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Pathogenesis and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections

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INTRODUCTION

The morbidity and mortality associated with several recent large outbreaks of gastrointestinal disease caused by Shiga toxin-producing *Escherichia coli* (STEC) has highlighted the threat these organisms pose to public health (10, 102, 114, 260). Such outbreaks have the potential to overwhelm acute-care resources, even in countries with advanced health care systems. Much attention has been focused upon this group of pathogens since their discovery, and there have been several excellent reviews covering either the field as a whole (156, 317) or specific aspects such as the toxin (232, 340), its structure and function (89, 139), its interaction with host cell receptors (193), and clinical aspects of disease (334). Our capacity to control STEC disease in humans and to limit the scale of outbreaks is dependent upon prompt diagnosis and identification of the source of infection. In recent years, there have been significant advances in our understanding of the pathogenesis of STEC infection, and these are contributing to the development of improved diagnostic methods, as well as to the development of therapeutic and preventative strategies. It is these aspects of STEC infection that will be the principal focus of this review.

It is now 20 years since Konowalchuk et al. (175) reported the feature which distinguishes STEC from other classes of pathogenic *E. coli*, namely, the production of a toxin with a profound and irreversible cytopathic effect on Vero (African green monkey kidney) cells. Of the 10 toxic strains in this initial study, 7 had been isolated from infants with diarrhea, suggesting the possibility of a role for this new Verotoxin (VT) in the pathogenesis of gastrointestinal disease. In the early 1980s, verotoxigenic *E. coli* strains were linked to cases of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (160, 282). Moreover, verotoxigenic *E. coli* strains associated with two outbreaks of HC belonged to a previously rare serotype (O157:H7) (61, 282), as did two of eight verotoxigenic isolates from HUS patients reported by Karmali et al. (160). While it is now recognized that STEC strains belonging to a very diverse range of serotypes are capable of causing serious human disease, O157:H7 is a dominant STEC serotype in many parts of the world and historically has been the type most commonly associated with large outbreaks (127, 156, 317).

O'Brien et al. purified and characterized the cytotoxin produced by one of Konowalchuk's isolates (strain H30; serotype O26:H11) and found that it had strikingly similar structure and biological activity to Shiga toxin (Stx) produced by *Shigella dysenteriae* type 1 (233, 234). Moreover, it could be neutralized by anti-Stx (233, 234), resulting in the new nomenclature of Shiga-like toxin (SLT). SLT and VT nomenclature systems have been used interchangeably in the literature since this time. The situation has been complicated further by the subsequent recognition that there are two major types of SLT/VT (SLT-I and SLT-II or VT1 and VT2), with additional sequence variants within these types, as discussed below. In an attempt to avoid further confusion, Calderwood et al. (54) have proposed a rationalization of nomenclature (reproduced in Table 1), which recognizes that all of these toxins have a high degree of structural and functional homology and so belong to a Shiga toxin family. The rationalized nomenclature system will be used throughout this review; we also use STX as a generic abbreviation for the Shiga toxin family as a whole and *STX* to denote all *stx*-related genes.

TABLE 1. Nomenclature of members of the Shiga toxin family, as proposed by Calderwood et al. (54)[a]

Previous nomenclature	Proposed new nomenclature	
	Gene	Protein
Shiga toxin (Stx)	*stx*	Stx
Shiga-like toxin I (SLT-I) or verotoxin 1 (VT1)	stx_1	Stx1
SLT-II or VT2	stx_2	Stx2
SLT-IIc or VT2c	stx_{2c}	Stx2c
SLT-IIe or VT2e	stx_{2e}	Stx2e

[a] Reprinted from reference 54 with permission of the publisher.

Types, Structure, and Mode of Action of STX

Initial recognition of the presence of multiple STX types arose from the observation that anti-Stx could not neutralize the cytotoxicity of some STEC strains (308, 327). Conversely, crude antisera raised against nonneutralizable strains did not neutralize Stx. These antibody neutralization studies demonstrated that some STEC strains produced only the anti-Stx neutralizable toxin (now referred to as Stx1), others produced only the nonneutralizable toxin (now designated Stx2), while yet others produced both (308, 327). Additional studies also demonstrated that STEC isolates from piglets with edema disease produce a variant of Stx2 (designated Stx2e) (207). Although it was neutralized by polyclonal anti-Stx2, this variant could be distinguished from Stx2 on the basis of its lack of cytotoxicity for HeLa cells.

Members of the Stx family are compound toxins (the holotoxin is approximately 70 kDa), comprising a single catalytic 32-kDa A subunit and a multimeric B subunit (7.7-kDa monomers) that is involved in the binding of the toxin to specific glycolipid receptors on the surface of target cells (232). Biochemical cross-linking analysis suggested that the holotoxins of both Stx and Stx1 include five B-subunit monomers (85), and this was confirmed by X-ray crystallographic analysis of purified B subunits (322). Additional crystallographic analysis of the Stx holotoxin demonstrated that the B subunits form a pentameric ring, which encircles a helix at the C terminus of the single A subunit (97). This was consistent with the results of mutational analysis of the C-terminal region of the 293-residue Stx A subunit, which demonstrated that a sequence of nine nonpolar amino acids from residues 279 to 287 was essential for holotoxin assembly (121). These residues form an alpha-helix that penetrates the pore in the centre of the B pentamer; flanking charged residues appear to stabilize this interaction (145).

The eukaryotic cell surface receptor for members of the STX family is globotriaosylceramide (Gb_3; Galα[1→4]Galβ[1→4]Glc-ceramide) (194, 360). The exception to this is the variant toxin Stx2e, which recognizes globotetraosylceramide (Gb_4; GalNAcβ[1→3]Galα[1→4]Galβ[1→4]Glc-ceramide) preferentially over Gb_3 (79, 289). The interaction between the Stx1 B subunit and its receptor has been studied extensively by molecular modelling, site-directed mutagenesis, and crystallographic analysis. Phe30 was shown to play a critical role. Two possible Gb_3 binding sites have been identified on either side of this residue; one site is near the cleft between adjacent B monomers, while the other is a shallow indentation on the B subunit surface opposed to the plasma membrane (71, 193, 230, 231). A third putative Gb_3 binding site has also recently been identified in the vicinity of Trp34 (23).

Once bound to a target cell membrane, toxin molecules are thought to be internalized by a process of receptor-mediated endocytosis; this has been recently reviewed by Sandvig and van Deurs (294). Briefly, internalization involves the formation of a clathrin-coated pit within the cell membrane, which subsequently pinches off to form a sealed coated vesicle with toxin

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bound to the intenral surface. Subsequent intracellular trafficking has a major impact on the biological effects of STX. In some cells, the toxin-bound vesicles undergo fusion with cellular lysosomes, resulting in toxin degradation. However, in cells which are particularly sensitive to STX, the endosomal vesicles containing toxin-receptor complexes undergo retrograde transport via the Golgi apparatus to the endoplasmic reticulum before being translocated to the cytosol (291–293). During this process, the A subunit is nicked by a membrane-bound protease furin (107), generating a catalytically active 27-kDa N-terminal A1 fragment and a 4-kDa C-terminal A2 fragment, which remain linked by a disulfide bond. This disulfide bond is subsequently reduced, thereby releasing the active A1 component (294). Saleh et al. (288) have proposed that a signal sequence-like hydrophobic domain at the C terminus of the A1 fragment may function in the translocation process by directing insertion into the endoplasmic reticulum membrane. The released A1 fragment has RNA *N*-glycosidase activity and cleaves a specific *N*-glycosidic bond in the 28S rRNA, a property shared by the plant toxin ricin (93, 298, 315). This cleavage presents elongation factor 1-dependent binding of the aminoacyl-tRNA to the 60S ribosomal subunit (93, 131, 240), thereby inhibiting the peptide chain elongation step of protein synthesis and ultimately causing cell death.

Structure and Organization of *STX* Genes

The nucleotide sequences of the genes encoding Stx from *S. dysenteriae*, as well as Stx1 and Stx2 from *E. coli*, were determined in the late 1980s (55, 78, 142, 143, 177, 325). The operons had a common structure consisting of a single transcriptional unit, encoding first the A subunit followed by the B subunit. The *stx* B-subunit gene has a stronger ribosome binding site than that of the A-subunit gene, resulting in increased translation of B subunits, thereby satisfying the 1:5 A/B-subunit stoichiometry of the holotoxin (119). The predicted amino acid sequences were 315, 315, and 318 amino acids long for the A subunits of Stx, Stx1, and Stx2, respectively, and 89 amino acids for the B subunits of all three toxins. Both A and B subunits had hydrophobic N-terminal signal sequences characteristic of secreted proteins, and the predicted M_r values for the processed A and B subunits were in accordance with previous estimates based on analysis of purified toxins. Interestingly, a 21-bp region of dyad symmetry spanning the −10 region was found upstream of *stx* and stx_1, and this motif is thought to be associated with iron regulation of toxin expression (55, 78, 143, 177). Comparison of the deduced amino acid sequences indicated that Stx and Stx1 were virtually identical (there was a single amino acid difference in the A subunit) whereas Stx2 had only 56% identity to the other toxins for both the A and B subunits (142). Interestingly, a significant degree of amino acid homology was also observed between the A subunits of STX and the plant toxin ricin, which has an identical mode of action (55, 177). The most highly conserved regions were subsequently shown to be part of the active (catalytic) site (128, 379).

In 1988, the sequence of an operon encoding the variant toxin Stx2e, associated with piglet edema disease, was reported (118, 368). The deduced amino acid sequence of the A subunit of Stx2e was 1 amino acid longer than that of Stx2 and exhibited 94% homology to it. The B subunit of Stx2e was 2 amino acids shorter than that of Stx2, and there was only 87% homology. Studies involving the construction of chimeric Stx2/Stx2e operons demonstrated that the variations in the B subunit of Stx2e with respect to Stx2 were responsible for the reduced cytotoxicity of Stx2e for HeLa cells, which lack its preferred receptor, Gb_4 (367). Subsequent site-directed mutagenesis and molecular modelling studies have demonstrated that two amino acids in the mature Stx2e B subunit (Gln64 and Lys66) are critical for the distinct glycolipid binding specificity of the toxin (231, 350).

A number of other variant forms of Stx2 and also Stx1 have since been reported for human STEC isolates, illustrating the diversity of the Stx family (106, 138, 189, 214, 242, 250, 256, 258, 259, 305). One particular subgroup of Stx2 variants contains specific B-subunit amino acid differences with respect to classical Stx2 (Asp16→Asn and Asp24→Ala), which correlate with a lower binding affinity for the receptor Gb_3 and reduced in vitro cytotoxicity for Vero cells (191). In view of this functional distinction, these toxins are now considered a separate subgroup and have been designated Stx2c.

Studies in the early 1980s established that Stx1 and Stx2 were encoded on a variety of bacteriophages (236, 309, 316). However, toxin-converting bacteriophages have not been isolated from *S. dysenteriae* type 1 or from STEC strains associated with piglet edema disease (156). Several of the additional variant *STX* genes from other human STEC strains also do not appear to be phage encoded, although it is possible that they are carried on defective phage particles (256, 258, 259). One study has identified an IS element adjacent to an stx_1 operon in an O111:H^- STEC strain (253); there was no duplication of target sequence at the insertion site, which raised the possibility that the segment of DNA containing the toxin gene was part of a transposon. However, the direct involvement of such mobile elements in transmission of *STX* genes has yet to be demonstrated. Involvement of bacteriophages and transposons may help to explain why many STEC strains readily lose their *STX* genes after subcultivation in vitro (154).

Clinicopathological Features of STEC Disease

It is now recognized that there is a very broad spectrum of human disease associated with STX-producing organisms. STEC-related disease may involve either sporadic cases or large outbreaks involving a common contaminated food source. Some individuals infected with STEC may be completely asymptomatic, in spite of the presence of large numbers of organisms as well as free toxin in the feces (50, 91). Very little is known of the true incidence of asymptomatic carriage. One study of Canadian dairy farm families (a group with high environmental exposure) detected carriage of STEC in about 6% of individuals (375). However, there has been little or no large-scale surveillance of healthy urban populations. Reported examples of asymptomatic carriage have usually been detected as a consequence of targeted testing of family contacts of persons with clinical STEC disease (284, 357). Many STEC-infected patients initially suffer a watery diarrhea, but in some this progresses within 1 or 2 days to bloody diarrhea and HC (235, 281, 282). Severe abdominal pain is also frequently reported. In a proportion of patients, STEC infection progresses to HUS, a life-threatening sequela characterized by a triad of acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia (158, 160). Some individuals with HUS experience neurological symptoms including lethargy, severe headache, convulsions, and encephalopathy (340). Although HUS occurs in all age groups, its incidence is higher in infants, young children, and the elderly. Indeed, it is a major cause of acute renal failure in the pediatric population (156, 317). The age distribution of HUS may be a consequence of the immunological naivety of young children and declining immune system function in the elderly (156), although age-related differences in receptor expression may contribute

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(193). Improved clinical management and pediatric renal dialysis techniques have reduced the mortality associated with HUS from about 50% to less than 10% over the last two to three decades (156). Nevertheless, a significant number of survivors (approximately 30%) suffer a range of permanent disabilities including chronic renal insufficiency, hypertension, and neurological deficits (156, 334). STEC infection can also result in a variant form of HUS, sometimes referred to as thrombotic thrombocytopenic purpura (TTP). This "diarrhea-associated TTP" is more common in adults than in children. The pathological features are essentially the same, but it differs from the typical form of HUS in that patients are more often febrile and have marked neurological involvement (156, 224). However, there is another form of TTP without a diarrheal prodrome, which is not associated with STEC infection.

Whether STEC-associated diarrheal disease progresses to life-threatening complications depends upon an interplay between bacterial and host factors. In an outbreak setting, the age of infected persons will have a significant influence on the proportion of infected persons who develop HUS, as well as the mortality rate. Characteristics of individual STEC strains will also have a major impact, and these are discussed below. Notwithstanding these considerations, studies of large outbreaks caused by O157:H7 STEC indicate that roughly 5 to 10% of individuals with diarrhea progress to HUS (114, 115, 156).

Epidemiology of STEC Disease

Species and serotype distribution of STX producers. It has been recognized for a number of years that STEC strains causing human disease belong to a very broad range of O:H serotypes. Karmali (156) listed 32 O serogroups (approximately 60 distinct O:H types), and the list has grown considerably since then (109, 179, 267). Although not represented in the initial group of STEC isolates described by Konowalchuk (175), serotype O157:H7 was the first STEC type to be linked to outbreaks of HC and HUS (156, 282). In many parts of the world, STEC strains belonging to this serotype (as well as O157:H$^-$) appear to be the most common causes of human disease. However, the relative ease of isolation of this serotype on the basis of its inability to ferment sorbitol may be contributing to an overestimation of its prevalence with respect to other STEC serotypes. Other common STEC serogroups include O26, O91, O103, and O111, and in several studies, non-O157 STEC serotypes such as these have been the predominant cause of human disease (39, 109, 267). There have been several reports of multiple STEC serotypes being isolated from a single patient, and in such circumstances, the contribution of each type to the pathogenesis of disease is difficult to ascertain (39, 260, 344). When one of the isolated types is O157, there is a (perhaps mistaken) tendency to ignore the potential etiological significance of the other(s). Other members of the family *Enterobacteriaceae* are known to produce STX and to cause serious gastrointestinal disease and HUS in humans. The most notable of these is *S. dysenteriae* type 1, the causative agent of bacillary dysentery, which is frequently complicated by HUS (232). It is the principal cause of HUS in parts of Africa and Asia (16, 37). Disease due to *S. dysenteriae* type 1 may be particularly severe, because the organism is capable of invading the colonic mucosa, and this might result in more efficient delivery of Stx to the bloodstream, as well as significant endotoxemia. Stx2-producing *Citrobacter freundii* also causes diarrhea and HUS in humans, including one outbreak in a German child care centre (304, 348). Haque et al. (124) have described the production of an Stx1-related cytotoxin by strains of *Aeromonas hydrophila* and *A. caviae*, as judged by stx_1-specific PCR and neutralization of Vero cytotoxicity with Stx1 antiserum. *Enterobacter cloacae* has also been associated with transient expression of an stx_2-related gene, although its role in disease is unproven (254, 263).

Sources of STEC. Cattle have long been regarded as the principal reservoir of STEC strains, including those belonging to serotype O157:H7. However, epidemiological surveys have revealed that STEC strains are also prevalent in the gastrointestinal tracts of other domestic animals, including sheep, pigs, goats, dogs, and cats (31, 58, 156, 180, 377). Estimation of the incidence of carriage of STEC is complicated by the fact that fecal shedding may be transient and is almost certainly influenced by a range of factors including diet, stress, population density, geographical region, and season (72, 180). Serological studies have suggested that the vast majority of cattle have been exposed to STEC at some point during their lives (72, 269). STEC isolates from animal sources include the important human disease-causing serotypes, as well as a number of O:H types that have yet to be associated with human infections (31, 72, 156).

While many domestic animals carrying STEC are asymptomatic, certain STEC strains are capable of causing diarrhea in cattle, particularly calves (117, 317). STEC strains have also been detected in cats and dogs with diarrhea (1, 123). Natural and experimental infection of calves with a O111 STEC strain results in colitis with attachment and effacement of the colonic mucosa (306). Other studies involving experimental infection with O157:H7 STEC showed that both adult cattle and calves could be transiently colonized but only neonatal calves developed significant intestinal lesions (76, 77).

Piglet edema disease, on the other hand, is a serious, frequently fatal STEC-related illness. It is characterized by neurological symptoms including ataxia, convulsions, and paralysis; edema is typically present in the eyelids, brain, stomach, intestine, and mesentery of the colon. This disease is associated with particular STEC serotypes (most commonly O138:K81, O139:K82, and O141:K85) (133, 223, 228); these types are not associated with human disease and produce Stx2e. The B subunit of this toxin has a different glycolipid receptor specificity from that of other members of the STX family; it alters the tissue tropism of the toxin, accounting for the distinctive clinical presentation of edema disease, as discussed below.

STEC can potentially enter the human food chain from a number of animal sources, most commonly by contamination of meat with feces or intestinal contents after slaughter. In a Canadian survey of local and imported ground beef, 4 to 16% of samples (depending on the source) were culture positive for STEC. However, 15 to 40% of cultures were cytotoxic for Vero cells, and this may be a more accurate reflection of the proportion which were actually contaminated (72). A Belgian survey, which included more exotic meats, found STEC in samples of beef, lamb, deer, wild boar, ostrich, partridge, antelope, and reindeer (268). One of the more common sources of human STEC infection is hamburger patties made from ground beef, and a number of outbreaks of O157:H7 infection have been linked to this source (156). Ground beef may pose a particular risk for two reasons. First, the prevalence of highly pathogenic STEC strains such as O157:H7 may be higher in cattle than in other animal species. Second, STEC contaminating the surface of meat becomes evenly distributed during the mincing process, and unless hamburger patties are thoroughly cooked, STEC organisms in the center may not be exposed to lethal temperatures. There is a potential for massive outbreaks when hamburgers are sold by fast-food restaurant chains using a common source of ground-beef patties and standardized (sub-

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optimal) cooking procedures. Such an outbreak occurred in the western United States in late 1993 (114); over 700 people became ill, and there were over 50 cases of HUS with four fatalities. Other proven food sources of STEC infection include raw or inadequately pasteurized dairy products, fermented or dried meat products such as salami and jerky, and fruit and vegetable products which presumably had come into contact with domestic animal manure at some stage during cultivation or handling (9, 62, 63, 108, 166, 213, 221, 260, 348). The largest outbreak of STEC disease yet reported occurred in Sakai, Japan, in 1996 and involved over 6,000 cases of HC and over 100 cases of HUS (102); the most likely source appears to have been radish sprouts in mass-prepared school lunches.

Person-to-person transmission of STEC is well documented during outbreaks and may also account for a significant proportion of sporadic cases (115, 277). In a study of patients with O157 infection, the median duration of fecal shedding of STEC was 2 to 3 weeks, but 13% of patients shed O157 for more than 1 month (the maximum was 124 days) and were clinically asymptomatic during the latter stages (155). Thus, there is ample scope for secondary transmission, which may involve direct hand-to-hand contact (e.g., among children in day care centres) or could be indirect, e.g., via contaminated water used for swimming (8, 165). The sources of sporadic cases of STEC infection are often difficult to pinpoint, because of the lack of epidemiological correlation, but there is no reason to propose that the source of these infections differs from those listed above.

PATHOGENESIS

Production of a potent STX is essential for many of the pathological features as well as the life-threatening sequelae of STEC infection. However, pathogenesis is a multistep process, involving a complex interaction between a range of bacterial and host factors. Orally ingested STEC (often in very low initial doses) must initially survive the harsh environment of the stomach and then compete with other gut microorganisms to establish intestinal colonization. STEC organisms remain in the gut, and so STX produced in the lumen must be first absorbed by the intestinal epithelium and then translocated to the bloodstream. This permits delivery to the specific toxin receptors on target cell surfaces inducing both local and systemic effects. An overview of the steps involved in this process is provided below.

Colonization of the Gut

STEC strains are a diverse group in terms of their capacity to cause serious disease in humans, and their ability to adhere to intestinal epithelial cells and to colonize the human gut is undoubtedly one of the key determinants of virulence. Estimates of the infectious dose for some STEC strains (O111:H$^-$ and O157:H7) are of the order of 1 to 100 CFU (114, 260); these estimates are many orders of magnitude lower than that for enterotoxigenic *E. coli* (ETEC) or enteropathogenic *E. coli* (EPEC) strains. At present, the processes involved in establishment and maintenance of gut colonization by STEC are poorly understood. However, an increased knowledge of the mechanisms at the cellular and the molecular level and identification of the bacterial products involved may provide targets for vaccination strategies, or opportunities for therapeutic intervention.

Acid resistance of STEC. An important feature of STEC strains that may impact upon their capacity to colonize the human gut, particularly at low infectious doses, is resistance to the acidity of the stomach. It is now known that exposure of certain enteric bacteria, including *E. coli*, to low pH induces an acid tolerance response (110), and this has been shown to increase the survival of O157:H7 STEC in mildly acidic foods (187). A distinct phenotype referred to as acid resistance has also been described and is mediated by *rpoS*, which encodes a stationary-phase sigma factor. This factor regulates genes enabling stationary-phase *E. coli* organisms to survive for extended periods below pH 2.5 (111). Interestingly, Waterman and Small (365) have recently reported heterogeneity in acid resistance phenotype among STEC strains which correlated with mutations in *rpoS*. Such differences may contribute to apparent differences in infectivity of STEC strains.

Epithelial cell adherence phenotypes. Having survived the harsh conditions of the stomach, STEC must establish colonization of the gut by adhering to intestinal epithelial cells. It is generally assumed that the colon and perhaps also the distal small intestine are the principal sites of STEC colonization in humans, although this has not been demonstrated directly. In vitro adherence of STEC has been examined by using several different epithelial cell lines under a range of experimental conditions, and several adherence phenotypes have been described. However, interpretation of the significance of these studies is complicated by the fact that adherence to nonpolarized epithelial cells in tissue culture (even those of human colonic origin) may not be an accurate reflection of molecular interactions that occur between STEC and human colonic epithelium in vivo. Even within STEC strains belonging to serotype O157:H7, there is heterogeneity in adherence, and this may reflect differences in mechanisms. Indeed, Sherman et al. (313) reported marked quantitative differences (up to 250-fold) in the adherence of five O157:H7 strains to both HEp-2 (human laryngeal epithelioma) and Henle 407 (human colonic carcinoma) cell lines. Some strains adhered in a diffuse fashion, with bacteria distributed evenly over the surface of the epithelial cells (diffuse adherence [DA]). Other strains formed tight clusters or microcolonies at a limited number of sites on the epithelial surface (localized adherence [LA]). Moreover, a given strain did not necessarily exhibit the same pattern of adherence on both cell lines (313). A LA phenotype is also exhibited by EPEC and is mediated by type IV fimbriae (bundle-forming pili), which are encoded by a cluster of 14 *bfp* genes carried by the EAF plasmid (319, 324). However, STEC strains do not carry this plasmid and lack *bfp* genes (32, 371, 373). McKee and O'Brien (210) also described a distinct pattern of adherence of O157:H7 STEC to HCT-8 (human ileocecal) cells, which they termed "log jam." Since adherence occurred principally at junctions between the cells, it is possible that this is a consequence of interaction with the basolateral surface. Moreover, the log jam phenotype was also observed in some commensal *E. coli* strains. The best-characterized STEC adherence phenotype, however, is intimate or attaching and effacing (A/E) adherence. This property is exhibited by a subgroup of STEC strains and is discussed in more detail below.

STEC strains have also been examined for their capacity to invade epithelial cells. STEC strains differ from certain other enteric pathogens (e.g., salmonellae, shigellae, and EPEC) in that they are unable to efficiently invade HEp-2 and Henle 407 cells (239, 313). However, O157:H7 STEC strains were taken up by T24 bladder cells and HCT-8 cells, although individual STEC strains varied in their invasive capacity. The invasion process was dependent upon both bacterial protein synthesis and host cell microfilaments (239). However, McKee and O'Brien (210) reported that the level of uptake of O157:H7 STEC by HCT-8 cells was significantly lower than that of EPEC or *Shigella flexneri* strains and no greater than that of a

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commensal *E. coli* strain. Thus, the clinical relevance of this property is questionable.

Role of the 60-MDa plasmid in STEC adherence. The involvement of the 60-MDa STEC plasmid, referred to as pO157, in the adherence of O157:H7 STEC was initially suggested by Karch et al. (150). These investigators found that the presence of the plasmid correlated with expression of fimbriae and adherence to Henle 407 but not HEp-2 cells. However, subsequent studies have produced conflicting results (98, 148, 347), and there is no consistent in vitro evidence for a role for pO157 in STEC adherence. The discordant findings may be attributable to differences in growth or assay conditions, as well as to differences between O157:H7 strains, and possibly also to the fact that the large plasmid itself appears to be heterogeneous, even within serotype O157:H7 (21, 86). The potential contribution of pO157 to pathogenesis has also been examined in animal models. Tzipori et al. (353) found that the presence or absence of the plasmid had no effect on the capacity of STEC strains to colonize the colon or to cause A/E lesions in gnotobiotic piglets. On the other hand, Wadolkowski et al. (361) demonstrated that both O157:H7 strain 933 and its plasmid-cured derivative 933cu could individually colonize the gut of streptomycin-treated mice but that 933cu could not establish colonization when used together with 933. Although the same strains were used in the above piglet experiments, competitive colonization studies were not performed. Therefore, it is not possible to determine whether the apparent contribution of pO157 is influenced by host species. Moreover, the degree to which either of these animal models reflects colonization mechanisms in humans is uncertain. Clearly, more research is needed to determine whether pO157 or related plasmids play a role in adherence of STEC to colonic epithelium.

Attaching and effacing adherence. It has been known for more than a decade that certain strains of STEC are capable of causing A/E lesions on enterocytes (96, 312). A/E lesions involve ultrastructural changes, including loss of enterocyte microvilli and intimate attachment of the bacterium to the cell surface. Beneath the adherent bacteria, there is accumulation of cytoskeletal components, resulting in the formation of pedestals; this is recognizable by electron microscopy and by fluorescence microscopy after staining with phalloidin-fluorescein isothiocyanate (172). The capacity to produce A/E lesions was initially recognized in EPEC strains, and recent studies have elucidated the molecular events involved in their generation, as reviewed by Donnenberg et al. (81). All of the genes necessary for generation of A/E lesions in EPEC are located on a 35.5-kb "pathogenicity island" termed the locus for enterocyte effacement (LEE), which is inserted at 82 min in the *E. coli* chromosome. Binding of EPEC to epithelial cells (initially via the bundle-forming pili) triggers intracellular signals including release of inositol triphosphate, phosphorylation of myosin light chains, and tyrosine phosphorylation of certain proteins in the epithelial cell membrane (81). In contrast to earlier reports, generation of A/E lesions does not require changes in intracellular Ca^{2+} levels (17). LEE includes a cluster of genes (*sepA* to *sepI*) which encode a type III secretion system. This machinery is responsible for secretion of other LEE-encoded proteins, including EspA, EspB, and EspD, which are necessary for initiation of the signal transduction events referred to above. LEE also includes the *eaeA* gene, which encodes intimin, a 939-amino-acid outer membrane protein (OMP) which mediates intimate attachment to the enterocyte (81, 181). Interestingly, Kenny et al. (168) have recently reported that the receptor for intimin is also encoded by LEE. This protein was previously referred to as Hp90 but has now been renamed Tir (translocated intimin receptor). Tir is secreted from EPEC as a 78-kDa species, and efficient delivery into the host cell is dependent upon the type III secretion system and other LEE-encoded secreted proteins. Tyrosine phosphorylation of Tir after insertion into the epithelial cell membrane increases its apparent size to 90 kDa, a phenomenon which can be reversed by alkaline phosphatase treatment. However, tyrosine phosphorylation is not essential for intimin binding, at least in vitro (168).

The mechanism whereby STEC strains generate A/E lesions is less well characterized but is essentially analogous to that for EPEC. STEC strains displaying the A/E phenotype have a LEE homolog (208), which, although not yet fully characterized, contains a copy of *eaeA*, whose 934-amino-acid product has 83% amino acid identity to EPEC intimin (27, 380). The STEC LEE also encodes a Tir homolog (93a), as well as a type III secretion system. Jarvis and Kaper (144) demonstrated that the latter was responsible for the secretion of proteins with masses of 100 to 110, 37, and 24 kDa, which reacted with sera from HUS patients. N-terminal amino acid sequencing identified the 37-kDa protein as an EspB homolog. Ebel et al. (90) demonstrated that secretion of a range of proteins by STEC was influenced by both growth temperature and culture medium, and N-terminal sequence analysis also identified homologs of EspA and EspB. Production of EspB was induced at 37°C and in serum-free tissue culture medium. Interestingly, sequence analysis indicated that EspB homologs from O157:H7 and O26:H^- STEC strains had only 80% amino acid homology.

However, there are some differences between EPEC and STEC, as well as gaps in our knowledge. For example, STEC strains do not usually exhibit the LA pattern of adherence to enterocytes to the same degree as EPEC strains. They lack the *bfp* genes found in EPEC, and the factors which mediate initial interaction with enterocytes are not yet fully characterized, as discussed below. Like EPEC, interaction of STEC with the host cell triggers increases in intracellular levels of inositol triphosphate but does not appear to result in tyrosine phosphorylation of Tir (137).

Studies with *eaeA*-negative O157:H7 STEC mutants have shown that, like EPEC intimin, STEC intimin is essential for the generation of cytoskeletal rearrangements in HEp-2 cells in vitro (82, 196). Donnenberg et al. (82) demonstrated that such mutants had also lost the capacity to adhere intimately to the colonic epithelium of piglets. These properties were reconstituted by transformation with a plasmid carrying either STEC or EPEC *eaeA*, indicating that the two genes were functionally homologous. Interestingly, however, further studies with the same constructs in a gnotobiotic piglet model demonstrated that the source of *eaeA* significantly influenced the nature and distribution of the A/E lesions in the piglet intestine (352). The *eaeA* mutant STEC strain reconstituted with EPEC *eaeA* colonized and caused A/E lesions in the distal half of the small intestine, as well as on the surface cells of the large intestine, a pattern typical of EPEC infection (351). This strain also caused more severe diarrhea than the wild-type STEC, which colonized the cecum and colon only but caused A/E lesions on both crypt and surface epithelial cells. The STEC *eaeA* gene was only partially capable of reconstituting these properties in the *eaeA* mutant STEC strain, a possible consequence of polar effects of the mutation. It seems likely that the marked differences in tissue tropism displayed by these otherwise isogenic strains, as well as the difference in severity of symptoms induced is a consequence of heterogeneity of the primary amino acid sequences of intimin from EPEC and STEC. The two molecules are virtually identical for the first ca. 700 amino acids, but the C-terminal portion (about 25% of the total

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length) is quite divergent, displaying only about 50% homology; this region is involved in binding to the epithelial cell (96a).

Similar studies have also been conducted by McKee et al. (209), using a derivative of the same O157:H7 STEC with an in-frame *eaeA* deletion, which eliminates possible complications due to polar effects of the mutation. Complementation of this *eaeA* mutant STEC with plasmids encoding an intact copy of *eaeA* demonstrated unequivocally that *eaeA* is essential for the LA adherence phenotype in HEp-2 cells. This gene was also essential for colonization of the piglet cecum and colon, generation of A/E lesions on enterocytes, and mediation of colitis, as judged by histological testing. However, the fact that the same plasmids could not complement the in vitro HEp-2 adherence phenotype in the *eaeA* insertion-deletion STEC mutant or confer adherence upon a wild-type *eaeA*-negative STEC strain indicated that an additional gene(s) downstream from *eaeA* was essential (209). This region of the STEC LEE is now known to include *esp* homologs. In a subsequent study, McKee and O'Brien (211) reconstituted HEp-2 adherence by exogenous addition of either of two purified His_6-EaeA fusion proteins. Both proteins were N-terminally truncated, and the smaller comprised only the carboxyl two-thirds of the protein. Interestingly, the fusion proteins also enhanced the adherence of an *eaeA*-negative wild-type STEC to HEp-2 cells but without conferring the capacity to generate cytoskeletal rearrangements. No enhancement of adherence of *E. coli* K-12 was observed.

There is no doubt that there is a strong association between carriage of *eaeA* and the capacity of STEC strains to cause severe human disease such as HC and HUS. Several studies have shown that the proportion of $eaeA^+$ strains from such sources is much higher than among STEC isolates from animals. Moreover, the presence of *eaeA* in animal isolates is most commonly associated with known human-virulent strains such as those belonging to serogroups O157, O26, O111, etc. (21, 31, 32, 195, 290, 372). An additional potential complication in the elucidation of the role of intimin in the pathogenesis of human disease is introduced by the significant sequence heterogeneity of the C-terminal portion of the protein. Heterogeneity between STEC and EPEC intimin accounts for marked differences in tissue tropism, as discussed above, but heterogeneity also occurs within STEC strains. For example, there was approximately 25% amino acid sequence divergence over the last 250 residues of intimin from O157:H7 and O111:H8 STEC strains, and additional sequence variation between *eaeA* genes from O111:H8 and O111:H11 STEC strains was detected by PCR (195). In another study, Wieler et al. (371) found that *eaeA* probe-positive STEC strains from only 8 of 17 O serogroups tested were PCR positive with primers based on the 3′ portion of O157:H7 *eaeA*. Such differences have been used as the basis for serotype-specific assays for STEC, as discussed below, but it is not known whether the variations affect the biological activity or receptor specificity of intimin.

Notwithstanding the above, a significant minority of human STEC isolates, including those from patients with HC and HUS, do not contain *eaeA*, indicating that intimin is not essential for human virulence (21, 195). These strains do not produce cytoskeletal rearrangements and A/E lesions in vitro, although at least some are capable of microvillus effacement (89). The possibility remains that these strains produce additional, as yet uncharacterized virulence factors to compensate for the absence of *eaeA*. Interestingly, Wieler et al. (371) found that only 65% of *eaeA* probe-positive bovine STEC isolates were positive by fluorescent actin staining of infected HEp-2 cells and, furthermore, that only 19% were positive for *espB* by PCR or even by low-stringency hybridization. Thus, the presence of *eaeA* does not necessarily imply that a given STEC strain is capable of production of functional intimin and generation of A/E lesions.

Other adherence mechanisms. Factors implicated in the adherence of other enteric pathogens include fimbriae, OMPs, and lipopolysaccharide (LPS). Studies by Sherman and Soni (311) showed that antibodies to whole cells or outer membranes, but not to H7 flagella, significantly inhibited the adherence of O157:H7 STEC to HEp-2 cells. Moreover, exogenous addition of OMP extracts inhibited adhesion in a concentration-dependent manner but addition of isolated flagella and LPS did not. Subsequent studies demonstrated that polyclonal antiserum raised against a purified 94-kDa OMP also blocked adhesion (310). This protein was subsequently shown to be distinct from intimin (88). Recently, an 8-kDa O157:H7 OMP has also been implicated in adherence. A Tn*phoA* insertion mutant deficient in production of this protein had a significantly reduced adherence to Henle 407 cells in vitro and was less able to colonize chicken ceca than was the wild-type O157:H7 STEC. Furthermore, preincubation with a monoclonal antibody specific for the 8-kDa OMP blocked subsequent in vitro adherence of the bacteria (382). In another recent study, Tarr et al. (335) described the isolation of a chromosomal *E. coli* O157:H7 gene, designated *iha*, which appears to encode the capacity to adhere to HeLa cells. The gene was found in all 20 O157:H7 STEC strains tested and in 4 of 5 $eaeA^+$ non-O157:H7 human STEC isolates but was not found in 10 *eaeA*-negative meat isolates. Interestingly, however, *iha* was not part of the LEE, and the 696-amino-acid product of this gene is a surface protein with approximately 40% homology to IrgA, an iron-regulated protein of *Vibrio cholerae*. It is not yet known whether *iha* encodes the capacity of STEC to adhere to epithelial cells of intestinal origin. Maneval et al. (204) have also recently reported the isolation of 21-kDa fimbrial subunits from O157:H7 and O26:H11 STEC strains. N-terminal sequence analysis indicated a degree of homology to *Bordetella pertussis* and *E. coli* F17 fimbriae. However, there was evidence of antigenic variation between fimbriae from the two STEC strains, and their role in adherence remains to be determined.

Two further studies have directly examined the role of LPS O-antigen side chains in adherence of O157:H7 STEC strains. In both studies, Tn*phoA* mutagenesis was used to construct STEC strains deficient in O-antigen biosynthesis, and these were found to be hyperadherent to HEp-2 cells in vitro (41, 74). The enhancement of adherence might be due to increased exposure of one or another of the above-mentioned OMPs on the bacterial surface, although it is possibly an artifact of the gross disturbance of cell surface hydrophilicity due to loss of O antigen.

Adherence mechanisms of non-O157 STEC. The mechanism of adherence of the class of STEC responsible for piglet edema disease to intestinal cells has been studied extensively (133). These strains do not generate A/E lesions, and adhesion to isolated porcine intestinal villi is mediated by a specific fimbrial adhesin referred to as F107. The gene cluster encoding F107 biosynthesis has been cloned, and the structural gene encoding the 15-kDa fimbrial subunit (designated *fedA*) has been sequenced (134). *fedA* has been found in the majority of edema disease STEC isolates but is also present in a small number of ETEC strains associated with postweaning diarrhea in piglets (132).

There are comparatively few studies in the literature, however, which have examined the adherence of non-O157 STEC strains from humans. Willshaw et al. (373) found that 13 of 48

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non-O157/O26 human isolates exhibited a LA phenotype on HEp-2 cells; all of these were *eaeA*$^+$ but a further 5 *eaeA*$^+$ STEC strains were LA negative. Nishikawa et al. (229) examined the effect of growth conditions on adherence of O157 and O111 STEC to HEp-2, Henle 407, and CaCo-2 (human colonic carcinoma) cells and concluded that although adherence was mannose resistant, prior growth in metabolizable sugars resulted in catabolite repression of adherence. Dytoc et al. (89) have studied the adhesion phenotype of an *eaeA*-negative STEC strain belonging to serotype O113:H21. This piliated strain adhered to rabbit ileal brush border membranes and to both Hep-2 and Henle 407 cells in a diffuse pattern; adherence was resistant to D-mannose. Although this strain was capable of microvillus effacement in vivo, it did not cause the cytoskeletal rearrangements and intimate A/E lesions typical of *eaeA*$^+$ STEC. In a recent study (261), the adherence of a range of STEC isolates from patients linked to an outbreak of HUS and diarrhea (caused by contaminated fermented sausage) was compared with that of apparently nonvirulent STEC strains also isolated from the implicated food source in a quantitative Henle 407 model. The adherence of STEC strains from HUS patients was significantly greater than that of STEC strains found in the contaminated food source but not in any patients. Other STEC strains from sporadic HUS cases, which included an *eaeA*-negative O48:H21 strain, also displayed enhanced adherence. These studies support the hypothesis that an enhanced capacity to adhere to intestinal cells is one of the factors which distinguishes human-virulent STEC strains from those of lesser clinical significance.

Role of STX in Pathogenesis of Disease

Uptake and translocation of STX by intestinal epithelial cells. Studies with rabbits have shown that STX has direct enterotoxic properties which result from selective targeting of Gb_3-containing absorptive villus epithelial cells in the ileum. Interestingly, this susceptibility of rabbit intestinal cells is age related and correlates with upregulation of net Gb_3 biosynthesis in the third week of life (218, 219). It is possible that the diarrhea seen in human STEC infections is due at least in part to direct exposure of enterocytes to STX in the gut lumen. However, the presence of Gb_3 in human enterocytes has yet to be demonstrated. Other studies suggest that many of the gastrointestinal pathological findings may be caused by systemic toxin. Intravenous injection of Stx1 into rabbits caused diarrhea with edematous and hemorrhagic lesions in the mucosa and submucosa of the cecum (280). Tashiro et al. (336) also demonstrated that local intra-arterial injection of Stx1 or Stx2 caused hemorrhagic lesions in the rat small intestine. In both studies, microvascular endothelium appeared to be the principal cytotoxic target. Since STEC strains appear to be unable to invade gut epithelial cells to any significant extent, the generation of systemic sequelae must presumably involve translocation of STX produced by colonizing bacteria from the gut lumen to underlying tissues and the bloodstream. One possible route might be through lesions in the mucosal barrier caused either by the direct effects of STX or other factors such as intimin or perhaps through gaps between adjacent epithelial cells. An alternative route from gut lumen to tissues might be through intact epithelial cells. This possibility has been examined by using polarized human colonic carcinoma cells (CaCo-2A and T84) grown on collagen-coated polycarbonate membranes (4). When grown for extended periods, these cells form tight junctions and the monolayers exhibit high transepithelial electrical resistance. For both cell lines, a significant proportion of active Stx1 added to the culture medium on the apical side was translocated to the medium on the basolateral side over 24 h; during this time, there was no toxin-induced damage to the epithelial barrier as judged by electrical resistance. This process appeared to be energy dependent, since it was blocked by low temperature or an uncoupler of oxidative phosphorylation. The total amount of Stx1 that could be translocated appeared to be saturable, suggesting the involvement of a cellular receptor, but this is unlikely to be the specific STX receptor Gb_3, since T84 cells lack this glycolipid. Moreover, induction of Gb_3 synthesis in CaCo-2A cells by treatment with sodium butyrate actually reduced toxin translocation. Thus, at least Stx is capable of translocation across intestinal epithelial cells without apparent cellular disruption via a transcellular pathway (4).

Interaction of STX with its glycolipid receptor. Once having crossed the epithelial barrier and presumably entered the bloodstream, STX targets tissues expressing the appropriate glycolipid receptor. The specificity of this interaction and the distribution of receptors among various cell types has a major impact on the pathogenesis of disease, both in humans and in various animal models, as recently reviewed by Lingwood (193). High levels of Gb_3 are found in the human kidney, particularly in the cortical region, the principal site of renal lesions in patients with HUS (47). However, overall levels were higher in adult kidneys than in infant kidneys, which contrasts with the age susceptibility to the disease. A subsequent study involving overlaying human renal sections with fluorescence-tagged Stx1 indicated that in tissue from adults, the toxin bound principally to distal convoluted tubules, particularly those closely apposed to a glomerulus, whereas in infants, overall Stx1 staining was less intense, in parallel with Gb_3 content. However, toxin bound to glomeruli as well as to distal tubules, which is consistent with the increased susceptibility to HUS in this age group (192). Tesh et al. (342) found similar levels of Gb_3 in the cortex and medulla of human, baboon, and mouse kidneys, as well as comparable binding of Stx1; both receptor and bound toxin were associated primarily with tubular epithelial cells. Although the pathological findings of HUS in humans are strongly indicative of significant endothelial cell (EC) disturbance (discussed below), there is also evidence for the involvement of other renal cell types in pathogenesis of disease. For example, Takeda et al. (332) reported that HUS patients have elevated levels of markers of tubular injury during the early stages of disease. Tubular necrosis is also seen in a proportion of patients with HUS (120, 279), and a significant number of patients with HUS present with acute anuric renal failure consistent with tubular necrosis but without obvious signs of coagulopathy. In rabbits, intravenous administration of Stx1 results in vascular damage (thrombotic microangiopathy), particularly in the cecum, colon, and central nervous system (280, 383). EC in these tissues were also the principal sites of uptake of ^{125}I-labelled Stx1. However, rabbit renal tissue was unaffected and did not bind toxin (280), which is consistent with its lack of Gb_3 (383).

The most dramatic demonstration of the contribution of STX receptor specificity to the disease process involved site-directed mutagenesis of the gene encoding the Stx2e B subunit, such that Gln64 and Lys66 were changed to Glu and Gln, respectively (the analogous residues in Stx2). These amino acid substitutions altered the predominant in vitro binding specificity of the mutant toxin from Gb_4 to Gb_3, that is, to the same receptor binding phenotype as Stx2. They also changed the relative cytotoxicity of the mutant Stx2e for various cell lines, in accordance with their Gb_3 and Gb_4 content (350). When the mutant Stx2e was injected intravenously into pigs, the distribution of toxin to the various organs was different from that

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obtained with wild-type Stx2e; the former was targeted to tissues containing Gb_3, while the latter bound extensively to erythrocytes (RBCs) (which contain Gb_4) and delivery to specific tissues was influenced by regional blood flow in addition to Gb_4 content. Differences in the clinical characteristics of toxin-induced disease were also observed, but there was no obvious effect on the nature of the histological lesions (49).

Notwithstanding the above, the susceptibility of a given cell type to STX is not determined solely by its total Gb_3 content, and recent studies indicate that the lipid moiety of Gb_3 has a significant influence on the interaction of the oligosaccharide head group with toxin (170, 193, 265). Stx1 exhibits optimum binding to Gb_3 with a fatty acyl chain length of 20 to 22 carbons, while for Stx2c the optimum chain length is 18. For both toxins, receptor binding is increased when the fatty acid is unsaturated. As discussed previously, three distinct sites on the Stx1 B subunit have now been postulated to interact with Gb_3 (23, 71, 193, 230, 231). Thus, if the lipid moiety of Gb_3 influences the conformation of the oligosaccharide component, this could in turn alter the binding affinity for one site on the B subunit relative to the others. Moreover, variations in the B subunit among members of the STX family may result in a preference for one receptor binding site over another, which would then result in differential specificity for Gb_3 receptor subsets on the basis of the lipid moiety (193). This may result in different specific activities for STX toxins against given cell types, as well as in vivo variations in tissue specificity, affecting the pathological findings and the 50% lethal dose (LD_{50}), as discussed below.

Effects of the lipid moiety on toxin-receptor interactions may also explain why some Gb_3-containing cell lines are refractory to STX cytotoxicity. As discussed above, receptor-bound STX is internalized and undergoes retrograde transport via the Golgi apparatus and endoplasmic reticulum and is ultimately released into the cytosol in sensitive cells. However, the intracellular trafficking is different and Stx-receptor complexes are internalized and confined in lysosomes in the insensitive cells (294). Interestingly, treatment of such cell lines with sodium butyrate induces retrograde transport and sensitivity to toxin and also results in changes in the fatty acyl component of Gb_3 (293). Thus, the lipid moiety of Gb_3 may influence susceptibility of cell types to STX (193).

It has also been suggested that interaction of STX with glycolipid receptors on the surface of RBCs may play a role in the pathophysiology of HUS. The human P blood group antigens are glycolipids and include Pk (which is Gb_3), P (which is Gb_4), and P1 (a neolactoceramide which also has a terminal Galα1-4Gal moiety); the relative exposure of these antigens on the RBC surface varies with the blood group. On the basis of their observed association between particular P phenotypes and the outcome of HUS, Taylor et al. (338) hypothesized that STX binding by RBCs might remove toxin from the circulation, thereby protecting sensitive Gb_3-containing tissue types. Bitzan et al. (44) have demonstrated by immunofluorescence that purified Stx1, Stx2, Stx2c, and Stx2e all bind in vitro to RBCs, with the affinity varying in accordance with the P phenotype and toxin type. Enhanced binding of Stx1, Stx2, and Stx2c to RBCs of the P1 relative to the P2 phenotype was attributed to increased surface exposure of Gb_3, as well as direct binding of toxin to the P1 antigen. However, direct binding of STX to RBCs has yet to be demonstrated in cases of human STEC disease, and a more recent study by Orr et al. (243) did not report a HUS-protective association with the P1 phenotype. The significance of toxin-RBC interactions is likely to be dependent upon the relative affinity for receptors on RBCs and those on target tissues; low-affinity receptors on RBCs might mediate the transport of STX to tissues (e.g., the kidney) with high-affinity receptors. The importance of the lipid moiety of receptor glycolipids is also suggested by the report that RBCs of patients with HUS have a lower nonhydroxylated fatty acyl Gb_3 content than do those from healthy controls or patients with STEC diarrhea without HUS (226). However, it is not known whether this apparent association with HUS susceptibility is a direct consequence of altered affinity of STX for either RBC or other target tissues.

Effects of STX on endothelial cells. It is now generally agreed that the major portion of the histopathological lesions associated with both HC and HUS is a consequence of the interaction of STX with endothelial cells. The typical features of HUS include swollen and detached glomerular EC, and deposition of fibrin and platelets in the renal microvasculature (particularly in the glomerulus) (279). Capillary occlusion results in reduced blood flow to the kidneys and hence to renal insufficiency and may also cause physical damage to RBCs. Thrombotic lesions are also observed systemically, particularly in the microvasculature of the bowel, brain, and pancreas. Analogous histopathological lesions in the brain and gastrointestinal tract are seen in animal models of STX-induced disease (280, 336), although there is evidence that in both mice and rabbits, neurological damage by Stx2-related toxins involves direct neuronal injury in addition to microangiopathy (100, 101, 217). Although the precise molecular mechanisms whereby microangiopathic lesions are generated are not fully elucidated, STX may prevent the production of molecules critical for maintenance of the procoagulant-anticoagulant balance of the endothelium and/or an imbalance between vasodilators and vasoconstrictors produced by these cells (149).

Initial in vitro studies were performed with cultured EC derived from large vessels (umbilical or saphenous veins). These cells were shown to be susceptible to Stx/Stx1 and Stx2, as judged by inhibition of protein synthesis, detachment of the cells from the substratum, and loss of viability (237, 343). These EC were much less sensitive to Stx than were Vero cells, which was attributed to their relatively low levels of Gb_3 (343). However, the susceptibility of large-vessel EC to Stx was significantly increased if tumor necrosis factor alpha (TNF-α) was coadministered or if EC were preincubated with the cytokine (198, 343). Subsequent studies demonstrated similar enhancement of Stx cytotoxicity by preincubation of EC with LPS or interleukin-1β (IL-1β) (163, 199, 356). All three factors increase the binding of Stx1 to EC, and for TNF-α and IL-1β, this was shown to be a consequence of upregulation of Gb_3 on the cell surface (163, 356). Subsequent studies demonstrated that TNF-α induces the expression of a galactosyltransferase involved in the biosynthesis of Gb_3 (355). However, there appear to be differences in the intracellular signalling pathways involved in sensitization, since Louise et al. (201) have recently shown that the effects of LPS and TNF-α on EC are mediated by protein kinase C of class I/II and III, respectively, whereas the sensitization due to IL-1β is protein kinase C independent. Interestingly, Louise et al. (197) have shown that sodium butyrate can also sensitize human umbilical vein EC to Stx through up-regulation of Gb_3 receptors. They suggested that during STEC infection, intestinal damage might result in increased exposure of EC to butyrate, which is present in high concentrations in the colon, and thereby contribute to the pathogenesis of EC damage.

Interpretation of in vitro studies of EC in tissue culture is complicated by the fact that cells isolated from different human tissues exhibit different properties. For example, Keusch et al. (169) have reported differences in the responses of human umbilical and saphenous vein EC to Stx1, cytokines, and bu-

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tyrate. More significantly, Obrig et al. (238) found that levels of Gb_3 in renal microvascular EC were 50 times higher than in umbilical vein EC and that the former were also 1,000 times more sensitive to Stx. Furthermore, the Gb_3 content of renal EC and their sensitivity to Stx were not further increased by preincubation with either TNF-α or LPS. A similar high sensitivity to Stx and lack of enhancement of susceptibility by IL-1β and TNF-α has also recently been reported for human intestinal microvascular EC (2). However, human cerebral EC appear to behave like umbilical vein EC, exhibiting a low baseline sensitivity to Stx, which is stimulated by preincubation with IL-1β and TNF-α (130). Notwithstanding the above, caution must be exercised when comparing studies. Differences in experimental procedures used for isolation, purification, and establishment of EC cultures, and possible contamination with other cell types, may affect the results. Monnens (220), for example, has recently reported that highly purified human glomerular microvascular EC are not susceptible to Stx1 unless they are preincubated with TNF-α. In a separate study, Kohan et al. (173) also reported increased susceptibility of glomerular EC to Stx1 after preincubation with either TNF-α, IL-1β, or LPS.

From the above, it seems probable that development of maximum EC damage during STEC infection requires both STX as well as host and/or bacterial inflammatory mediators to upregulate receptor expression. Endotoxemia has been reported in association with HUS due to *S. dysenteriae* (176) but may be of lesser magnitude in cases caused by STEC strains, which are less invasive than shigellae. Karpman et al. (162) have reported a higher incidence of systemic symptoms and glomerular pathological changes after intragastric inoculation of O157:H7 STEC in LPS-responder mice than in LPS-nonresponder mice, but the relevance of this to human disease is again uncertain, since many of the animals were bacteremic. Although elevated IL-1β and IL-8 levels have been found in the plasma of HUS patients, TNF-α has only occasionally been detected in plasma (94, 136). However, Karpman et al. (161) reported marked elevation of IL-6 and TNF-α levels in the urine of HUS patients during the acute phase of illness relative to those in healthy controls. Lack of correlation with cytokine levels in paired samples of plasma and urine suggested that the kidney itself was the site of production. Inward et al. (136) have also reported higher levels of IL-8 in urine than plasma in HUS patients. These findings are consistent with studies with transgenic mice carrying a reporter for TNF-α synthesis; injection of Stx resulted in induction of TNF-α in the kidneys but not in any other tissues (125). Kohan et al. (173) have reported Stx1-induced production of TNF-α, IL-1β, and IL-6 by human glomerular EC and proximal tubular epithelial cells. Monocytes and macrophages are also a potential source of proinflammatory cytokines, and production of TNF-α, IL-1β, IL-6, and IL-8 is induced by treatment with Stx1 (272, 341, 358). Levels of monocyte chemoattractant protein 1 (MCP-1) are also significantly elevated in the urine of HUS patients, and immunohistochemical studies have demonstrated MCP-1 expression, as well as infiltration of monocytes, in glomeruli of HUS patients (359). Local production of TNF-α and IL-1β by such cells, as well as by other renal cell types, is likely to ensure maximal induction of Gb_3 expression in glomerular EC, enhancing susceptibility to STX. Robinson et al. (285) have also reported that Stx1 binds to human glomerular mesangial cells and inhibits mitogenesis. These cells are involved in modulation of the glomerular filtration rate and elaborate a range of hormones, cytokines, and growth factors. Thus, it is possible that direct effects of STX on mesangial cells also contribute to the acute renal failure associated with HUS.

The presence of elevated IL-8 levels in the plasma of HUS patients (94) may also be significant, because IL-8 it is a powerful selective activator and chemoattractant of polymorphonuclear leukocytes (PMN). HUS patients have increased PMN counts on presentation, and high levels correlate with poor clinical outcome (mortality or permanent nephropathy) (73, 215, 363). Fitzpatrick et al. (94) reported that levels of α_1-antitrypsin-complexed elastase (a marker of PMN activation and degranulation) correlated with but lagged slightly behind those of IL-8 and that both of these parameters were highest in fatal cases. Moreover, there is evidence of oxidative damage and PMN in the glomeruli of HUS patients at autopsy (95, 337). Thus, cytokine-mediated PMN activation may play an important role in the pathophysiology of HUS. To cause inflammatory damage to the endothelium, however, PMN must be in close proximity to it. IL-1β and TNF-α upregulate the expression of adhesion molecules on the EC surface, and IL-8 and platelet-activating factor also mediate flattening of rolling PMN, promoting strong adherence to the EC (337). Products such as elastase released from adherent PMN could then degrade the extracellular matrix, resulting in detachment of EC from the basement membrane, a common histopathological feature of HUS. Interestingly, Morigi et al. (222) have also shown that purified Stx1 is a strong promoter of adhesion of leukocytes to cultured EC. The adhesive response was comparable to that achieved with IL-1β and was even greater when EC were preexposed to TNF-α. Thus, local production of TNF-α in the kidneys might increase the susceptibility of EC to inflammatory damage from adherent leukocytes as well as enhancing the direct cytotoxic actions of Stx.

Influence of STX type on pathogenesis. Epidemiological studies have indicated that STEC strains producing Stx2 only are more commonly associated with serious human disease, such as HUS, than those producing Stx1 alone or Stx1 and Stx2 (171, 244). One possible explanation for this is that the level of transcription of stx_2 in vivo is higher than that of stx_1. Transcription of stx_1 is known to be iron repressible in vitro, and its promoter region includes a recognition site for the *fur* gene product (56, 366). However, iron levels in the gut are very low, and so it is likely that transcription of stx_1 will be fully derepressed in vivo. Sung et al. (329) have shown that in vitro transcription of stx_2-related genes is constitutive and at a level commensurate with that of derepressed stx_1. Mühldorfer et al. (225) also found that stx_2 promoter activity was unaffected by osmolarity, pH, oxygen tension, acetates, iron level, or carbon source but that there was a slight effect of growth temperature. However, treatment with mitomycin, which induces the lytic cycle of *STX*-converting bacteriophages, has been shown to significantly increase both Stx1 and Stx2 production in lysogenized *E. coli* strains (6, 126). For Stx2, this increase appears to be due to a combination of amplification of stx_2 as the phage DNA is replicated and an increase in stx_2 promoter activity mediated by a phage-encoded positive regulatory factor (225). This increase in Stx2 production may be clinically significant, since mitomycin is used to treat certain neoplastic disorders and these patients are at increased risk of HUS (185).

The link between Stx2 production and HUS may be a direct consequence of increased in vivo toxicity of Stx2, or, alternatively, carriage of stx_2 may simply be a clonal marker of STEC strains producing some additional virulence factor. The former alternative is consistent with in vitro studies which demonstrated that human renal microvascular EC were approximately 1,000 times more sensitive to the cytotoxic action of Stx2 than of Stx1 (200). Binding studies suggested that there were differences in the number of potential binding sites for Stx1 and Stx2 on the renal EC surface, presumably a function

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of heterogeneity of the lipid moiety of Gb_3. Thus, preferential binding of Stx2 to Gb_3 receptor subpopulations which are more efficiently internalized and processed would account for the massively increased cytotoxicity.

Increased in vivo toxicity of Stx2 is also supported by studies involving a streptomycin-treated mouse model of toxin-induced renal tubular damage. Streptomycin treatment reduced the normal facultative intestinal flora of the mice and facilitated colonization with orally administered *E. coli* strains (361). Oral infection of mice with an O157:H7 STEC strain producing Stx1 and Stx2 resulted in fatal cortical tubular necrosis. Death could be prevented by passive immunization with monoclonal anti-Stx2 but not with anti-Stx1 (361, 362). Oral challenge with *E. coli* K-12 strain DH5α carrying cloned stx_2 but not stx_1 was also capable of inducing fatal tubular damage. The level of toxin production was clearly important, since challenge with *E. coli* K-12 strain DH5α carrying stx_2 on a low-copy-number vector was not lethal (362). Tesh et al. (339) subsequently demonstrated that purified Stx2 had an approximately 400-fold lower LD_{50} for mice than did Stx1 by both the intravenous and intraperitoneal routes. Interestingly, immunofluorescent staining showed that both toxins were capable of binding to Gb_3 receptors on renal tubular cells, and in a solid-phase in vitro binding assay, Stx1 actually exhibited a 10-fold greater affinity for Gb_3 than did Stx2.

Assessment of the impact of STX type on the capacity of STEC to cause severe disease is complicated by the fact that there is a significant degree of naturally occurring amino acid sequence variation, particularly among Stx2 types from different strains (106, 138, 189, 214, 242, 258, 259, 305). Lindgren et al. (190) compared the oral virulence of a number of Stx2-producing clinical isolates for streptomycin-treated mice. In this study, two strains belonging to serotype O91:H21 were exquisitely virulent ($LD_{50} < 10$ CFU, compared with $>10^{10}$ CFU for other strains tested). Although the capacity to grow in mouse small intestinal mucus appeared necessary for maximal virulence, it was not the sole determinant, since at least one other strain tested grew well under these conditions yet was of low virulence; the production of a particularly mouse-virulent Stx2-related toxin appeared to be a likely explanation for this observation. Assessment of the contribution of specific toxins to the virulence of Stx2-producing STEC strains is further compounded because many isolates produce more than one Stx2-related toxin (305). Indeed, one of the highly virulent O91:H21 isolates referred to above carried three stx_2-related genes, while the other isolate carried two genes (190); strains carrying one stx_1 gene and two stx_2-related genes have also been described (260). One strength of the streptomycin-treated mouse model is that the oral virulence of *E. coli* K-12 strains expressing different cloned stx_2-related genes can be compared. Interestingly, clones expressing stx_2 genes from the O91:H21 STEC strains were no more virulent than those expressing classical stx_2 in this mouse model; in addition, the purified toxins had indistinguishable intraperitoneal LD_{50} (191). Moreover, sequence analysis indicated that the B subunits of the O91:H21 toxins were identical to that of Stx2c, which contains amino acid differences in the B subunit, resulting in a 100-fold-lower cytotoxicity for Vero cells compared with that of Stx2 (191).

Studies involving otherwise isogenic *E. coli* DH5α derivatives expressing a different set of naturally occurring Stx2 variants have, however, detected differences in virulence in the streptomycin-treated mouse model (251, 252). The least virulent (and least verocytotoxic) clone produced an Stx2-related toxin with an amino acid substitution (Arg176→Gly) in a region of the A subunit known to be important for the catalytic activity of the toxin. Interestingly, clones producing two other toxins (designated Stx2/O48 and Stx2/OX3b) had high cytotoxicity for Vero cells, but the latter was more virulent when fed to streptomycin-treated mice, as judged by the median survival time (but not by the overall survival rate). Subsequent studies demonstrated that the increased virulence of clones producing Stx2/OX3b was a function of the combination of A-subunit residues Met4 and Gly102 (252). A clone producing another Stx2 variant (designated Stx2/OX3a), which had the B-subunit amino acid sequence variations characteristic of the Stx2c subgroup, had similar oral virulence to clones producing Stx2/O48, even though it was approximately 500-fold less verocytotoxic (251), in agreement with the earlier finding of Lindgren et al. (191). However, when the model was modified by withdrawal of streptomycin selection and reintroduction of the normal gut flora after 3 days, the mortality rate of mice challenged with the more verocytotoxic clone was significantly greater (262).

Recently, Melton-Celsa et al. (212) reported that the Stx2-related toxins purified from one of the highly mouse-virulent O91:H21 strains referred to above exhibited 10- to 1,000-fold greater cytotoxicity for Vero cells after incubation in the presence of mouse proximal small intestinal mucus or human colonic mucus. In marked contrast, no stimulation in cytotoxicity was observed when extracts containing Stx1, Stx2, Stx2c, or Stx2e were similarly treated. Activation of the Stx2-related O91:H21 toxins by mucus also appeared to be a function of the A subunit, because the B subunit is identical to Stx2c, which was not activatable. However, the A-subunit amino acid differences (with respect to Stx2c) common to both the activatable toxins (Ser291 and Glu297) are not, on their own, sufficient for activation, since they are also found in Stx2e, which is not activatable. At present, the significance of activation of some Stx2-related toxins by intestinal mucus to pathogenesis of human disease is uncertain. Clearly, it is not essential, since serious human disease frequently results from infection with STEC strains producing nonactivatable toxins. However, Melton-Celsa et al. (212) have suggested that production of an activatable toxin may increase virulence and/or compensate for the absence of another separate virulence factor, since both the O91:H21 STEC strains studied lack *eaeA*. Interestingly, Baker et al. (18) have reported differences in the virulence for gnotobiotic piglets among O157:H7 STEC strains, all of which were positive for stx_1, stx_2, and *eaeA*. However, whether this is a consequence of differences in toxin sequence or accessory virulence factors is unknown (discussed below).

It should also be emphasized that the link between Stx2 production and the capacity of an STEC strain to cause HUS is not absolute. STEC strains producing only Stx1 are capable of causing HUS, as are strains of *S. dysenteriae* type 1 which also produce only Stx. Indeed, Stx has recently been shown to cause medullary tubular injury and proteinuria in isolated perfused rat kidneys (314), although the toxicity of Stx2 in this model system was not investigated.

Putative Accessory Virulence Factors

Enterohemolysin. Beutin et al. (33) observed that a high proportion of STEC strains (89% of those tested) had a novel hemolytic phenotype which was distinct from that associated with the *E. coli* alpha-hemolysin (Hly). Strains producing this enterohemolysin (subsequently designated EHEC-Hly) are not hemolytic on standard blood agar but produce small, turbid hemolytic zones on washed sheep RBC agar (supplemented with Ca^{2+}) after overnight incubation. Unlike alpha-hemolysin which is chromosomally encoded, EHEC-Hly was found to be encoded by the 60-MDa "virulence plasmid" (pO157) of the

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O157:H7 strain EDL933 (302). Subsequent sequence analysis of DNA cloned from pO157 demonstrated the presence of an operon, consisting of four open reading frames, with approximately 60% homology to the *E. coli* alpha-hemolysin operon (*hlyCABD*) and operons encoding other members of the RTX family of pore-forming cytolysins. The O157 enterohemolysin operon was therefore designated EHEC-*hlyCABD* (300, 303). Within the alpha-hemolysin operon, *hlyA* is the structural gene for the hemolysin, which is synthesized as an inactive precursor and converted to its active form by the product of the *hlyC* gene. The secretion of HlyA is signal peptide independent and mediated by both a specific membrane translocator system encoded by the *hlyB* and *hlyD* genes and an unlinked OMP, TolC (369). EHEC-HlyA, with a predicted size of 107 kDa, is not efficiently secreted by *E. coli* EDL933. However, *trans* complementation with *hlyBD* significantly increases export, resulting in an alpha-hemolysin-like phenotype (300). Schmidt et al. (303) have suggested that the poor secretion of EHEC-HlyA by wild-type strains could be due to a specific amino acid difference in the ATP binding cassette (ABC) domain of EHEC-HlyB compared with HlyB (Asn and Gly, respectively).

Schmidt et al. (300), using a PCR assay to detect EHEC-*hlyA*, surveyed diarrheagenic *E. coli* isolates and found that all 22 STEC O157:H7 strains and 12 of 25 STEC strains of other serotypes were positive. All non-STEC diarrheagenic strains were negative for EHEC-*hlyA*. Although PCR-positive strains hybridized with the CVD419 probe (a commonly used marker for the large virulence plasmid [186]), analysis of this probe indicated that part of the EHEC-*hlyA* and EHEC-*hlyB* genes were included. Thus, there seems little doubt that there is a correlation between STX and enterohemolysin production (particularly in O157 STEC strains). Schmidt and Karch (301) have also shown that an EHEC-*hlyA* homolog from an O111:H$^-$ STEC strain had 99.4% homology to O157 EHEC-*hlyA*. Within this STEC serotype, 16 of 18 isolates from patients with HUS were both enterohemolytic and positive for EHEC-*hlyA*. In contrast, only 4 of 18 isolates from patients with diarrhea had this phenotype and genotype, suggesting the possibility of a direct association between enterohemolysin production and the capacity of a given STEC strain to cause more serious disease. Interestingly, regardless of the presence or absence of EHEC-*hlyA*, all the O111:H$^-$ STEC strains tested carried large plasmids (301). A possible role for EHEC-Hly in pathogenesis is also consistent with the finding that 19 of 20 convalescent-phase sera from patients recovering from HUS due to O157 STEC contained antibodies to EHEC-Hly, compared with only 1 of 20 sera from age-matched controls (300).

The manner in which EHEC-Hly might contribute to the pathogenesis of STEC disease is not understood. One possibility is that hemoglobin released by the action of EHEC-Hly provides a source of iron, thereby stimulating the growth of STEC in the gut (184). However, the biological effects of members of the RTX toxin family are not confined to lysis of RBCs, and cytotoxicity has been reported for a range of cell types (369). Moreover, sublytic concentrations of *E. coli* alpha-hemolysin have been shown to mediate the release of leukotrienes from granulocytes and IL-1β from cultured monocytes and to reduce the binding of chemotactic factors by neutrophils (36, 299). Of potential interest regarding the pathogenesis of HUS is the finding that alpha-hemolysin is cytotoxic for endothelial cells (330) and increases superoxide production by rat renal tubular cells (164). Intravenous administration of alpha-hemolysin was also reported to induce granulocytopenia and thrombocytopenia in monkeys (354). Notwithstanding this, there appear to be substantial differences in the target cell specificities of alpha-hemolysin and EHEC-Hly. Both toxins lysed sheep and human RBCs and a bovine lymphoma cell line, but, unlike alpha-hemolysin, EHEC-Hly did not lyse two human lymphocyte cell lines (26). Interestingly, a major area of sequence divergence between HlyA and EHEC-HlyA is the 70 residues at the N terminus that includes the region believed to be important for target cell specificity (26). Thus, although it is tempting to speculate that EHEC-Hly might be involved in modulation of inflammatory processes associated with EC damage or might act in synergism with STX or LPS, direct studies with purified EHEC-Hly are needed to define the tissue specificity and biological properties of this toxin.

Serine protease (EspP). Another putative virulence factor also encoded on pO157 is the recently described extracellular serine protease EspP (extracellular serine protease, plasmid encoded) (52). The *espP* gene encodes a 1,300-amino-acid protein, which is subsequently subjected to N- and C-terminal processing during the secretion process. The mature form has an apparent size of 104 kDa. The amino acid sequence of the N terminus of the mature protein indicated that EspP may be the same as a 104-kDa secreted protein found in an O26:H$^-$ STEC strain by Ebel et al. (90). The deduced amino acid sequence of the complete O26 protein was subsequently shown to be virtually identical to the sequence of EspP (80). EspP has significant homology (approximately 70% overall) to EspC, a 110-kDa EPEC secreted protein, and to a lesser degree to immunoglobulin A1 (IgA1) proteases of *Haemophilus influenzae* and *Neisseria* spp. The region of homology includes the serine-containing proteolytic active site. EspP did not exhibit IgA1 protease activity in vitro but was capable of cleaving pepsin, an activity which was inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride. EspP was also able to cleave human coagulation factor V. Thus, Brunder et al. (52) have suggested that secretion of EspP by STEC colonizing the gut could result in exacerbation of hemorrhagic disease. Djafari et al. (80) also reported that EspP is cytotoxic for Vero cells. A role for EspP in pathogenesis is also consistent with the presence of antibodies to the protease in sera from five of six children with STEC infection but not in sera from age-matched controls. Nevertheless, EspP was produced by only four of six O157 and one of two O26 isolates but by none of two O103 STEC clinical isolates tested, indicating that it is not a universal virulence factor of STEC (52).

Heat-stable enterotoxin. A final virulence factor that might contribute to the pathogenesis of the watery diarrhea often seen during the early stages of STEC infection is the enterotoxin EAST1 (encoded by *astA*). This is a 39-amino-acid enterotoxin that was initially recognized in certain strains of enteroaggregative *E. coli* (EAggEC) and is distinct from the heat-stable toxins produced by ETEC (296). Recently, Savarino et al. (297) demonstrated that an *astA*-specific probe hybridized with all 75 O157:H7 STEC strains tested, as well as with 8 of 9 O26:H11 strains and 12 of 23 STEC strains of other serotypes. This probe also hybridized with 41, 41, and 22% of EAggEC, ETEC, and EPEC strains, respectively. Enterotoxicity for rabbit ileal tissue was confirmed by testing culture ultrafiltrates (<10 kDa) of three of the *astA*$^+$ O157:H7 strains in Ussing chambers. In these three strains, there were two copies of *astA*, which were located on the chromosome (297).

DIAGNOSIS

There are a number of difficulties associated with the diagnosis of STEC infection. In the early stages of infection, there may be very large numbers of STEC in feces; in many cases, the STEC constitutes more than 90% of the aerobic fecal flora

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(260). However, as disease progresses, the numbers may drop dramatically. In patients with HUS, the typical clinical signs may become apparent only a week or more following the onset of gastrointestinal symptoms, at which time the numbers of STEC may be either very small or the bacteria may have been eliminated from the gut altogether. Also, in some cases, diarrhea is no longer present and only a rectal swab is available at the time of admission to hospital, limiting the amount of specimen available for analysis. For these reasons, diagnostic tests should preferably be very sensitive and require minimal specimen volumes. Also, the clinical presentation of STEC disease is sometimes confused with other conditions such as inflammatory bowel disease, appendicitis, intussusception, and *Clostridium difficile* infection. Thus, rapid diagnosis is important to prevent unnecessary invasive and expensive surgical and investigative procedures or administration of antibiotic therapy which may be contraindicated for STEC infection (248, 334). Diagnostic procedures are based on detection of the presence of *STX* or STX in fecal extracts or fecal cultures, and/or isolation of the STEC (or other STX-producing organisms). Since these procedures differ in complexity, speed, sensitivity, specificity and cost, diagnostic strategies must be tailored to the clinical circumstances and the resources available.

Testing for STX

Tissue culture cytotoxicity assays. The profound sensitivity of Vero cells to STX was first observed by Konowalchuk et al. (175), and cytotoxicity for this cell line remains the "gold standard" for confirmation of putative STX-producing isolates. Since Vero cells have a high concentration of Gb_3 and Gb_4 (the preferred receptor for Stx2e) in their plasma membranes, they can be used to detect all known STX variants. HeLa cells have also been used, but this cell line lacks Gb_4 and therefore is less sensitive to Stx2e. It is not practical or economical to test filtrates of individual colonies from a fecal culture plate to isolate STEC, because these organisms frequently comprise less than 1% of the aerobic flora. However, the Vero cell cytotoxicity assay is an effective means of demonstrating the presence of STX in cell extracts of feces or fecal culture filtrates. The assay involves treatment of Vero monolayers (usually in 96-well trays) with sterile extracts or filtrates (serially diluted in some cases) and examining cells for cytopathic effect after 48 to 72 h of incubation (156, 318). Historically, this assay has played an important role in establishing a diagnosis of STEC infection, particularly where subsequent isolation of the causative organism has proven to be a difficult task. When testing such crude samples, the sensitivity is influenced by the abundance of STEC, the total amount and potency of the STX produced by the organism concerned, and the degree to which the particular STX is released from the bacterial cells. Karmali et al. (157) found that treating mixed fecal cultures with polymyxin B to release cell-associated STX improved the sensitivity of the Vero cell assay to the point where it could reliably detect STEC at a frequency of 1 CFU per 100. Clearly some STEC strains produce very high levels of toxin, and these can be detected at even lower frequencies. However, the converse also applies. Since the presence of cytoxicity in a crude filtrate could be due to other bacterial products or toxins, positive samples should always be confirmed (and typed) by testing for neutralization of cytotoxicity by specific (preferably monoclonal) antibodies to Stx1 or Stx2 (317).

Although a valuable diagnostic method, detection of STX by tissue culture cytotoxicity is labor-intensive, time-consuming, and cumbersome. Not all microbiology laboratories perform tissue culture work with Vero cell monolayers available on demand. Moreover, rapid diagnosis is important, and the results of cytotoxicity testing are generally not available for 48 to 72 h. Development of a Vero cell line carrying the gene encoding firefly luciferase might potentially overcome the latter drawback (122). Since luciferase has a very short in vivo half-life, it is a sensitive marker of STX inhibition of protein synthesis. By using luciferase, equivalent or lesser amounts of STX were detected by diminution of chemiluminescence after 6 h of incubation compared to detection by the conventional 72-h cytotoxicity assay. Another potentially useful modification that eliminates the cost of filter-sterilizing bacterial extracts before testing involves overlaying confluent Vero monolayers with tissue culture medium containing 0.9% agarose (249). Putative STEC colonies can then be inoculated directly onto the surface of each well and cytotoxicity in the underlying monolayer can be assessed after 48 h. Although this technique worked well with pure STEC isolates, its sensitivity has yet to be assessed with crude fecal cultures.

ELISAs for the direct detection of STX. During the past decade, a number of enzyme-linked immunosorbent assays (ELISAs) have been developed for the direct detection of Stx1 and Stx2 in fecal cultures. Like verocytotoxicity, these play a potentially important role in diagnosis because they can detect the presence of STEC (or other STX-producing species), regardless of serogroup. Such assays can also be used to confirm toxin production by putative STEC isolates where tissue culture facilities are unavailable. Most of the published ELISA methods involve a sandwich technique with immobilized monoclonal or affinity-purified polyclonal antibodies to the toxins as capture ligands. After incubation with cultures, bound toxin is detected with a second STX-specific antibody followed by an appropriate anti-Ig–enzyme (usually alkaline phosphatase) conjugate (84, 87). Some assays involve an STX detection antibody directly conjugated to the enzyme (174) or a biotinylated detection antibody which is used with a streptavidin-enzyme conjugate (19, 321). Another variation in the ELISA protocol involves the use of purified Gb_3 (or a deacylated derivative) or hydatid cyst fluid (containing P_1 glycoprotein, which also binds STX) to coat the solid phase (7, 14, 15, 24, 182, 183). Two STX ELISAs are commercially available in kit form (Premier EHEC [Meridian Diagnostics, Inc., Cincinnati, Ohio] and LMD [LMD Laboratories, Carlsbad, Calif.]).

Tests of pure isolates show that the specificities of the various STX ELISAs are in close agreement with the results of verocytotoxicity assays (15, 24, 87, 321). Also, Law et al. (183) reported a specificity of 99.7% when fecal cultures were tested by STX ELISA and the results were compared with isolation of STEC. On the other hand, Ball et al. (19) found that 6 of 13 ELISA-positive pure isolates (4 *E. coli* strains, 1 *Citrobacter freundii* strain, and 1 *Enterobacter cloacae* strain) from fecal cultures were negative when subsequently tested for verocytotoxicity. Beutin et al. (34) also reported false-positive reactions with several strains of *Pseudomonas aeruginosa* using the Premier EHEC kit; this included one ATCC strain which had previously been tested as negative by the manufacturer.

The sensitivity of the various ELISAs is affected by a number of variables including the avidity of the antibodies used and the type and amount of STX produced by a given strain. ELISAs are generally less sensitive than the verocytotoxicity assay. Downes et al. (87) concluded that ELISA sensitivity was inadequate to reliably detect low levels of STX found in direct fecal extracts. However, the amount of free STX present in primary fecal cultures is generally greater, particularly when broths are supplemented with polymyxin B and mitomycin to enhance the release of Stx1 and Stx2, respectively. Under such circumstances, ELISAs were reported to be capable of detecting the

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presence of Stx1-producing organisms comprising less than 1% of total flora (15, 182) and Stx2-producing organisms at an abundance of less than 0.1% (182). Thus, under optimal conditions, STX ELISAs can provide a reliable primary screen for the presence of STEC strains (including non-O157 strains) in fecal cultures as long as the specimen is obtained fairly early in the course of infection. Studies of comparative specificity and sensitivity carried out to date indicate that commercially available STX ELISA kits are likely to be of considerable utility for laboratories without access to more specialized diagnostic procedures, particularly for detection of non-O157 STEC strains (5, 167, 202, 245, 247). However, reports of false-positive ELISA reactions indicate that independent confirmation of STX production or the presence of *STX* genes would be prudent.

A reverse passive agglutination test for the detection of STX production is also commercially available (Oxoid, Unipath Ltd., Basingstoke, United Kingdom). The test involves incubating serially diluted polymyxin B extracts of putative STEC cultures with Stx1- and Stx2-specific antibody-coated latex particles and observing for agglutination after 24 h. Beutin et al. (35) found that this assay detected toxin production (of the appropriate type) in strains containing stx_1, stx_2, and stx_{2c} but did not detect toxin produced by the strains carrying stx_{2e}. Since a number of Stx2 and Stx2c producers gave positive reactions only when undiluted extracts were tested (29), this method is not sufficiently sensitive for screening primary fecal cultures for the presence of STX-producing organisms.

Detection of *STX* Genes

Hybridization with DNA and oligonucleotide probes. The availability of cloned stx_1 and stx_2 genes enabled the development of DNA probes for the detection of STEC (227, 374). Initially, probes labelled with ^{32}P or ^{35}S were used for testing large numbers of fecal *E. coli* isolates or the direct screening of colonies on primary isolation plates for the presence of *STX* genes by colony hybridization (307, 345). These procedures were both highly sensitive and specific, and when stringent washing conditions were used, strains carrying stx_1, stx_2, or both could be differentiated. However, radioactively labelled probes had disadvantages for clinical laboratories, such as delays due to the need for long autoradiographic exposures, short probe half-life, and the problems associated with handling and disposal of radioisotopes. These problems have been largely overcome by the introduction of nonradioactive labels such as digoxigenin and biotin, and *STX* probes that use these have been used for detection of STECs without loss of sensitivity or specificity (345). The availability of nucleotide sequence data for *STX* genes has also permitted the design of synthetic oligonucleotide probes for detection of STEC (51, 152). Some oligonucleotide probes were based on sequences which are highly conserved among the various toxin genes and hence permitted detection of all types. Other probes were directed against less highly conserved regions, which, under the appropriate hybridization and washing conditions, distinguished between stx_1, stx_2, and stx_{2e} genes (51). Although hybridization with DNA or oligonucleotide probes is not a particularly sensitive means of screening broth cultures or fecal extracts for the presence of STEC, it is a powerful tool for distinguishing colonies containing *STX* genes from commensal organisms (discussed below).

PCR. Access to sequence data for the various *STX* genes has also permitted the design of a variety of oligonucleotide primer sets for amplification of *STX* genes by PCR. Crude lysates or DNA extracts from single colonies, mixed broth cultures, colony sweeps, or even direct extracts of feces or foods can be used as templates for PCR. *STX*-specific PCR products are usually detected by ethidium bromide staining after separation of the reaction mix by agarose gel electrophoresis. To date, some *STX* PCR assays have combined different primer pairs for stx_1 and stx_2, and in some cases stx_2 variants, in the same reaction, thereby directing the amplification of fragments which differ in size for each gene type (29, 50, 104, 147, 270). Other *STX* PCR assays use a single pair of primers based on consensus sequences. These primers are capable of amplifying all *STX* genes, with subsequent identification of the gene type requiring Southern or dot-blot hybridization with labelled oligonucleotides directed against type-specific sequences within the amplified fragment (153, 257, 276). Apart from increasing the sensitivity, secondary hybridization steps act as independent confirmation of the identity of the amplified product. Restriction fragment length polymorphism analysis of amplified portions of stx_2 genes has also been used to discriminate between stx_2 and stx_2 variants (188, 287, 349). In addition, PCR can be used for preparation of labelled DNA probes for use in hybridization reactions by amplification in the presence of, for example, digoxigenin-labelled nucleotides (140, 141).

The use of PCR technology permits the detection of *STX* genes from samples which are microbiologically complex (such as feces or foodstuffs), including samples containing nonviable organisms. PCR assays are potentially extremely sensitive; using serially diluted DNA extracted from an STEC isolate, Brian et al. (50) showed that amplification of less than 1,000 genomes resulted in visible stx_1 and stx_2 PCR products after ethidium bromide staining of agarose gels. When secondary Southern hybridization with a labelled probe was used to detect the PCR products, fewer than 10 *STX*-containing bacterial genomes per assay could be detected (50). In this study, the sensitivity was about 100-fold lower when the DNA template was prepared by direct extraction from feces seeded with known numbers of STEC. This was a consequence of the presence of inhibitors of *Taq* polymerase in the sample, which necessitated dilution before assay. Other studies have also shown suboptimal sensitivity when PCR is carried out directly on fecal extracts (274). Inhibitors of *Taq* polymerase are also present in meat. Begum and Jackson (28) showed that ground-beef homogenates had to be diluted 1,000-fold before assay. For both feces and food samples, the sensitivity of PCR assays is vastly increased if template DNA is extracted from broth cultures (28, 104, 257). Broth enrichment (which can involve as little as 4 h of incubation) serves two purposes; inhibitors in the sample are diluted, and bacterial growth increases the number of copies of the target sequence. Optimization of sensitivity is of paramount importance, because the number of STEC in the feces of patients with serious STX-related diseases or in suspected contaminated foodstuffs may be very small. Another consideration that may affect the performance of some PCR assays is the DNA sequence polymorphisms that are known to exist among *STX* genes; this is particularly so for stx_2-related genes, for which significant variation has been reported (106, 138, 189, 214, 242, 258, 259, 305). Sequence divergence between the primer and its target (particularly at the 3′ end of the primer) will significantly reduce the efficiency of annealing, with potentially dramatic effects on the sensitivity of the PCR reaction. When selecting or designing primers, care must be taken to avoid regions where sequence heterogeneity has been reported. PCR assays which use a single primer pair to amplify both stx_1 and stx_2 may be less susceptible to this potential complication. Target sequences which are conserved between otherwise widely divergent genes are likely to encode structur-

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ally important domains, and so random mutations will be strongly selected against.

The speed of diagnosis of STEC infection is also an important consideration in the clinical setting. The precise time required for a PCR assay varies with the amplification protocol itself (the number of cycles and incubation times at each temperature), the method used for DNA extraction, and the procedure for detection of the PCR products. The minimum time required for direct PCR analysis of an unenriched fecal sample analyzed by agarose gel electrophoresis could be as little as 4 to 6 h. Inclusion of a broth enrichment step and the use of a more sophisticated DNA purification procedure would increase this time to 12 to 24 h, while hybridization of PCR products with *STX* probes could add another day. The cumulative increase in sensitivity resulting from each additional step must be balanced against the increase in time; this equation will vary depending on the particular clinical or epidemiological context.

It has often been argued that PCR should be confined to reference laboratories, because it is labor-intensive and requires highly skilled staff. However, an increasing number of clinical laboratories are now routinely using PCR for a range of applications. The risk of cross-contamination of samples leading to false-positive PCR results can also be minimized by the physical separation of pre- and postamplification procedures and other precautions such as the use of plugged micropipette tips. Unlike the STX-specific antibodies and other specialized reagents needed for ELISAs, custom-made oligonucleotide primers are inexpensive and universally available and have a very long shelf life. Modern versatile PCR thermal cyclers are no more expensive than ELISA plate readers and can handle assays in 96-well format for laboratories that have a high specimen throughput. Moreover, a variety of alternatives to agarose gel electrophoresis have been developed for high-volume, sensitive, semi-automated detection of PCR products (for example, Q-PCR and TaqMan [Perkin-Elmer Applied Biosystems, Foster City, Calif.] and PCR-ELISA [Boehringer, Mannheim, Germany]).

PCR for detection of other STEC markers. PCR has also been used for the detection of genes encoding accessory virulence factors, such as *eaeA* and EHEC-*hlyA*, in STEC isolates (105, 300). This information may be of significance, because as discussed previously, there is a link between the presence of these genes and the capacity of an STEC isolate to cause serious human disease (21, 300, 301). Thus, a child presenting with acute diarrhea who is infected with a STEC isolate that is also positive for *eaeA* and EHEC-*hlyA* is likely to be at increased risk of developing complications such as HUS. Fratamico et al. (99) combined previously described *STX*-specific (153) and *eaeA*-specific (105) PCR primer pairs with those specific for a portion of the 60-MDa virulence plasmid from an O157:H7 STEC in a multiplex assay. They concluded that this assay was suitable for the identification of STEC strains belonging to serogroup O157. However, the O157 virulence plasmid primers actually recognize a portion of the EHEC-*hlyA* gene, which is not confined to serogroup O157. Thus, this particular multiplex PCR will also be capable of identifying a significant proportion of potentially virulent non-O157 STEC strains. Gannon et al. (103) have recently described two other multiplex PCR formats based on *STX*-specific and two distinct *eaeA*-specific primer sets, as well as a primer pair specific for a portion of the $fliC_{h7}$ gene, which encodes the H7 antigen. The two *eaeA*-specific primer sets recognized either the highly conserved 5′ portion of *eaeA* or a region at the (variable) 3′ end of the gene which was specific for O157 strains and a small number of other serogroups (105). Collectively, these assays could distinguish between EPEC and STEC strains, identify STEC strains that were likely to be of increased virulence, and identify those likely to belong to serotype O157:H7 (103). Another multiplex PCR to detect O157:H7 STEC uses stx_1- and stx_2-specific primer pairs in combination with primers that recognize a single-base mutation in the β-glucoronidase-encoding *uidA* gene (60). This mutation in *uidA* results in a β-glucoronidase-negative phenotype, a feature strongly associated with O157:H7 strains (discussed below).

While O157:H7 strains are the most common cause of serious STEC disease in Europe and North America, O111:H^- STEC strains are also a common cause of disease and in some regions (e.g., Australia) are the most frequent cause of HUS. Louie et al. (195) also exploited the heterogeneity of the 3′ end of *eaeA* to design two PCR assays, one of which was specific for *eaeA* from O157 STEC and O55 EPEC strains. The other primer pair gave a positive reaction with 16 of 22 O111 STEC strains but not with O111 EPEC strains or 72 STEC strains belonging to six other serogroups, including O157. Sequence heterogeneity of *eaeA* within O111 STEC is presumably responsible for the fact that six of these strains (all of which were O111:H11) were PCR negative even though they hybridized to a probe specific for the conserved region of *eaeA* (195). A more specific O111 PCR assay has recently been described which directly targets a group-specific portion of the O-antigen-encoding *rfb* locus of *E. coli* O111 (260); this region (designated ORF 3.4) is thought to encode the biosynthesis of GDP-colitose (25). We have also recently described two multiplex PCR assays for the detection and characterization of STEC. The first uses four primer pairs and detects the presence of stx_1, stx_2 (including variants of stx_2), *eaeA*, and EHEC-*hlyA*. The second assay combines the above O111 *rfb*-specific primers with primers directed at a type-specific portion of the O157 *rfb* locus (the *rfbE* gene); the latter primer pair does not give a false-positive reaction with *E. coli* O55 sequences (255).

Isolation of STX-Producing Bacteria

Although a substantial amount of information on the causative STEC strain can be obtained by molecular analysis of mixed cultures, isolation of the STEC strain must be considered the definitive diagnostic procedure. Apart from confirming the molecular data, isolation permits additional characterization of STEC by a variety of methods, including O:H serotyping, phage typing, restriction fragment length polymorphism, pulsed-field gel electrophoresis, and amplification-based DNA typing. While this characterization may have limited clinical application, it is of great importance from an epidemiological point of view, particularly in an outbreak setting.

Culture and immunological methods for O157 STEC. For many years, sorbitol-MacConkey agar culture (SMAC) has been the most commonly used method for isolation of STEC, because of the predominance of O157:H7 and O157:H^- strains as etiological agents of human disease in North America and Europe. Most of these strains are unable to ferment sorbitol, which distinguishes them from the majority of fecal *E. coli* belonging to other serotypes (178, 205, 370). Sorbitol-MacConkey agar plates are inoculated with the fecal specimen and examined after 18 to 24 h of incubation for the presence of colorless, sorbitol-negative colonies. Individual colonies are tested by slide or tube agglutination with O157 and H7 antisera. Several commercial latex reagents for O157 antigen and one for H7 antigen are also available and have been shown to be both accurate and sensitive compared with standard serological tests (320). It is still necessary to confirm STX production in tissue culture or ELISAs (as discussed above), since not all O157 strains are toxin producers.

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The sensitivity of SMAC is limited by the capacity to recognize nonfermenting colonies against the background of other organisms on the plate; this is particularly difficult when the O157 strain forms less than 1% of the flora. However, Chapman et al. (65) improved the isolation rate of O157 STEC by supplementing SMAC with cefixime, to inhibit *Proteus* spp., and rhamnose, which is fermented by most sorbitol-negative non-O157 *E. coli* strains (O157 strains generally do not ferment rhamnose). Zadik et al. (381) have reported a further improvement in O157 isolation rates by using SMAC supplemented with cefixime and potassium tellurite (CT-SMAC). Although screening fecal cultures on SMAC is inexpensive and involves minimal labor and equipment, it will primarily detect STEC belonging to serogroup O157. Serious STEC disease has been associated with many other serogroups (147), and although some are sorbitol negative (241), most are sorbitol positive. Thus, the efficacy of SMAC will vary in accordance with the local STEC serotype prevalence. In one study, SMAC resulted in the isolation of *E. coli* O157 from 80% of fecal samples which were positive for STX by direct cytotoxicity (283), whereas in another study, SMAC was positive for only 30% of verocytotoxin-positive samples (273). An additional consideration is that Stx2-producing, sorbitol-positive *E. coli* O157:H$^-$ isolates have been associated with both outbreaks and sporadic cases of HUS in Germany. In one study, 17 of 44 HUS patients had Stx-producing *E. coli* O157 strains that were sorbitol fermenters (116). Such strains have also recently been isolated from patients with HUS in the Czech Republic (40). Moreover, these strains are very sensitive to tellurite, which argues strongly against the use of CT-SMAC for isolation of STEC.

Another biochemical feature that distinguishes O157:H7 STEC strains from other *E. coli* strains is failure to produce β-D-glucuronidase (178, 275). This enzyme can be readily detected fluorigenically with the substrate 4-methylumbelliferyl-β-D-glucuronide or colorimetrically on plates supplemented with 5-bromo-6-chloro-3-indolyl-β-D-glucuronide. Thompson et al. (346) examined 188 serogroup O157 strains by this method; 166 isolates were glucuronidase negative and STX positive, while the remaining 22 isolates were all glucuronidase positive and STX negative. Again, this method is not useful in cases of STEC-related disease where the causative organism is of a serogroup other than O157. Moreover, the 17 sorbitol-positive, Stx2-producing O157 isolates from HUS patients described by Gunzer et al. (116) were also glucuronidase positive.

Recently, a commercial agar medium (Rainbow Agar O157 [Biolog Inc., Hayward, Calif.]) containing selective agents for *E. coli* and chromogenic substrates for β-D-glucuronidase and β-galactosidase has become available for the isolation of STEC. Glucuronidase-negative, galactosidase-positive O157 strains appear as black colonies on this medium, whereas commensal *E. coli* strains are pink. It has also been claimed that some non-O157 STEC strains overproduce β-galactosidase relative to β-D-glucuronidase on this medium, giving the colonies a distinctive intermediate color. To date, analyses of the efficacy of this medium for the detection of either O157 or non-O157 STEC have been confined to testing of foods, particularly raw-meat samples and fruit juices (135, 295), and its utility for clinical diagnosis has yet to be reported. Again, it should be emphasized that isolation of a putative O157 strain is not a definitive diagnosis in itself, since isolates must be tested to confirm STX production.

Park et al. (246) have used direct immunofluorescent staining of fecal specimens with polyclonal anti-O157–fluorescein isothiocyanate as an alternative to SMAC for the detection of *E. coli* O157. This was at least as sensitive as SMAC and moreover involved only a 2-h turnaround time. Commercial ELISAs for rapid detection (less than 1 h) of the presence of O157 antigen in fecal specimens are also available (LMD and Premier *E. coli* O157 [Meridian Diagnostics, Inc.]). Both of these ELISAs have similar sensitivity to SMAC (202, 248) and, importantly, are capable of detecting sorbitol-fermenting O157 STEC strains, should they be present. Both the immunofluorescence assays and ELISAs, however, require confirmation by culture or by demonstration of STX in the sample.

Culture methods for non-O157 STEC. As discussed above, *E. coli* strains belonging to a large range of serotypes, as well as other bacterial species, can produce STX and cause disease in humans. Regrettably, there is no definitive biochemical characteristic which distinguishes STEC strains belonging to serogroups other than O157 from commensal fecal *E. coli* strains, a fact which significantly complicates the isolation of such organisms. As discussed, however, nearly all O157 STEC strains and a significant proportion of non-O157 STEC strains produce EHEC-Hly. These organisms can be distinguished phenotypically from alpha-hemolytic strains. Strains producing EHEC-Hly are not hemolytic on standard blood agar but produce small, turbid hemolytic zones on washed sheep RBC agar (supplemented with Ca^{2+}) after 18 to 24 h of incubation at 37°C. Alpha-hemolytic strains form large, clear zones of hemolysis on standard blood agar or washed sheep RBC agar plates after only 4 to 6 h of incubation at 37°C. Production of EHEC-Hly has a high positive predictive value, since Beutin et al. (33) found no enterohemolytic strains among 267 fecal *E. coli* isolates which did not produce STX. However, the predictive value of a negative EHEC-Hly result is low. Schmidt and Karch (301) investigated the enterohemolytic genotypic and phenotypic profiles of 36 O111:H$^-$ STEC strains isolated from patients with HUS and diarrhea. Twenty strains were positive for EHEC-Hly; two additional strains were positive for EHEC-*hlyA* by DNA hybridization and PCR but were not hemolytic. The remaining 14 O111:H$^-$ STEC strains were negative by both DNA hybridization and plating. The fact that a significant proportion of disease-causing STEC strains (44% in this particular study) are EHEC-Hly negative diminishes the usefulness of washed sheep RBC agar as a "generic" screen for primary isolation of STEC.

Comprehensive isolation of STEC. The only comprehensive means of isolating STEC or other STX-producing organisms involves direct analysis of colonies on agar plates with either nucleic acid probes or antibodies, and a variety of protocols for this purpose have been described (129, 146, 216, 260, 266). This is a labor-intensive process and should be carried out only for specimens which have tested positive in screens for STX (by cytotoxicity or ELISA) or for *STX* (by PCR). Colonies from agar plates are directly blotted onto a suitable membrane (e.g., nitrocellulose for immunoblots or positively charged nylon for hybridization). A carefully aligned replicate of the filter is made and then processed and reacted with an antibody or nucleic acid probe by standard procedures. Theoretically, up to several hundred discrete colonies can be tested on a single filter, although this may require dilution and replating of primary cultures. Alternatively, colonies from primary isolation plates can be selected and inoculated into 96-well microtiter trays containing broth. This is a time-consuming step (15 to 20 min per tray), but the 96-well format enables the subsequent use of semi-automated machinery to make replicate copies of trays and, after incubation, to transfer aliquots onto appropriate filters; the trays are also convenient for preservation of the isolates at −70°C. Hull et al. (129) have directly compared the sensitivity and specificity of immunoblotting and DNA probing for the detection of STEC colonies. Cytotoxicity testing indi-

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cated that if the results disagreed between the two methods, it was almost always a consequence of a false-positive immunoblot reaction. This was more prevalent when polyclonal rather than monoclonal antibodies were used. When measured against DNA probing, the immunoblot had a sensitivity of 88% and a specificity of 89% for the identification of STX-positive colonies. Occasional false-positive results with colony immunoblotting have also been reported by Milley and Sekla (216). Immunoblot techniques have the additional disadvantage of having to grow colonies on special media in order to optimize the production and/or release of Stx (129, 216).

Immunomagnetic separation for isolation of STEC. Immunomagnetic separation (IMS) techniques have been developed to assist in the isolation of STEC (principally O157) from low-abundance specimens. The procedure involves coating magnetic beads with anti-LPS antibody and mixing them with broth cultures or suspensions of feces or suspect food homogenates. The beads and bound bacteria are then trapped in a magnetic field, the unbound suspension is decanted, and the beads are washed. After additional binding and washing cycles, the beads are plated and the resultant colonies are tested for reactivity with the appropriate O antiserum and, more importantly, for STX production. Several studies have examined the utility of a commercial magnetic bead reagent (Dynabeads anti-*E. coli* O157 [Dynal, Oslo, Norway]) for the isolation of *E. coli* O157. IMS was 100 times more sensitive than direct culture on either cefixime-rhamnose SMAC or CT-SMAC for isolation of *E. coli* O157 from bovine feces (66). It was also more effective than either plate medium for isolation from human fecal samples (64). As might be expected, the enrichment procedure is not 100% efficient, and a number of sorbitol-nonfermenting, non-O157 bacterial strains (of several species) also adhered to the beads. The most comprehensive investigation of the efficacy of IMS for isolation of *E. coli* O157 is that of Karch et al. (151). IMS allowed the detection of O157 strains at 10^2 CFU per g of feces in the presence of 10^7 coliform background bacteria. When tested on fecal samples from HUS patients, IMS followed by culture on CT-SMAC resulted in the isolation of *E. coli* O157 in 18 of 20 samples, compared with only 12 by colony hybridization and 7 by direct culture on CT-SMAC. The principal drawback of IMS is, of course, its serogroup specificity. However, it is an extremely valuable enrichment technique, particularly in circumstances where STEC strains of known serogroups are being deliberately targeted, for example when testing foods suspected of being the source of an outbreak. During an outbreak of HUS, we isolated STEC strains belonging to serotype O111:H$^-$ from the feces of 13 HUS patients and from cultures of two samples of fermented sausage suspected of being the source of the outbreak (260). Cultures from an additional five HUS patients and three sausage samples were weakly positive by PCR for both *STX* and O111-specific sequences, suggesting the presence of small numbers of the causative organism. However, attempts to isolate O111:H$^-$ STEC by colony hybridization were unsuccessful even after up to 1,000 colonies from each sample were tested. Magnetic beads were therefore coated with anti-O111 LPS and used to enrich the cultures by IMS, which enabled the isolation of the causative O111:H$^-$ STEC from three of the previously negative fecal cultures and two more of the sausage cultures (260).

Serological Diagnosis of STEC Infection

Diagnosis of STEC-related disease can be particularly problematic when patients present late in the course of disease, because the numbers of STEC in feces may be extremely small and hence undetectable even by PCR. In such circumstances, the etiology of infection may be established by serological means. Antibodies to STX or LPS have been proposed as markers of recent infection, but unless geographically matched and age-matched baseline data for the healthy population are available, caution must be exercised when interpreting results for single serum specimens. Ideally, acute- and convalescent-phase sera should be tested for rising or falling antibody titers. Barrett et al. (20), using ELISAs specific for antibodies to Stx1, Stx2, and O157 LPS, studied serological responses from 83 patients linked to two outbreaks of *E. coli* O157:H7 diarrhea and from 66 healthy controls. Only about 25% of patients associated with the first outbreak (caused by a strain producing Stx1 and Stx2) had antibodies to Stx1, but 11% of control sera were also positive for anti-Stx1, as were 14% of sera from patients associated with the second outbreak, which was caused by a strain producing Stx2 only. Antibodies to Stx2 were not detected in any of the patient or control sera. However, Yamada et al. (378) found that sera from five of six HUS patients tested had detectable antibody levels to Stx2 (measured by ELISA). The higher rate of seroconversion to Stx2 in this study than in that of Barrett et al. (20) may reflect increased immune responses in patients with severe systemic disease compared with those in patients with uncomplicated diarrhea. Karmali et al. (159) found that 7.7% of 790 sera from healthy controls in Toronto were seropositive for Stx1 in a tissue culture cytotoxicity neutralization assay; seropositivity was age related and peaked at 19% in the 20- to 30-year age group. Smaller samples of control sera from Dutch, Japanese, and Indian populations had overall Stx1 seropositivity rates ranging from 1 to 10%. Karmali et al. (159) also found that only a minority of patients infected with a Stx1-producing organism were seropositive. In both the above studies, similar results were obtained by using either ELISAs or cytotoxicity neutralization assays. Recently, Reymond et al. (278) described a Western immunoblot assay for Stx1 antibodies which was more specific and sensitive than either ELISA or cytotoxicity neutralization methods and proposed this as a more useful tool for seroepidemiological studies. However, an earlier study found no antibodies to Stx1 or Stx2 in sera from 30 HUS patients by using either ELISA or Western immunoblot methods (67). Thus, it appears that serological responses to STX are unlikely to be helpful as a diagnostic test.

More encouraging results were obtained by testing for antibodies to LPS, although this diagnostic approach suffers from the disadvantage of being able to target only specified serogroups. Barrett et al. (20) reported that 52% of patients associated with the two O157 outbreaks referred to above were ELISA positive for O157 LPS antibodies, and if only culture-confirmed cases were considered, this rate increased to 92%. Moreover, the background seropositive rate in healthy controls was lower for O157 LPS (3%) than for Stx1 (11%). Chart et al. (68) also showed that 44 of 60 sera from HUS patients were ELISA positive for anti-O157, including 9 of 9 sera from patients from whom O157 STEC was cultured; none of 16 control sera contained anti-O157. Similar findings have also been reported by Greatorex and Thorne (113).

Bitzan et al. (42, 43) described passive hemagglutination assays for anti-LPS involving sheep erythrocytes coated with LPS from a range of serogroups including O157. They demonstrated that 22 of 27 acute-phase sera from patients with HUS had high anti-O157 titers (>1:4,096) but negligible titers against the other serogroups tested. In contrast, sera from only 4 of 249 healthy individuals were positive, mostly with titers ranging from 1:4 to 1:256 (42). Yamada et al. (378) also demonstrated the utility of anti-LPS passive hemagglutination as-

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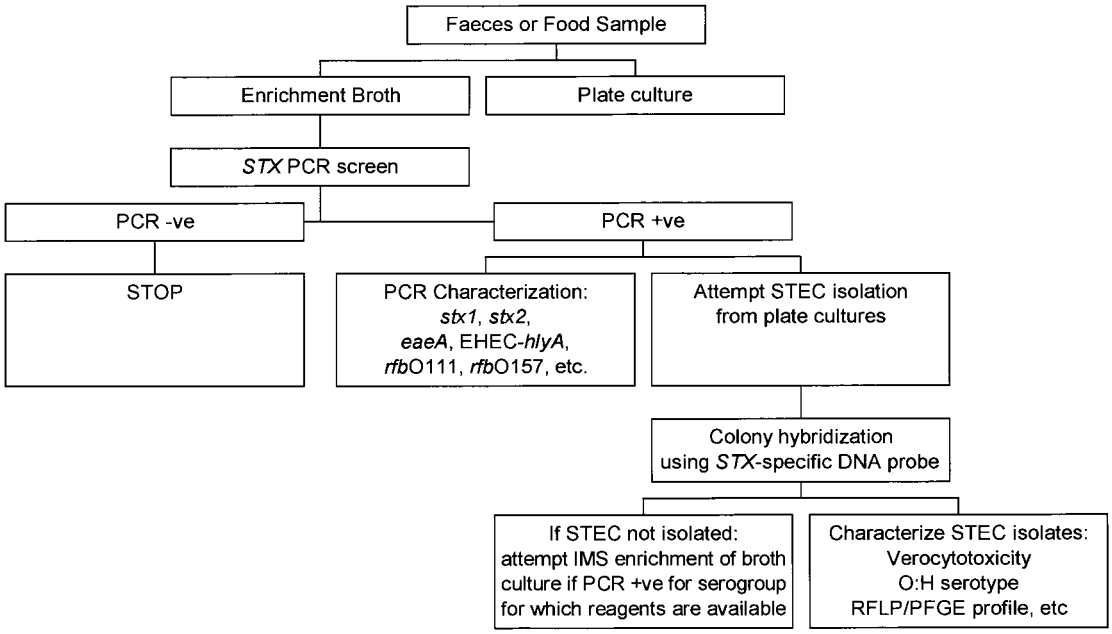

FIG. 1. Flow chart for the molecular microbiological diagnosis of STEC infection.

says specific for serogroups O26, O111, O128, and O157 for establishing a diagnosis of STEC infection in HUS patients from whom STEC had not been isolated. Acute-phase sera from all eight patients tested were positive for anti-O157. Several of the above studies showed that anti-LPS titers fell rapidly during the immediate post-acute phase, and so elevated titers in a single specimen may indeed be a reliable indicator of current or very recent infection (42, 43, 68, 378).

The utility of serodiagnosis is not confined to O157 infections. Caprioli et al. (57) used Western blotting to demonstrate the presence of antibodies to O111 LPS in sera from a cluster of HUS patients in northern Italy thought to constitute a common source outbreak. We have also used Western blotting to demonstrate the presence of anti-O111 in sera from three HUS patients linked to an outbreak caused by fermented sausage contaminated with STEC O111:H$^-$ (260). Unlike the other 18 HUS patients in this outbreak, attempts to demonstrate the presence of viable O111:H$^-$ STEC in fecal cultures, either by isolation or by PCR for *STX* and an O111-specific portion of the *E. coli rfb* region, were unsuccessful. Interestingly, two patients associated with this outbreak yielded both O111:H$^-$ and O157:H$^-$ STEC isolates and sera from these individuals were positive for both O111 and O157 antibodies.

Strategies for STEC Diagnosis and Surveillance

Ultimately, decisions about the selection of STEC diagnostic methods will involve striking a balance between speed, specificity, sensitivity, and cost of the alternatives. Ideally, clinical microbiology laboratories should screen all fecal samples for which bacterial culture is requested for the presence of STEC, not just samples which are bloody. A comprehensive Belgian study involving *STX* PCR screening of all 10,241 samples received for analysis over a 3-year period found that STEC strains were the third most prevalent bacterial pathogens detected (after *Salmonella* spp. and *Campylobacter* spp.) (267). STEC strains were much more commonly detected than either *Yersinia* spp. or *Shigella* spp. The importance of universal screening by methods which are not serogroup restricted is emphasized by the fact that in this study, non-O157 STEC strains were three times more prevalent than O157 strains. For both O157 and non-O157 STEC strains, the majority of isolates were from patients with uncomplicated, apparently nonbloody diarrhea. The probability of isolating the causative STEC strain from patients with severe disease such as HUS is also significantly greater during the diarrheal prodrome. Comprehensive screening would also provide early warning of outbreaks of infection with highly virulent STEC strains, since a cluster of HUS cases would invariably be preceded by a much larger cluster of cases of diarrhea. Such early warning would undoubtedly facilitate epidemiological and further microbiological investigations, increasing the likelihood of identifying the source in time to prevent additional, potentially life-threatening cases. Comprehensive screening would also provide an accurate estimate of the extent of endemic disease and provide a bench mark for monitoring the efficacy of intervention programs aimed at lowering the overall incidence.

PCR analysis of primary fecal cultures is probably the most sensitive and specific means of screening for the presence of STEC and other *STX*-carrying bacteria, and a flow chart for a molecular microbiological diagnostic workup based on this is shown in Fig. 1. For laboratories that lack PCR capability, direct screening of fecal extracts or cultures for verocytotoxicity, although slower, is a highly satisfactory alternative. Although methods such as STX ELISA, SMAC, EHEC-Hly detection, etc., are useful in certain circumstances, they are suboptimal as stand-alone primary screens for reasons relating to either poor sensitivity or inability to detect all STEC strains. Nevertheless, it is preferable for laboratories that have neither PCR nor tissue culture facilities to screen fecal samples by a combination of at least two of the other methods (e.g., STX ELISA plus SMAC, or STX ELISA plus washed sheep RBC agar) than not to screen at all. It would also be prudent, however, for such laboratories to refer negative specimens from suspected HUS cases to a reference laboratory.

All samples and cultures which test positive after screening should be sent to a reference laboratory for confirmation and

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attempted isolation of STEC if adequate resources are not available on-site. Given the widespread instability of *STX* genes during subculture (154), it is important that initial samples and primary cultures be referred in addition to putative STEC isolates. It is at the isolation stage where use of the specialized plate media referred to above may save time by directing attention to suspect colonies, particularly where they are in low abundance. However, if such media rather than nonselective plates are used, it is essential to test a range of colony types and not just those with the STEC-associated phenotype. Given the sensitivity of PCR screens, a proportion of genuine STEC-positive specimens may not yield an isolate even after heroic attempts. It may still be possible to obtain meaningful additional information about the causative organism in such circumstances. PCR analysis will indicate the toxin type and whether virulence-related genes such as *eaeA* and EHEC-*hlyA* or genes associated with important serogroups (O157 and O111) are also present in the sample. However, the interpretation of this information is complicated by the possibility that the composite genotypic profile represents the sum of genotypes of more than one STEC organism. At least for cases of HUS, information on the likely infecting serogroup can also be obtained by serological tests for anti-LPS antibodies.

Special considerations for testing of foods. Methods for the detection of STEC in foods have focused upon serogroup O157, because this was the cause of all reported major outbreaks until the early 1990s. The infectious dose of the O157:H7 STEC strain responsible for the large outbreak in the western United States in 1993 has been estimated to be of the order of 10 to 100 CFU (114). *E. coli* O157:H7 has justifiably been declared an adulterant by regulatory authorities in the United States, and as such there is a zero tolerance for this particular STEC organism in foods.

However, food-borne outbreaks of Stx-related disease (including HUS) have now been unequivocally attributed to STEC strains belonging to other serogroups, including O111 (57, 260) and O104 (63), and to *Citrobacter freundii* (348). Furthermore, outbreaks of STEC disease are not necessarily caused by contamination with a single "outbreak" strain and may indeed be microbiologically complex (260). There is also no reason to believe that sporadic cases of STEC disease, which have been associated with a vast array of STEC serogroups, are not principally food-borne infections. Both the food industry and regulatory authorities need to be cognizant of the potential hazards posed by all these bacteria, particularly as contaminants of foods destined for human consumption without further cooking or processing. When such foods are being analyzed for the presence of STEC, it is essential to use the most sensitive methods. Analysis of the food source responsible for the outbreak of HUS in South Australia in 1995 referred to above suggested that there may have been as little as 1 O111:H^- STEC organism per 10 g, implying that the infectious dose for a susceptible child may be of the order of 1 to 10 organisms. Of note, the food source was identified only by PCR analysis of enrichment cultures.

There is, however, a problem with interpretation of the significance of the presence of any STX-producing organism in a food. While any such organism is likely to be of clinical significance when detected in feces of symptomatic patients, foods may contain STEC strains that have yet to be associated with human disease. The properties which distinguish these strains from human-virulent STEC are not completely understood. Clearly, STEC strains which are positive for *eaeA* (and perhaps also EHEC *hlyA*) should be regarded as highly significant, but a proportion of serious human infections, including HUS, are caused by STEC strains which are *eaeA* and/or EHEC *hlyA* negative. Until definitive criteria are established, we propose that all STEC-contaminated foods be considered potentially hazardous.

PROSPECTS FOR TREATMENT AND PREVENTION OF STEC INFECTION

Specific Therapeutic Intervention

Intensive supportive therapy to maintain homeostasis (e.g., peritoneal dialysis or hemodialysis, fluid balance, and treatment of hypertension) is, of course, the mainstay of management of HUS. However, the availability of rapid and sensitive methods for the diagnosis of STEC infection early in the course of disease has created a window of opportunity for additional specific therapeutic intervention. The objectives of therapeutic strategies would be threefold: (i) to limit the severity and/or duration of gastrointestinal symptoms, (ii) to prevent life-threatening systemic complications such as HUS, and (iii) to prevent the spread of infection to close contacts.

Antibiotics. Antibiotic therapy might be expected to satisfy all three of the above goals. However, doubts have been raised as a consequence of retrospective studies of its efficacy in preventing the progression of STEC infection from diarrhea or bloody diarrhea to HUS. Such analyses have been compounded by variations in the types of antibiotics used, the timing of commencement of therapy in relation to onset of symptoms, and the possibility that the severity of disease may have influenced the decision to implement therapy. Nevertheless, the bulk of these studies suggested either that there was no significant benefit associated with administration of antibiotics or that therapy (either during or preceding infection) actually increased the risk of developing HUS (59, 70, 264, 282). However, in one study, HUS patients who had been given antibiotics during the diarrheal prodrome had milder illness (206). Examination of antibiotic use in two recent large O157:H7 STEC outbreaks in Scotland and Japan have also produced conflicting findings. Stewart et al. (323) found a significant association between prior antibiotic usage and subsequent development of HUS. On the other hand, Takeda et al. (333) found that the proportion of patients who progressed from bloody diarrhea to HUS was significantly lower when antibiotics had been administered within 3 days of the onset of symptoms, compared with untreated patients or those given antibiotics later in the course of infection. Very few prospective studies have been performed, but Proulx et al. (271) found that administration of trimethoprim-sulfamethoxazole to patients infected with O157 STEC (albeit late in the course of infection) did not prevent progression to HUS.

Apart from the lack of unequivocal evidence for clinical benefit, there are theoretical arguments against the use of antibiotics. First, although STX is extracellular, much of the toxin remains associated with the STEC cell surface. Thus, antibiotics which result in cell lysis might actually increase the amount of free STX in the gut lumen available for systemic absorption. Moreover, in vitro studies have shown that treatment of O157:H7 STEC with subinhibitory concentrations of antibiotics results in a significant increase (up to 50-fold) in the amount of free STX in the culture medium (364, 376). The effect was most pronounced with antibiotics such as trimethoprim-sulfamethoxazole and ciprofloxacin, which interfere with bacterial DNA synthesis, and correlated with increased induction of toxin-converting bacteriophages (376). Second, Cordovez et al. (75) noted a high rate of antibiotic resistance amongst STEC, and so empirical treatment with an

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inappropriate drug might confer a selective advantage on the STEC over other members of the gut flora and cause overgrowth. The same risk/benefit considerations are also relevant when considering whether to administer antibiotics either to asymptomatic STEC carriers to limit the spread of infection or to uninfected close contacts of patients with proven infection to prevent acquisition. Indeed, the case for prophylaxis is weakened by reports of patients becoming infected with O157:H7 STEC while undergoing therapy for an unrelated condition with an antibiotic to which the STEC was sensitive (334). More extensive randomized controlled trials are required to determine whether there is a role for antibiotic prophylaxis or therapy in STEC disease.

There are also sound reasons for not administering antimotility agents to patients with STEC diarrheal disease, since these would be expected to impede the elimination of STEC from the gut and thereby extend the exposure to STX. Indeed, retrospective analyses have shown that administration of these agents to patients with O157:H7 infection extended the duration of bloody diarrhea and increased the risk of developing HUS and central nervous system lesions (30, 69). At present, the risks or benefits of administration of other antidiarrheal agents such as kaolin or bismuth are not known.

Therapeutic strategies directed against STX. As discussed above, STX is principally responsible for the clinical manifestations of STEC infection, particularly the serious systemic complications. Thus, in vivo binding or neutralization of STX is an attractive therapeutic alternative, particularly in view of the possible risks associated with the use of antibiotics. Such strategies have the potential to limit the severity or duration of disease but would not, of course, be expected to reduce STEC transmission. Therapeutic strategies exploiting the high degree of specificity and the strength of the interaction between STX and its glycolipid receptor are currently being developed. One particularly promising agent is Synsorb-Pk (Chembiomed, Edmonton, Canada), which consists of the oligosaccharide component of Gb_3 (Galα[1→4]Galβ[1→4]Glc-) covalently linked via an 8-carbon spacer to silica particles derived from diatomaceous earth (11). Synsorb-Pk was capable of binding and neutralizing Stx1 and Stx2 in polymyxin B extracts of STEC cultures and in feces from patients with HC and/or HUS. A phase I clinical trial did not detect any adverse effects associated with oral administration, and Synsorb-Pk retained its STX binding capacity even after passage through the human gastrointestinal tract (13). Armstrong et al. (13) have proposed that if treatment with this agent is started early enough in the course of STEC diarrhea, the major portion of free STX in the gut lumen will be bound, thereby limiting further systemic absorption and subsequent development of HUS. Compounds capable of binding STX in the gut might also play a role as an adjunct to antibiotic therapy. Preliminary studies with streptomycin-treated mice have shown that while oral Synsorb-Pk did not protect the mice from oral challenge with highly virulent STEC, it did delay death by 1 day (286). Randomized, double-blind trials of Synsorb-Pk versus placebo, aimed at determining the clinical efficacy of Synsorb-Pk in humans with STEC infection, are in progress.

A likely limitation of Synsorb-Pk or other orally administered agents is that they would not be expected to be active against Stx that has already been absorbed from the gut. Although absorption of toxin into the blood has never been directly demonstrated in humans or animals, it is inferred by the generation of vascular lesions at remote sites. Thus, parenterally administered agents may have advantages, particularly since the total amount of absorbed toxin is only a minute fraction of that present in the gut lumen. Recent studies indicate that water-soluble receptor analogs may be suitable for this purpose. Boyd et al. (46) synthesized a galabiosyl analog dimerized via the *meta* position of benzene, which is capable of blocking both Gb_3 binding sites on the Stx1 B subunit. The analog also protected Vero cells against Stx1 cytotoxicity and to a lesser extent against Stx2.

As described above, parenteral administration of monoclonal anti-Stx2 antibody protects mice from fatal renal damage following oral challenge with STEC. Clearly, murine Igs are not suitable for parenteral administration to humans. However, anti-Stx1 and anti-Stx2 monoclonal antibodies have now been "humanized" by cloning variable-region cDNA from the mouse hybridoma cell lines into a mammalian expression vector, such that chimeric mouse variable region/human IgG1/kappa antibodies are produced (92). In vitro characterization of these antibodies is in progress. An alternative to the humanized monoclonal antibodies that could be considered is hyperimmune human Ig from donors who have been vaccinated with STX toxoid, once such antigens become licensed for use.

A common feature of all these proposed therapeutic agents is that efficacy is likely to depend upon early commencement of treatment, i.e., before significant amounts of STX have been absorbed from the gut. Indeed, preliminary analysis of clinical trials of Synsorb-Pk suggests that therapy may have to begin within 3 days of onset of symptoms (12). This underscores the need for rapid and sensitive diagnostic procedures. However, such agents may also be valuable in the outbreak setting, where persons are known to have been exposed to an implicated vehicle, or in settings such as child care centers or nursing homes, where there is a high risk of secondary transmission from a proven sporadic or index case. In these circumstances, a high proportion of the at-risk population could receive treatment even before any symptoms become apparent and thereby gain maximum benefit from the therapeutic agent.

Immunization against STEC Disease

There are a variety of ways in which immunization strategies might be used to reduce the incidence and/or effect of STEC infection in humans. One option is to vaccinate livestock to reduce the STEC load and minimize entry into the human food chain. However, this would require vaccination of a very large number of animals and the identification of STEC factors responsible for colonization of livestock; at present these are poorly understood. Options for human vaccination include blockade of the transmission or acquisition of STEC infection and interruption of the pathogenic process. Strategies for achieving these are discussed below.

Vaccines based on STX. Vaccines based on STX are likely to be effective in preventing HUS in persons infected with STEC and may also have a significant impact on the severity of gastrointestinal symptoms. This confidence is underpinned by the results of studies of the protective efficacy of vaccines based on Stx2e for protection of pigs from edema disease. MacLeod and Gyles (203) demonstrated that immunization with Stx2e toxoided with glutaraldehyde protected piglets from intravenous challenge with an otherwise lethal dose of purified toxin. Gordon et al. (112) found that Stx2e toxoided with formaldehyde was immunogenic in piglets but that there appeared to be residual in vivo toxicity. However, adverse reactions were not observed when piglets were immunized with an immunogenic derivative of Stx2e with a defined amino acid substitution (Glu167→Gln) in the active-site region of the A subunit. Immunization with this "genetic toxoid" has recently been shown to protect piglets from edema disease after oral challenge with a Stx2e-producing strain of *E. coli* (45).

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Clark et al. (71) described a derivative of Stx1 with a Phe30→Ala substitution in the B subunit, which exhibits reduced binding to Gb_3 and is 10^5-fold less cytotoxic for Vero cells than is native Stx1. This potential vaccine antigen has recently been shown to be highly immunogenic in rabbits, but marked systemic toxicity was observed when larger doses of antigen were administered, indicating that the Phe30→Ala substitution had a much smaller effect in vivo than in vitro (22). The authors suggested that differences between lipid moieties of Gb_3 from Vero cells and rabbit tissues might account for their finding and concluded that additional mutations would be required to reduce the toxicity to acceptable levels for use in human vaccines. Interestingly, a similar discrepancy between effects on Vero cell cytotoxicity and LD_{50} was previously observed for amino acid substitutions in a similar region of the Stx2 B subunit (191).

An alternative means of circumventing the problem of residual toxicity is to use just the B subunit as an immunogen. Several B subunit-specific Stx/Stx1-neutralizing monoclonal antibodies have been prepared (326), and neutralizing polyclonal antibodies were isolated from hyperimmune sera by affinity chromatography with immobilized B subunit (85). Boyd et al. (48) demonstrated that immunization of rabbits with purified Stx1 B subunit confers protection against challenge with an otherwise lethal intravenous dose of purified Stx1.

Recently, much interest has centered upon the vaccine potential of live-attenuated enteric bacteria expressing foreign antigens. These vaccine strains are capable of limited invasion of the gut mucosa, and they deliver the foreign antigens directly to Peyer's patches, resulting in strong humoral and secretory immune responses. Su et al. (328) described the construction of fusions of the complete *stx* B-subunit gene, or N-terminal segments thereof, with the gene for the *E. coli* OMP LamB. The *stx* sequences were inserted at a point in the *lamB* gene which encoded a surface-exposed loop of the protein. These constructs directed the expression of the hybrid LamB/StxB protein on the surface of *E. coli* K-12 and wild-type *Salmonella typhimurium*. However, when attenuated *S. typhimurium aroA* vaccine strains were used, the recombinant antigen was confined to the cytoplasm, presumably a consequence of membrane export defects in these strains. In spite of this, oral immunization of mice with these latter constructs resulted in the production of significant levels of Stx B subunit-specific antibodies, both in serum and in intestinal washings. Acheson et al. (3) have also expressed $stxB_1$ in a *Vibrio cholerae* vaccine strain. Sera from rabbits immunized orally with this strain contained significant levels of Stx1 B-subunit-specific antibodies, which were capable of neutralizing Stx1 holotoxin in vitro. There was also evidence of protection against enterotoxic effects of purified Stx1 in ileal loops constructed from immunized animals.

It is presumed that human anti-STX vaccines will need to be bivalent to provide protection against both Stx1- and Stx2-producing STEC strains. Polyclonal antisera raised against Stx/Stx1 do not neutralize the verocytotoxicity of Stx2 and vice versa (308, 327). Interestingly, however, Bielaszewska et al. (38) recently reported evidence for cross-neutralization in vivo in a rabbit model of STX toxemia. As expected, sera from rabbits immunized with either Stx1 or Stx2 toxoids did not neutralize the heterologous toxin but there was a degree of cross-reaction as judged by ELISA. Moreover, when rabbits were challenged with homologous or heterologous ^{125}I-labelled holotoxins, both toxins were cleared by the liver and spleen rather than being directed to the usual Gb_3-containing target tissues (the brain and the gastrointestinal tract), which occurs in unimmunized animals. This cross-protective property appeared to be mediated by the A subunit. Thus, inclusion of both Stx1 and Stx2 antigens may be more important for B-subunit vaccine formulations.

Vaccines to prevent gut colonization by STEC. While vaccines based on STX would be expected to prevent the serious systemic complications of STEC disease, they would not be expected to prevent colonization of the gut by STEC and so would have little effect on the transmission of disease in the community. Vaccines directed against colonization factors would be expected to be more effective, but at present our knowledge of these factors is incomplete. STEC intimin (EaeA) may be an appropriate vaccination target, at least for the majority of human pathogenic STEC strains which produce it. Indeed, Butterton et al. (53) constructed a *V. cholerae* vaccine strain carrying a chromosomally integrated copy of *eaeA* which expressed intimin on its surface. Oral immunization of rabbits with this strain elicited a serum antibody response to intimin. However, the C-terminal portion of intimin exhibits marked amino acid sequence heterogeneity among different STEC strains (195), and it remains to be seen whether this will limit cross-protection.

Vaccines directed against LPS may also be effective against STEC, since experience with other enteric pathogens suggests that LPS-specific serum IgG is likely to leak into the gut lumen in quantities sufficient to block colonization. Phase I clinical trials of O157 O antigen conjugated to a protein carrier (to enhance immunogenicity) have shown that such vaccines are safe and immunogenic (331). It should be emphasized, however, that LPS-based vaccines will provide only serotype-specific protection, and so the overall efficacy will be limited by the prevalence of O157 STEC in a given region. It may not be economically viable to include more than three or four additional serotypes in an LPS conjugate vaccine formulation. One way of partially addressing this problem is to broaden coverage by using STX B subunits as the carrier for the O157 O antigen; such a conjugate has also recently been constructed (331).

CONCLUDING REMARKS

In this review, we have attempted to summarize the current state of knowledge on the biology of STEC disease in humans, with particular reference to pathogenic mechanisms and diagnostic procedures. Research in recent years has been revolutionized by the availability of purified toxins, monoclonal antibodies, and cloned genes and by the development of DNA sequencing and amplification technologies. Additional benefits will undoubtedly be derived from access to the complete genome sequences of representative STEC strains in the near future. The pathogenesis of STEC disease is clearly multifactorial and involves several levels of interaction between bacterium and host. These include colonization of the gut, generation of diarrhea and intestinal lesions, absorption of STX and interaction of the toxin with host tissues, generation of inflammatory responses, and perturbation of EC function. Each stage of this process provides potential opportunities and targets for the development of therapeutic and preventative strategies. Although our understanding is still far from complete, we now know enough to begin to make informed decisions about the control of STEC disease in humans.

While not diminishing the importance of O157 STEC strains as major causes of disease in many parts of the world, it is now clear that non-O157 STEC strains make a significant contribution to the overall disease burden. However, it is likely that not all STEC strains found in animal reservoirs are pathogenic for humans, and some of the genetic markers associated with human virulence have been identified. Rapid and sensitive meth-

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ods for detecting STEC strains with these characteristics have now been developed. It is increasingly being recognized that the direct and indirect costs of human STEC disease are substantial. Therefore, it is likely that comprehensive microbiological surveillance programs, which would provide early warning and limit the scale of outbreaks, will ultimately be cost-effective, as will vaccination programs. However, control of STEC disease will also benefit from an improved understanding of the biology of STEC in animal reservoirs, and alterations in farm management practices may reduce the entry of these bacteria into the human food chain. Measures are also required to maximize the microbiological safety of our food. Meat products can be made safe by thorough cooking; dairy products and fruit juices can be pasteurized. However, salad vegetables, which have been the source of several serious outbreaks, present a problem in the absence of food irradiation.

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