. Bacteriol. **166**:375–379.
45. **Jacewicz, M., H. Clausen, E. Nudelman, A. Donohue-Rolfe, and G. T. Keusch.** 1986. Pathogenesis of *Shigella* diarrhea. XI. Isolation of a *Shigella* toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. J. Exp. Med. **163**:1391–1404.
46. **Jacewicz, M., and G. T. Keusch.** 1983. Pathogenesis of *Shigella* diarrhea. VIII. Evidence for a translocation step in the cytotoxic action of Shiga toxin. J. Infect. Dis. **148**:844–854.
47. **Kaper, J. B., H. Lockman, M. M. Baldini, and M. M. Levine.** 1984. Recombinant nontoxinogenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates. Nature (London) **308**:655–658.
47a. **Jackson, M. P., J. W. Newland, R. K. Holmes, and A. D. O'Brien.** 1987. Nucleotide sequence analysis of the structural gene for Shiga-like toxin I by bacteriophage 933J from *Escherichia coli*. Microb. Pathogenesis **2**:147–153.
48. **Karch, H., P. Goroncy-Bermes, W. Opferkuch, H.-P. Kroll, and A. O'Brien.** 1985. Subinhibitory concentrations of antibiotic modulate amount of Shiga-like toxin produced by *Escherichia coli*, p. 239–245. *In* D. Adam, H. Hahn, and W. Opferkuch (ed.), Host-parasite relationship II. Springer-Verlag KG, Berlin.
48a. **Karch. H., J. Heesemann, and R. Laufs.** 1987. Phage-associated cytotoxin production by and enteroadhesiveness of enteropathogenic *Escherichia coli* isolated from infants with diarrhea in West Germany. J. Infect. Dis. **155**:707–715.
49. **Karch, H., J. Heesemann, R. Laufs, A. D. O'Brien, C. O. Tacket, and M. M. Levine.** 1987. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. Infect. Immun. **55**:455–461.
50. **Karch, H., N. A. Strockbine, and A. D. O'Brien.** 1986. Growth of *Escherichia coli* in the presence of trimethoprim-sulfamethoxazole facilitates detection of Shiga-like toxin producing strains by colony blot assay. FEMS Microbiol. Lett. **35**:141–145.
51. **Karmali, M. A., M. Petric, C. Lim, R. Cheung, and G. S. Arbus.** 1985. Sensitive method for detecting low numbers of Vero toxin-producing *Escherichia coli* in mixed cultures by use of colony sweeps and polymyxin extraction of Vero toxin. J. Clin. Microbiol. **22**:614–619.
52. **Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior.** 1985. The association between idiopathic hemolytic uremic syndrome and infection by Verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151**:775–782.
53. **Kashiwazaki, M., T. Ogawa, Y. Isayama, Y. Akaike, K. Tamura, and R. Sakazaki.** 1980. Detection of Vero cytotoxic strains of *Escherichia coli* isolated from diseased animals. Natl. Inst. Anim. Health Q. (Jpn.). **20**:116–117.
54. **Kashiwazaki, M., T. Ogawa, K. Nakamura, Y. Isayama, K. Tamura, and R. Sakazaki.** 1980. Vero cytotoxin produced by *Escherichia coli* strains of animal origin. Natl. Inst. Anim. Health A (Jpn.). **21**:68–72.
55. **Keenan, K. P., D. D. Sharpnack, H. Collins, S. B. Formal, and A. D. O'Brien.** 1986. Morphologic evaluation of the effects of Shiga toxin and *E. coli* Shiga-like toxin on the rabbit intestine. Am. J. Pathol. **125**:69–80.
56. **Keusch, G. T.** 1981. Receptor-mediated endocytosis of *Shigella* cytotoxin, p. 95–112. *In* J. L. Middlebrook and L. D. Kohn (ed.), Receptor-mediated binding and internalization of toxins and hormones. Academic Press, Inc., New York.
57. **Keusch, G. T., A. Donohue-Rolfe, and M. Jacewicz.** 1982. *Shigella* toxin(s): description and role in diarrhea and dysentery. Pharmacol. Ther. **15**:403–438.
58. **Keusch, G. T., A. Donohue-Rolfe, and M. Jacewicz.** 1985. *Shigella* toxin and the pathogensis of shigellosis, p. 193–214. *In* D. Evered and J. Whelan (ed.), Microbial toxins and diarrhoeal disease. Ciba Foundation Symposium 112. Pitman Publishers, London.
59. **Keusch, G. T., G. F. Grady, L. J. Mata, and J. McIver.** 1972. The pathogenesis of *Shigella* diarrhea. 1 Enterotoxin production by *Shigella dysenteriae* 1. J. Clin. Invest. **51**:1212–1218.
60. **Keusch, G. T., and M. Jacewicz.** 1973. Serum enterotoxin-neutralizing antibody in human shigellosis. Nature New Biol. **241**:31–32.
61. **Keusch, G. T., and M. Jacewicz.** 1977. The pathogenesis of *Shigella* diarrhea. VI. Toxin and antitoxin in *Shigella flexneri* and *Shigella sonnei* infections in humans. J. Infect. Dis. **135**: 552–556.
62. **Keusch, G. T., and M. Jacewicz.** 1977. Pathogenesis of *Shigella* diarrhea. VII. Evidence for a cell membrane toxin receptor involving β-4 linked N-acetyl-D-glucosamine oligomers. J. Exp. Med. **146**:535–546.
63. **Keusch, G. T., M. Jacewicz, and A. Donohue-Rolfe.** 1986. Pathogenesis of *Shigella* diarrhea: evidence for an N-linked glycoprotein *Shigella* toxin receptor and receptor modulation by β-galactosidase. J. Infect. Dis. **153**:238–248.
64. **Keusch, G. T., M. Jacewicz, M. M. Levine, R. B. Hornick, and S. Kochwa.** 1976. Pathogenesis of shigella diarrhea. Serum anticytotoxin antibody response produced by toxigenic and nontoxigenic *Shigella dysenteriae* 1. J. Clin. Invest. **57**:194–202.
65. **Kinsey, M. D., S. B. Formal, G. J. Dammin, and R. A. Giannella.** 1976. Fluid and electrolyte transport in Rhesus monkeys challenged intracecally with *Shigella flexneri* 2a. Infect. Immun. **14**:368–371.
66. **Konowalchuk, J., N. Dickie, S. Stavric, and J. I. Speirs.** 1978. Comparative studies of five heat-labile toxic products of *Escherichia coli*. Infect. Immun. **22**:644–648.
67. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18**:775–779.
68. **Koster, F., J. Levin, L. Walker, K. S. K. Tung, R. H. Gilman, M. M. Rahaman, M. A. Majid, S. Islam, and R. C. Williams, Jr.** 1977. Hemolytic-uremic syndrome after shigellosis. Relation to endotoxemia and circulating immune complexes. N. Engl. J. Med. **298**:927–933.
69. **Lasfarques, E., and A. Delaunay.** 1946. Cultures de tissus appliquées a la solution de problemes immunologiques. I. Etude du pouvoir nécrosant des toxines microbiennes *in vitro*. Ann. Inst. Pasteur (Paris) **72**:39–43.
70. **Levine, M. M., H. L. DuPont, S. B. Formal, R. B. Hornick, A. Takeuchi, E. J. Gangarosa, M. J. Snyder, and J. P. Libonati.** 1973. Pathogenesis of *Shigella dysenteriae* 1 (Shiga) dysentery. J. Infect. Dis. **127**:261–270.
71. **Levine, M. M., and R. Edelman.** 1984. Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: epidemiology and pathogenesis. Epidemiol. Rev. **6**:31–51.
72. **Maas, W. K.** 1981. Genetics of bacterial virulence, p. 341–360. *In* S. W. Glover and D. A. Hopwood (ed.), Genetics as a tool in microbiology. Thirty-first Symposium of the Society for General Microbiology. Cambridge University Press, Cambridge.
73. **Marques, L. R. M., M. A. Moore, J. G. Wells, I. K. Wachsmuth, and A. D. O'Brien.** 1986. Production of Shiga-like toxin by *Escherichia coli*. J. Infect. Dis. **154**:338–341.
74. **McIver, J., G. F. Grady, and S. B. Formal.** 1977. Immunization with *Shigella dysenteriae* type 1: evaluation of antitoxic immunity in prevention of experimental disease in Rhesus monkeys (Macca mulatta). J. Infect. Dis. **156**:416–421.
75. **Middlebrook, J. L., and R. B. Dorland.** 1984. Bacterial toxins: cellular mechanisms of action. Microbiol. Rev. **48**:199–221.
76. **Mohammad, A., J. S. M. Peiris, E. A. Wijewanta, S. Mahalingam, and G. Gunasekara.** 1985. Role of verocytotoxigenic *Escherichia coli* in cattle and buffalo calf diarrhoea. FEMS Microbiol. Lett. **26**:281–283.
77. **Moon, H. W., S. C. Whipp, R. A. Arzenio, M. M. Levine, and**

R. A. Gianella. 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41**:1340–1351.
78. **Newland, J. W., N. A. Strockbine, S. F. Miller, A. D. O'Brien, and R. K. Holmes.** 1985. Cloning of Shiga-like toxin structural genes from a toxin converting phage of *Escherichia coli*. Science **230**:179–181.
79. **O'Brien, A. D., and G. D. LaVeck.** 1982. Immunochemical and cytotoxic activities of *Shigella dysenteriae* 1 (Shiga) and Shiga-like toxins. Infect. Immun. **35**:1151–1154.
80. **O'Brien, A. D., and G. D. LaVeck.** 1983. Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. Infect. Immun. **40**:675–683.
81. **O'Brien, A. D., G. D. LaVeck, D. E. Griffin, and M. R. Thompson.** 1980. Characterization of *Shigella dysenteriae* 1 (Shiga) toxin purified by anti-Shiga toxin affinity chromatography. Infect. Immun. **30**:170–179.
82. **O'Brien, A. D., G. D. LaVeck, M. R. Thompson, and S. B. Formal.** 1982. Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. J. Infect. Dis. **1446**:763–769.
83. **O'Brien, A. D., and J. P. Nataro.** 1985. New concepts in the pathogenesis of enteropathogenic *Escherichia coli* diarrhea, p. 78–82. *In* L. Leive (ed.), Microbiology—1985. American Society for Microbiology, Washington, D.C.
84. **O'Brien, A. D., J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith, and S. B. Formal.** 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. Science. **226**:694–696.
85. **O'Brien, A. D., M. R. Thompson, P. Gemski, B. P. Doctor, and S. B. Formal.** 1977. Biological properties of *Shigella flexneri* 2A toxin and its serological relationship to *Shigella dysenteriae* 1 toxin. Infect. Immun. **15**:796–798.
86. **Obrig, T. G., T. P. Moran, and R. J. Colinas.** 1985. Ribonuclease activity associated with the 60S ribosome-inactivating proteins ricin A, phytolaccin and Shiga toxin. Biochem. Biophys. Res. Commun. **130**:879–884.
87. **Olsnes, S., and K. Eiklid.** 1980. Isolation and characterization of *Shigella shigae* cytotoxin. J. Biol. Chem. **255**:284–289.
88. **Olsnes, S., R. Reisbig, and K. Eiklid.** 1981. Subunit structure of *Shigella* cytotoxin. J. Biol. Chem. **256**:8732–8738.
89. **Olsnes, S., and K. Sandvig.** 1985. Entry of polypeptide toxins into animal cells, p. 195–234. *In* I. Pastan and M. C. Willington (ed.), Endocytosis. Plenum Publishing Corp., New York.
90. **Padhye, V. V., F. B. Kittell, and M. P. Doyle.** 1986. Purification and physiochemical properties of a unique Vero cell cytotoxin from *Escherichia coli* O157:H7. Biochem. Biophys. Res. Commun. **139**:424–430.
91. **Pai, C. H., R. Gordon, H. V. Sims, and L. E. Bryan.** 1984. Sporadic cases of hemorrhagic colitis associated with *Escherichia coli* O157:H7. Ann. Intern. Med. **101**:738–742.
92. **Pai, C. H., J. K. Kelly, and G. L. Meyers.** 1986. Experimental infection of infant rabbits with Vero toxin-producing *Escherichia coli*. Infect. Immun. **51**:16–23.
93. **Pickett, C. L., E. M. Twiddy, B. W. Belisle, and R. K. Holmes.** 1986. Cloning of genes that encode a new heat-labile enterotoxin of *Escherichia coli*. J. Bacteriol. **165**:348–352.
94. **Potter, M. E., A. F. Kaufmann, B. M. Thomason, P. A. Blake, and J. J. Farmer III.** 1985. Diarrhea due to *Escherichia coli* O157:H7 in the infant rabbit. J. Infect. Dis. **152**:1341–1343.
95. **Prado, D., T. G. Cleary, L. K. Pickering, C. D. Ericsson, A. V. Bartlett III, H. L. DuPont, and P. C. Johnson.** 1986. The relationship between cytotoxin production and clinical features in shigellosis. J. Infect. Dis. **154**:149–155.
96. **Raghupathy, P., A. Date, J. C. M. Shastry, A. Sudarsanam, and M. Jadhav.** 1978. Haemolytic-uraemic syndrome complicating shigella dysentery in south Indian children. Br. Med. J. **1**: 1518–1521.
97. **Reisbig, R., S. Olsnes, and K. Eiklid.** 1981. The cytotoxic activity of *Shigella* toxin. Evidence for catalytic inactivation of the 60 S ribosomal subunit. J. Biol. Chem. **256**:8739–8744.
98. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308**:681–685.
99. **Rothbaum, R., J. McAdams, R. Gianella, and J. C. Partin.** 1982. A clinicopathologic study of enterocyte-adherent *Escherichia coli*: a cause of protracted diarrhea in infants. Gastroenterology **83**:441–454.
100. **Rout, W. R., S. B. Formal, R. A. Giannella, and G. J. Dammin.** 1975. Pathophysiology of Shigella diarrhea in the rhesus monkey: intestinal transport, morphological, and bacteriological studies. Gastroenterology **68**:270–278.
101. **Sansonetti, P. J., H. d'Hauteville, C. Ecobichon, and C. Pourcel.** 1983. Molecular comparison of virulence plasmids in *Shigella* and enteroinvasive *Escherichia coli*. Ann. Microbiol. (Inst. Pasteur) **134A**:295–318.
102. **Sansonetti, P. J., T. L. Hale, G. J. Dammin, C. Kapfer, H. H. Collins, Jr., and S. B. Formal.** 1983. Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. Infect. Immun. **39**:1392–1402.
103. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1981. *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. Infect. Immun. **34**:75–83.
104. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
105. **Scotland, S. M., N. P. Day, and B. Rowe.** 1980. Production of a cytotoxin affecting Vero cells by strains of *Escherichia coli* belonging to traditional enteropathogenic serogroups. FEMS Microbiol. Lett. **7**:15–17.
106. **Seidah, N. G., A. Donohue-Rolfe, C. Lazure, F. Auclair, G. T. Keusch, and M. Chrétein.** 1986. Complete amino acid sequence of *Shigella* toxin B-chain. A novel polypeptide containing 69 amino acids and one disulfide bridge. J. Biol. Chem. **261**: 13928–13931.
107. **Sherwood, D., D. R. Snodgrass, and A. D. O'Brien.** 1985. Shiga-like toxin production from *Escherichia coli* associated with calf diarrhoea. Vet. Rec. **116**:217–218.
108. **Smith, W. H., P. Green, and Z. Parsell.** 1983. Vero cell toxins in *Escherichia coli* and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals, chickens and pigs. J. Gen. Microbiol. **129**:3121–3137.
109. **Strockbine, N. A., L. R. M. Marques, R. K. Holmes, and A. D. O'Brien.** 1985. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. Infect. Immun. **50**:695–700.
110. **Strockbine, N. A., L. R. M. Marques, J. W. Newland, H. W. Smith, R. K. Holmes, and A. D. O'Brien.** 1986. Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biological activities. Infect. Immun. **53**:135–140.
111. **Thompson, M. R., M. S. Steinberg, P. Gemski, S. B. Formal, and B. P. Doctor.** 1976. Inhibition of *in vitro* protein synthesis by *Shigella dysenteriae* 1 toxin. Biochem. Biophys. Res. Commun. **71**:783–788.
112. **Timmis, K. N., C. L. Clayton, and T. Sekizaki.** 1985. Localization of Shiga toxin gene in the region of *Shigella dysenteriae* 1 chromosome specifying virulence functions. FEMS Microbiol. Lett. **30**:301–305.
113. **van Heyningen, W. E.** 1971. The exotoxin of *Shigella dysenteriae*, p. 255–269. *In* S. Kadis, T. C. Montie, and S. J. Ajl (ed.), Microbial toxins, vol IIA. Academic Press, Inc., New York.
114. **van Heyningen, W. E., and G. P. Gladstone.** 1953. The neurotoxin of *Shigella shigae*. 1. Production, purification and properties of the toxin. Br. J. Exp. Pathol. **34**:202–216.
115. **Vicari, G., A. L. Olitzki, and Z. Olitzki.** 1960. The action of the thermolabile toxin of *Shigella dysenteriae* on cells cultivated in vitro. Br. J. Exp. Pathol. **41**:179–189.
116. **Weil, A. J.** 1943. Progress in the study of bacillary dysentery. J. Immunol. **46**:13–46.
117. **Wells, J. G., B. R. Davis, I. K. Wachsmuth, L. W. Riley, R. S. Remis, R. Sokolow, and G. K. Morris.** 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with

a rare *Escherichia coli* serotype. J. Clin. Microbiol. **18**:512–520.

118. **Wiley, R. G., A. Donohue-Rolfe, and G. T. Keusch.** 1985. Axonally transported *Shigella* cytotoxin is neuronotoxic. J. Neuropathol. Exp. Neurol. **44**:496–506.
119. **Willshaw, G. A., H. R. Smith, S. M. Scotland, and B. Rowe.** 1985. Cloning of genes determining the production of Vero cytotoxin by *Escherichia coli*. J. Gen. Microbiol. **131**:3047–3053.
120. **Yutsudo, T., T. Honda, T. Miwatani, and Y. Takeda.** 1986. Characterization of purified Shiga toxin from *Shigella dysenteriae* 1. Microbiol. Immunol. **30**:1115–1127.