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Antibody Therapy in the Management of Shiga Toxin-Induced Hemolytic Uremic Syndrome

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INTRODUCTION

In recent years, outbreaks and sporadic cases of disease attributable to Shiga toxin (Stx)-producing *Escherichia coli* (STEC), particularly *E. coli* O157:H7, have occurred with increasing frequency. Within the United States alone, it is estimated that annually there are ≈100,000 cases of STEC infection, of which some 73,000 are due to infection with O157:H7 (73). The most common sources of infection are food and water contaminated with animal and human effluents, of which cattle are considered the primary animal reservoir. Infection via contact with infected individuals, however, is also important.

Typically, individuals infected with STEC develop abdominal pain and mostly bloody diarrhea (hemorrhagic colitis) within 2 to 5 days following exposure. Although the illness usually resolves without sequelae, hemolytic uremic syndrome (HUS) can occur several days following the onset of bloody diarrhea in 5 to 10% of susceptible individuals, particularly children and the elderly. HUS, characterized by hemolytic anemia, thrombocytopenia, acute renal damage, and various degrees of central nervous system (CNS) complications, can result in death or chronic, irreversible renal dysfunction (36). Although HUS is not normally attributed to a single etiology,

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STEC-induced HUS is by far the most significant and the leading cause of acute renal failure in children.

STEC produce one or two genetically and antigenically distinct exotoxins designated Shiga toxin 1 (Stx1) and Stx2, of which Stx2 is the primary virulence factor for HUS. Currently there are no specific protective measures or therapy against STEC infection other than supportive therapy; the utility of antibiotics or antidiarrhetics is uncertain, and they may even be contraindicated (117, 138). Several excellent publications provide a comprehensive review of the current knowledge on these pathogens and the sequelae of STEC-induced HUS (2, 95, 102, 104, 119).

This communication reviews recent advances concerning HUS and the microbial toxins responsible for the syndrome and discusses the experimental evidence and rationale which, we believe, support the potential benefit of immune-based therapy against Stx2 as a means of protecting susceptible individuals at risk of developing STEC-induced HUS. Since the proposed immunotherapy is directed against HUS and is not expected to impact the gastrointestinal manifestations of the disease, the focus will be confined to HUS only.

SHIGA TOXIN: STRUCTURE AND MECHANISM OF ACTION

In the majority of STEC strains, the toxin genes are carried on lysogenic phages (86), known as toxin-converting phages. The Stx produced by *Shigella dysenteriae* type 1 is genetically and antigenically identical to STEC Stx1 (87). Stx2 is distinct genetically and antigenically from Stx1. By amino acid comparison, Stx1 and Stx2 are 56% homologous (49). Stx2 is the prototype of a family of toxins that are very similar to Stx2 and neutralized by polyclonal antibody against the Stx2 but have amino acid differences. Currently there are approximately 10 Stx2 gene variants (31, 47, 75, 94, 93, 100, 110, 111, 137). Stx2 is the most prevalent Stx genotype identified in STEC isolated from patients with HUS (26, 108), and Stx2c is the most common Stx2 variant associated with HUS (26). Stx2 variants other than Stx2c are found frequently in asymptomatic STEC carriers but can cause uncomplicated diarrhea (26) and, rarely, HUS (47, 103, 124).

In terms of basic structure, Stx1 and Stx2 are similar. The toxins consist of one enzymatically active A chain, 32,000 molecular weight and five B chains, approximately 7000 molecular weight, that are responsible for cell binding (19). Similar to the structure of cholera toxin, the A subunit can be proteolytically nicked into a 28-kDa A1 portion and a 4-kDa A2 polypeptide chain (106). In the native toxin molecule, the A1 and A2 fragments are held together by a disulfide bond. The A1 polypeptide is a 28S rRNA *N*-glycosidase and hence is responsible for the catalytic activity of the toxin (28, 106), inhibition of protein synthesis.

The B pentamer is responsible for toxin binding to the target cell (19). In the absence of an A subunit, the B subunits form pentamers which are capable of binding but not intoxicating host cells. The functional receptor of both Stx1 and Stx2 is the neutral glycolipid globotriasoylceramide [Gb_3; αGal(1–4)βGal (1–4)βGlc-ceramide] (48). Cells that are sensitive to Stx have Gb_3 in their cytoplasmic membranes. Cells can also be made sensitive to toxin by experimental insertion of Gb_3 into their membranes (55). Hence, it is well established that Gb_3 is a functional receptor for the toxins. The X-ray crystallographic structure of the B subunit complexed with a Gb_3 analogue revealed that the B subunit has three binding sites per arm and hence potentially 15 binding sites per pentamer (13). However, in a nuclear magnetic resonance study of the interaction of the B subunit with Gb_3 in solution, only one of the three binding sites was substantially occupied with the Gb_3 receptor (114).

There are differences between the two toxins, the most significant of which are differences in binding affinities to the Gb3 receptor and their regulation of expression. Although Stx1 and Stx2 have the same functional receptor, Stx1 has about 10-fold higher binding affinity than Stx2 (41, 42). In one study, Scatchard binding analysis gave a dissociation rate of 4.6×10^{-8} and 3.7×10^{-7} for Stx1 and Stx2, respectively (41).

A second approach with a BIAcore system, which measures real-time interactions between toxin and ligand, gave a somewhat lower dissociation rate for the two toxins, 2.2×10^{-7} and 1.04×10^{-6} for Stx1 and Stx2, respectively (81). In this study, the receptor, Gb3Cer, was in liposomes which were immobilized by covalent linkage to a dextran matrix on a sensor chip. Thus, the presentation and mobility of the Gb3 receptor may be in a different configuration than in a cell membrane and may explain the discrepancies in the dissociation rates. The BIAcore system also revealed that whereas Stx1 did have a greater association rate for the Gb3 receptor than Stx2, the dissociation from its receptor was slower with Stx2 than with Stx1, indicating that while Stx2 binds slowly to the receptor, it also dissociates at a slower rate. These characteristics may partially explain why Stx2 is more closely linked to vascular damage than Stx1. Systemic uptake, therefore, may be dependent on the high affinity of Stx1, which binds to gut receptors, and the rate of toxin transcellular process. In an infection with a strain producing both toxins, Stx1 probably competes with Stx2 in the initial binding steps, reducing the total amount of Stx2 that is systemically delivered. Once in the systemic circulation, Stx2 may be a more potent toxin than Stx1 at both killing mice (121) and potentially intoxicating human renal endothelial cells (63).

The second major known difference between the two toxins is how they are regulated. Stx1 is regulated by the level of iron in the medium. Toxin expression is greater under low-iron conditions than under high-iron conditions (8). Iron regulation involves a control gene, *fur*, whose protein product acts as a repressor of transcription. The level of iron in the growth medium has no effect on the regulation of Stx2. It is clear that phage induction plays a major role in the level of Stx2 expression (27, 78, 82). When the phage which carries the toxin genes is induced, due to both the increase in toxin genes and other phage-related factors there is a large increase in toxin production. In STEC strains growing in a rich culture medium, spontaneous phage induction contributes significantly to the level of Stx2 expression.

While there are significant similarities between Stx1 and Stx2 in basic structure, receptor recognition, and biochemical modes of action, there are considerable differences in the clinical impact in patients infected with STEC strains producing Stx1, Stx2, or both toxins. On the surface, one would predict that infection with a strain which produces both Stx1 and Stx2 puts the patient at the greatest risk of HUS development.

There is now compelling epidemiologic evidence showing that Stx2-producing strains are more closely linked with HUS development than either Stx1-producing strains or strains which produce both toxins (18, 58, 76, 89, 112).

One possible explanation that might link Stx2-producing strains to serious systemic complications is that strains expressing Stx2 may have other virulence factors that make them more virulent to the host. Our work (33) with the piglet infection model and isogenic mutant strains highlights the significance of the differences between Stx1 and Stx2. With *E. coli* O157:H7 strain 933, which produces Stx1 and Stx2, we generated isogenic strains that produce either Stx1 or Stx2 only and studied the effects of these strains in the piglet model. The wild-type 933, a double-toxin-producing strain, caused neurological complications in 33% of the orally challenged piglets. In contrast, infection with the isogenic strain producing only Stx2 caused CNS symptoms and lesions in 90% of the piglets, while infection with the isogenic strain producing only Stx1 caused no detectable CNS symptoms or lesions (33). Thus, infection of piglets with these isogenic strains showed that it was the nature of the toxin being produced that determined the systemic complication risk and not an additional virulence factor(s). These observations are consistent with epidemiologic data from HUS patients (76, 58, 89, 112) showing the contribution of strains expressing Stx2, Stx1 and Stx2, or Stx1.

MANIFESTATIONS OF STEC-INDUCED HUS

Diarrhea-associated HUS was first described as a discrete entity in 1955 by Gasser et al. (33). Although an infectious etiology was suspected from the beginning, based on the occasional clustering of cases and the seasonal pattern of occurrence, it was not until the breakthrough discoveries of Karmali et al. (52) in 1983 that HUS was definitively linked with antecedent enteral infection by STEC. Since then, because of several well-publicized outbreaks of food-borne infection and HUS, the disease has been prominently featured in both the lay press and the scientific literature. When it was first identified, the mortality of STEC-induced HUS was in excess of 25%. However, following the pioneering efforts of pediatric nephrologists, including Giannantonio in Argentina (29, 50) and Kaplan in South Africa (50), early initiation of dialysis therapy for the acute renal failure became routine procedure, and mortality during the acute illness fell precipitously to its current level of 3 to 5%. Nonetheless, STEC-induced HUS is still the most common cause of acquired acute renal failure in previously healthy children and remains a cause of considerable morbidity.

Clinical

The principal organ affected in STEC-mediated HUS is the kidney. This is presumed to be the consequence of the high level of renal blood flow and abundant baseline expression and high degree of inducibility of the Shiga toxin glycolipid receptor, Gb_3, within the glomerular microcirculation (51). These factors are more pronounced in younger children, accounting, in part, for the heightened susceptibility of pediatric patients to this disease (60). The severity of renal injury varies in degree from urinary abnormalities such as hematuria and proteinuria to acute renal failure. Approximately 40% of patients with STEC-induced HUS require temporary dialysis support until they recover from the acute episode (29). The second most important organ affected in the disease is the brain. Nearly all children manifest lethargy and irritability. However, more serious cerebral complications, including seizures, cortical blindness, and thrombotic strokes, occur in 5 to 10% of patients. These reflect a combination of factors such as vascular injury, hypertension, azotemia, hyponatremia, and hypocalcemia (116). A similar percentage of patients may develop life-threatening cardiopulmonary sequelae, including adult respiratory distress syndrome, congestive heart failure, and myocarditis. The occurrence of neurological and cardiovascular complications is associated with more severe STEC-induced HUS and a higher risk of mortality during the acute illness. Other organs that are frequently involved in STEC-induced HUS are the endocrine and exocrine pancreas, liver, gall bladder, gastrointestinal tract, and skin (116).

It is evident that STEC-induced HUS is a systemic illness potentially affecting every organ throughout the body. The manifestations of the disease arise as a consequence of two primary pathogenetic mechanisms: direct Stx-mediated injury to vascular endothelial cells leading to tissue ischemia and dysfunction (33, 59, 88), and a systemic inflammatory response triggered by Stx-mediated release of a wide range of cytokines and chemokines, including interleukin-6, interleukin-8, and tumor necrosis factor alpha (52, 53, 104). In view of these considerations, it is reasonable to anticipate that therapies which directly target the actions of Stx would have a favorable impact on the course of the acute disease.

Hematological

Microangiopathic anemia with erythrocyte fragmentation and thrombocytopenia are defining features of STEC-induced HUS. These problems may arise, in part, from Stx-mediated endothelial cell injury, leading to intravascular coagulation, fibrin deposition, and platelet adherence to microthrombi within the vascular lumen. Altered blood flow through these injured capillaries results in red blood cell destruction. In addition, Stx may directly inhibit marrow production of these cells and promote apoptosis of platelets (104). Other important hematological features of the disease include chemokine-induced leukocytosis, which may exacerbate disease as a consequence of leukocyte-derived oxygen free radicals and urate nephropathy. Early in the course of the disease, there is activation of the coagulation cascade, evidenced by prothrombin fragments 1 and 2, tissue plasminogen activator antigen, tissue plasminogen activator-plasminogen activator inhibitor type 1 complex, and D-dimers (10).

Immunological

A sustained, systemic inflammatory response is triggered by Stx following its absorption from the gastrointestinal tract. This is characterized by a rapid rise in the circulating level and urinary excretion of a variety of cytokines and chemokines. These abnormalities rapidly return to baseline values within 2 to 4 days of the onset of the illness, except for patients who experience more severe disease or death, in whom the cytokine

concentrations are persistently elevated (53, 104, 133). Coabsorption of lipopolysaccharide potentiates the inflammatory response and exacerbates the clinical disease in an experimental model of STEC-mediated HUS in baboons (115). There is a humoral response to the STEC type-specific lipopolysaccharide that does not appear to play an etiological role in the development of disease. However, serial assays of immunoglobulin A (IgA), IgG, and IgM antibody levels in the plasma and the saliva have been useful tools to confirm the occurrence of STEC-induced disease and to identify and trace the spread of the causative organism in diseases outbreaks (64).

Histopathological

The characteristic histopathological lesion in STEC-induced HUS is thrombotic microangiopathy, endothelial injury, and microthrombi (29). There is endothelial cell swelling, widening of the subendothelial space, deposition of fibrin-like material in this location, and narrowing of the capillary lumen. The lesions may contain fibrin, fibronectin, IgM, and C3 on immunofluorescence examination of the tissue. By electron microscopy, endothelial cell nuclei are prominent and the membrane fenestrata are damaged. These abnormalities are seen in the vascular bed of all organs. The gastrointestinal tract has additional features, including thrombosis of submucosal and intramural vessels, mucosal hemorrhage and ulceration, mucositis, and pseudomembrane formation.

Early Markers of Disease

STEC-induced HUS is an abrupt illness that generally occurs in previously healthy children. In most cases, oliguria and acute renal failure develop within 24 to 48 h of recognition of the syndrome (29, 125). Thus, there is a prevailing sense among some pediatric nephrologists and others who care for children with STEC-induced HUS that the die is cast regarding the likely severity and clinical course of the disease as soon as the patient is initially seen in HUS. Therefore, there is great interest in identifying early markers that identify children with STEC enteritis who are at risk of progressing to HUS prior to the development of established vascular injury and systemic inflammatory response. Potential candidates in this regard include more specific cytokines, such as HMG-B1, a macrophage-derived molecule that mediates the late lethality of endotoxin-induced shock in experimental animals (136). Others have proposed that serial assays of indices of the coagulation cascade, such as tissue plasminogen activator antigen and tissue plasminogen activator inhibitor 1 complexes may reveal disturbances prior to the onset of clinical disease (10).

Some investigators have proposed that diarrhea-induced dehydration, increased blood viscosity, and altered shearing forces within the glomerular microcirculation that is already damaged by Shiga toxin may be pivotal in the development of STEC-mediated HUS (119). Based on this proposal, they have advocated parenteral fluid therapy for all children with hemorrhagic colitis to promote adequate perfusion of the renal vasculature and to prevent STEC-mediated HUS. It remains to be determined whether direct measurements of blood rheology and flow characteristics can be used clinically to monitor children with STEC enteritis and identify those who are most at risk of developing HUS.

Finally, recent advances indicate that Stx can be detected within the plasma of patients with STEC enteritis, in free form or bound to polymorphonuclear leukocytes (122). The presence of Stx within the circulation for up to 1 week after the diagnosis of STEC-induced diarrhea suggests that delayed delivery of toxin to the microvasculature may exacerbate endothelial injury, organ damage, and the clinical manifestations of HUS (122). Interventions such as an effective antitoxin against Stx2 may block this process and ameliorate disease in children with HUS. Rapid assays of circulating levels of Stx may represent another method to identify those at highest risk of HUS during and after an episode of STEC infection.

CURRENT MANAGEMENT AND TREATMENT OF HUS

Because the etiology of STEC-induced HUS was unknown for so long, this left a therapeutic void into which clinicians leapt in an effort to treat the disease. Unfortunately, as outlined in the following section, none of these treatments have had any impact on the incidence and severity of STEC-induced HUS. This has led some clinicians to adopt a stance that there is no treatment for STEC-induced HUS except prevention. However, recent advances in the understanding of the pathobiology of Stx in these circumstances will hopefully justify renewed attempts to ameliorate the disease, with, for instance, antibody-based Stx treatment strategies.

Hematological

Based on laboratory data and histopathological evidence of activation of the clotting cascade and fibrin deposition in the vasculature of children with STEC-induced HUS, early trials were performed to test the effect of anticoagulants, such as heparin, and fibrinolytic agents, such as streptokinase and urokinase. None of these drugs ameliorated the disease and were associated with a significant increase in life-threatening hemorrhagic episodes. In addition, other studies have evaluated whether treatment with antiplatelet drugs, such as aspirin and dipyridamole, were useful in children with STEC-induced HUS. The justification for this intervention was the observation of platelet activation in this disease setting. However, once again, this hematology-based therapeutic strategy was ineffective (1).

Anti-inflammatory

As mentioned above, there is a systemic inflammatory response to Shiga toxin and lipopolysaccharide in children with STEC-induced HUS. In analogy to the circumstance of bacterial sepsis, efforts were made to treat the disease with steroids. In a randomized trial of pulse intravenous methylprednisolone involving 92 children, this anti-inflammatory therapy had no effect on the need for or duration of dialysis, the occurrence of seizures, or the nadir hematocrit (99). Only small studies have evaluated treatment with the antioxidant vitamin E, and no definitive statement can be made about its efficacy (1).

Microbiological

In view of the primary role of STEC enteritis as an antecedent, it would seem logical to treat the intestinal infection or early HUS with antibiotics. However, this is a very controversial subject. There have been two small clinical trials indicating that treatment of pediatric patients who have hemorrhagic enteritis with trimethoprim-sulfamethoxazole has no effect on the incidence of subsequent HUS (101, 96). In the notorious Jack-in-the-Box outbreak in the Pacific Northwest in December 1992 to January 1993, there was no relationship between prior treatment with antibiotics and trimethoprim-sulfamethoxazole, in particular, and the development of HUS (3). In contrast, in the 1996 outbreak of STEC enteritis in Sakai, Japan, administration of fosfomycin on day 2 after the onset of bloody diarrhea resulted in an 85% reduction in the relative risk of developing HUS (45). Finally, two recent epidemiological studies, one a national surveillance conducted by the Centers for Disease Control and the other a case-control study involving a network of participating centers in Washington, Idaho, Oregon, and Montana, indicated that antibiotic therapy of STEC enteritis resulted in a significantly higher risk of developing HUS (117, 138). This adverse outcome may reflect the effect of specific antimicrobial agents on phage induction and subsequent Stx gene expression and transcription (142). A recent meta-analysis of all studies of antibiotic therapy in STEC enteritis concluded that the impact of this treatment is still unknown and advocated the performance of a multicenter, randomized, placebo-controlled clinical trial (109). In any event, it is apparent that antibiotic therapy is not a panacea for STEC-mediated HUS.

Gastrointestinal STEC-Directed Therapies

In view of the key role of Stx in the pathogenesis of HUS, it was only logical to consider the potential efficacy of treatments designed to bind the toxin in the gastrointestinal tract, sequester it within the lumen, prevent its entry into the systemic circulation, and avoid the vascular injury and inflammation. SYNSORB Pk, a novel compound composed of diatomaceous earth covalently linked to the trisaccharide that mediates Shiga toxin binding by the endothelial cell, was the first drug in this class to be formally tested. Unfortunately, in a recently competed trial involving 145 children with STEC-induced HUS, the drug did not prevent death or serious extrarenal events and did not lower the need for dialysis or the duration of acute renal replacement therapy (126).

An analysis was completed for 145 children who received at least one dose of the study medication, of whom 96 had been treated with SYNSORB Pk and 49 with the corn meal placebo. There were no significant differences in the clinical or laboratory features at the time of entry into the study (Table 1). In most children, the study medication was started within 18 h after the diagnosis of STEC-induced HUS was made. Finally, the subjects generally received more than 75% of the scheduled doses of study medication and tolerated most of the doses by either the oral or nasogastric route. The two primary endpoints of the study were a reduction in the frequency of death and/or serious extrarenal events from 20 to 5% in the SYNSORB Pk-treated patients and a reduction in the need for dialysis from 50 to 25% of the study patients. Table 1 summarizes the outcome for the two principal clinical objectives in the intent-to-treat group of patients. There was no difference between the SYNSORB Pk-treated and placebo patients for either of the two primary endpoints. The occurrence of death and/or serious extrarenal complications was reasonably close to the expected rate of 20% based on a review of the literature. In addition, there was no disparity between the spectrum of these life-threatening events during the study and previous reports of children with STEC-induced HUS. Improvements in pediatric intensive care and standardized application of the criteria to initiate dialysis may account for the observation that the need for acute renal replacement therapy was lower than the projected rate of 50%. There were no serious adverse events that were considered definitely related to the study medication.

TABLE 1. Primary clinical outcomes in the HUS-SYNSORB clinical trial

Outcome	No. (%) of subjects	
	SYNSORB Pk (n = 96)	Placebo (n = 49)
Death/extrarenal events	13 (17)	9 (21)
Fatalities	3 (3)	1 (2)
Dialysis [duration of dialysis (days)]	40 (42) [5.2]	19 (39) [3.6]

Among the possible explanations for the failure of this study are inadequate delivery of the study drug to the segments of the intestine where STEC proliferate and release Stx because of nausea, vomiting, and paralytic ileus; unidentified factors within the gastrointestinal tract of children with STEC-induced HUS that interfered with SYNSORB Pk-Stx binding; low intraluminal levels of the toxin were below the avidity of the binding agent; and delivery of an oral Stx-binding agent to bind Stx in the gastrointestinal test after STEC-induced HUS was initiated too late in the disease course to make a significant difference in the outcome. The observation that only 36% of the children enrolled in this trial had microbiological confirmation of STEC infection or detection of free Stx in their stool at the time of randomization supports the fourth possibility. A full report of the study was recently published (126).

Other approaches include the use of X-ray crystallography and computer methods to model Stx binding to the Gb3 molecule; these compounds are molecularly engineered polymers of Gb3, designed to maximize this interaction (57, 84). In addition, nonpathogenic *Neisseria* strains have been genetically modified to express the Gb_3 molecule on their cell membrane (92). All of these reagents display more avid binding of Shiga toxin and have increased solubility, and they have been tested in animal models of STEC infection. However, based on the negative outcome of the SYNSORB Pk trial, it is unlikely that this therapeutic approach will be successful in patients with STEC-induced HUS.

Immunological

Based on the presence of high circulating levels of inflammatory cytokines and abnormalities in endothelial cell function, patients have been treated with plasma infusions (125).

However, this has no effect on the course of the disease. Although it has not been rigorously studied in a controlled clinical trial, plasmapheresis is also not recommended as therapy for STEC-induced HUS. This is in contrast to the widely accepted role of these therapies in atypical and familial forms of HUS. Finally, despite initial studies which suggested a benefit of intravenous administration of IgG, this was not confirmed in controlled clinical trial (107).

Overall, it is apparent that despite over 40 years of clinical research on this disease, no effective treatment has been developed for STEC-induced HUS. Some authorities suspect that no specific therapy is likely to work in newly diagnosed disease because the vascular injury and organ damage are already well established and initiation of therapy at this stage is a case of too little too late. However, with the recent observation that Stx is presented in the circulation of children with STEC-induced HUS in free form and bound to leukocytes for up to a week after the onset of disease and the pivotal role of the toxin in the pathogenesis of disease, the use of monoclonal antibodies against Stx to prevent HUS in children with STEC enteritis and to ameliorate disease in those with HUS is fully justified. The availability of human antibodies that are virtually devoid of adverse reactions underscores the viability of this therapeutic strategy.

USE OF SPECIFIC ANTIBODIES IN HUMAN THERAPY

Antibodies (chimeric, humanized, and fully human; Fig. 1) play an increasingly important role within the field of therapeutics. At present, more than a dozen monoclonal antibodies are either approved or marketed in the United States (105), with well over 70 more in various stages of development. Humanized and human antibodies have assumed greater prominence because of their markedly decreased or negligible immunotoxicity and side effects.

Similar to other toxin-induced diseases, such as tetanus and botulism (85), little endogenous serum antibody is induced against Stx1 or Stx2 following STEC infection (5, 112). Nonetheless, passively administered toxin-specific antibodies have been shown to be highly effective at preventing toxin-mediated diseases. Since specific antibodies against Stx too appear to completely neutralize the cytotoxicity of the toxin in cell culture and protect mice given a lethal dose (46, 90, 118), and such antibodies may protect children at risk of developing HUS if they are administered shortly after the onset of diarrhea, ideally before the onset of HUS. To test this concept, we used the gnotobiotic piglet infection model. In these experiments, infected piglets were fully protected against CNS symptoms when treated parenterally with Stx2-specific swine antiserum 24 h after challenge, shortly after the onset of diarrhea. These experiments showed that an infected host can still be protected when Stx-specific antibody is given well after the onset of diarrhea (20). The next step was to produce effective and safe Stx antibodies suitable for human therapy.

Chimeric and Humanized Antibodies

One approach has been to "humanize" existing murine monoclonal antibodies via replacement of the murine immunoglobulin constant region with and without framework regions with comparable human immunoglobulin components (21, 141). Stx-specific human-mouse chimeric monoclonal antibodies (Fig. 1) have been developed with the IgG1κ Stx1-specific murine monoclonal antibody 13C4 reported by Strockbine et al. (118) and the IgG1κ Stx2-specific murine monoclonal antibody 11E10 reported by Perera et al. (98). Structurally, such chimeric monoclonal antibodies retain the murine immunoglobulin elements required for antigen recognition, but the murine immunoglobulin elements not required for antigen recognition are replaced with counterparts derived from human immunoglobulins. Although the Stx1-specific mouse-human chimeric monoclonal antibody exhibited efficacy both in vitro and in vivo equivalent to that of the parent murine monoclonal antibody, 13C4, the Stx2-specific mouse-human chimeric monoclonal antibody was less effective both in vitro and in vivo than the parent murine monoclonal antibody, 11E10 (21).

The experience of Edwards et al. exemplifies a major difficulty in producing a chimeric antibody that retains the efficacy of the parent antibody (21). Furthermore, because chimeric mouse-human monoclonal antibodies retain portions of murine immunoglobulin elements, there is a risk that individuals who receive such antibodies may still develop antibodies against those administered. Thus, additional approaches have been utilized in an effort to produce fully human monoclonal antibodies, including immortalization of peripheral human

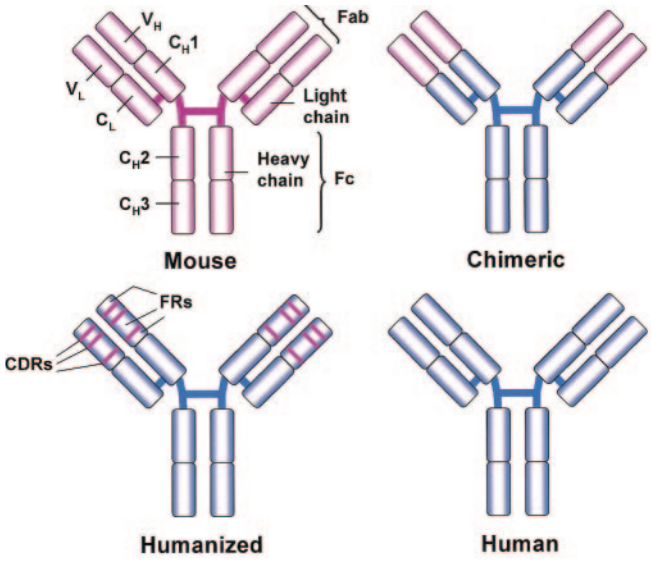

FIG. 1. Technologies used to produce antibodies for human therapy. Chimeric antibodies are made by combining the antigen-binding variable heavy- and light-chain domains (V_L and V_H) of the mouse antigen-specific monoclonal antibody with human constant region domains (C_H1, C_H2, and C_H3). Humanized antibodies are made by replacing the complementarity-determining regions (CDRs) (regions within the antigen-binding variable domains which react with the antigen) of human IgG molecule with the complementarity-determining regions of mouse antigen-specific monoclonal antibody. Transfer of one or more amino acid residues from the framework regions (FRs) is often required to create high-affinity humanized antibodies. Human antibodies are produced by conventional hybridoma technology with transgenic mice which bear human heavy- and light-chain immunoglobulin gene loci. Human antibodies are also obtained from single-chain variable fragments or Fab phage display libraries combined with a prefabricated human constant region.

lymphocytes isolated from immunized individuals via fusion with a murine myeloma or Epstein-Barr virus (22). This approach has had limited success due to safety concerns associated with immunizing humans with antigens not already licensed as vaccines. Although in vitro immunization has been attempted, large numbers of peripheral B cells are required, and in the absence of T cells, affinity maturation does not occur, resulting in low-affinity IgM antibodies. Furthermore, there is concern about the safety of antibodies isolated from human cells for in vivo human use, particularly those isolated from cell lines immortalized with Epstein-Barr virus.

Human Monoclonal Antibodies

In an effort to address these concerns, several investigators have developed mice which bear human heavy- and light-chain immunoglobulin gene loci introduced as minichromosomes or transgenes (7, 24, 61, 83, 121). One such mouse is the Hu-MAb-Mouse (GenPharm International, a subsidiary of Medarex, San Jose, Calif.), which contains one of three human heavy-chain transgenes designated HC2 (24), HCo7, and HCo12 and the human light-chain transgene KCo5 (24). The heavy-chain transgene constructs HC2, HCo7, and HCo12 are comprised of human immunoglobulin heavy-chain variable (V_H), diversity (D), and joining (J_H) segments along with the μ, γ_1, and/or γ_3 constant (C) region exons, the associated switch regions, the J_H intronic enhancer, and the rat 3′ heavy-chain enhancer. The light-chain transgene construct KCo5 is comprised of human immunoglobulin light-chain variable (V_k), joining (J_k), and constant (C_k) region segments.

Hu-MAb-Mouse mice are capable of expressing human but not murine antibodies. In response to immunization with an antigen, these mice express antibodies that are comprised entirely of human immunoglobulin structural elements; human antibodies of the IgMκ and IgG1κ and/or IgG3κ isotypes as a result of class switching; and human antibodies with evidence of extensive somatic mutation within the human variable regions encoding the expressed antibodies (40, 62, 127). Following immunization of a Hu-MAb-Mouse with a desired antigen, splenic B cells can be harvested and immortalized to produce human monoclonal antibodies bearing the attributes of affinity maturation (62). Although Hu-MAb-Mouse mice have only 10 to 50% of the normal level of B cells and only 20 to 80% respond to a given antigen with sufficiently high titers to be candidates for fusion to produce monoclonal antibodies, this problem can be overcome by immunizing multiple mice.

Production of Stx Human Monoclonal Antibodies

In collaboration with GenPharm International, we utilized Hu-MAb-Mouse mice to develop fully human monoclonal antibodies specific for Stx1 and Stx2. Hu-MAb-Mouse mice were immunized with Stx1 or Stx2 toxoid. Splenic B cells from mice with high titers of anti-Stx antibodies were harvested and fused to a murine myeloma to create murine hybridomas secreting human monoclonal antibodies (79, 80). With this method, we generated 37 Stx2-specific and 11 Stx1-specific human monoclonal antibodies (79, 80). These human monoclonal antibodies are comprised entirely of human immunoglobulin heavy- and light-chain structural elements. We demonstrated that these antibodies neutralize the cytotoxic and lethal activity of Stx1 and Stx2 in vitro and in vivo, respectively.

The choice of human rather than chimeric or humanized antibodies is obvious. In addition to issues of safety, half-life, and effector function, the process of humanizing murine antibodies often reduces their affinity considerably, which adversely impacts efficacy. Our approach, to immunize transgenic mice rather than humanize one or two murine antibodies, resulted in the production of a large number of hybridomas, from which we selected the most effective against the toxins and their variants. The availability of a large panel also provides future options to use a mixture of several monoclonal antibodies should this be required either to further improve efficacy through synergy or to enlarge the spectrum of activity against Stx variants. Since these antibodies are human to begin with, their affinity, specificity, and efficacy are not prone to change during the process of humanization, as is usually the case.

PRECLINICAL EVALUATION OF STX-SPECIFIC ANTIBODIES

Given the low incidence of HUS in the population, even among those who present with bloody diarrhea, the need for an appropriate animal model in which to conduct preclinical evaluation to determine efficacy and the likely required optimal therapeutic dose is critical. Various animal models have been used to investigate the efficacy of immunotherapeutic reagents and the systemic effect of Stx on the mammalian host, including HUS.

Animal Models for HUS

Infection models. Naturally occurring HUS-like disease has been described in greyhounds (15, 23) and in rabbits (32). However, the association between the condition in dogs known as idiopathic cutaneous and renal glomerular vasculopathy and STEC has been neither fully established nor experimentally confirmed since these initial reports (15, 43). The recent report of bloody diarrhea and HUS-like kidney lesions in laboratory rabbits from which unique STEC were isolated (15) may be a significant step toward the development of an animal model for HUS. Toxin production was demonstrated, and histopathological findings of erosive and necrotizing enterocolitis with adherent bacterial rods, proliferative glomerulonephritis, tubular necrosis, and fibrin thrombi within small vessels and capillaries were observed in the affected animals. These systemic microvasculature changes indicated that affected rabbits developed thrombotic microangiopathy, the hallmark of STEC-induced HUS (32). The reproduction of this condition experimentally will presumably follow. Clearly, this model is likely to be very useful for investigating the pathogenesis of STEC-induced HUS. Until more information on this model is available, its suitability for testing therapeutic or preventative measures will have to wait.

The ferret model described by Woods and colleagues (140) requires treatment with streptomycin. While there was no evidence of enterocolitis in ferrets challenged orally with STEC, glomerular lesions and thrombocytopenia were observed in a proportion (23%) of them. Given the percentage of animals

affected, this model, as described, is less than optimal for testing methods of prevention or treatment of systemic complications induced by STEC.

Models for parenteral administration of Stx. The systemic effect of Stx administered parenterally has been characterized in many mammalian hosts (77). It would probably be true to state that the administration of sufficient amounts of Stx systemically to any mammalian host is likely to result in serious and ultimately lethal consequences. In the majority of them, this involves vascular injury, which invariably includes the glomerular endothelium and often the tubular epithelium as well. Such studies are useful in shedding light on the nature of the interactions between Stx and the target cell receptor, target cell and organ, and body system. This includes studies in baboons infused with Stx1, which appears to link the microvascular thrombosis of the damaged renal glomerular tissue to a localized inflammatory response in the kidney and to show that the systemic thrombocytopenia, anemia, and schistocytosis possibly arise secondarily. This group also showed that while Stx2 given intravenously to baboons caused the development of progressive thrombocytopenia, hemolytic anemia, and azotemia with glomerular thrombotic microangiopathy at necropsy, baboons given the same dose of Stx1 showed no symptoms or renal lesions (115). In another study with the mouse model, investigators concluded that the interaction between the neuroendocrine and immune systems modulates the level of renal damage (35), which may or may not apply to HUS in humans.

Models for Testing the Efficacy of Immunotherapy

Two animal models are currently being used to test the efficacy of therapeutic agents against HUS. They include the gnotobiotic piglet and the mouse models.

Piglet infection model. Piglets are the only species other than humans that are naturally susceptible to the systemic effects of STEC proliferating in the gastrointestinal tract. A variant of Stx2, designated Stx2e, is responsible for the well-characterized, naturally occurring, mostly fatal systemic illness known as edema disease in swine (66, 70). The neonatal piglet is uniquely relevant as a model for human infections with STEC. In piglets, as in humans, STEC cause intestinal infection and diarrhea, attributed in part to the colonic attaching and effacing ability of these bacteria (17, 25, 128, 131, 132, 129). This attribute requires expression of the locus of enterocyte effacement region genes of the STEC. Expression of the locus of enterocyte effacement region genes and the resulting intestinal lesions are critical for intestinal colonization and production of clinically significant amounts of Stx at the site of mucosal injury, facilitating Stx uptake from the intestine into the bloodstream, where it causes systemic complications, including the characteristic CNS and other symptoms (17, 129, 131).

As in humans, Stx2 is believed to be the principal toxin responsible for the systemic complications in these animals. Similarly, piglets challenged orally with STEC develop diarrhea within 16 to 20 h and CNS symptoms attributed to Stx2 within 48 to 72 h (79). This interval between the onset of diarrhea and the onset of systemic, vascular-induced CNS complications somewhat mimics the situation in children, in whom HUS develops on average 6.5 days after the onset of diarrhea (120).

The clinical signs and lesions observed in pigs given Stx2e intravenously (66, 67) or infected orally with Stx2 (17, 25, 128, 129, 131, 132) or Stx2e-producing *E. coli* (71) are similar and include ataxia, convulsions, paddling of limbs, tremors, and coma along with cerebral hemorrhage and edema (17, 25, 30, 67, 71, 128, 129, 130, 131, 132). Even though neurological signs occur rarely in humans (39, 130), the histopathological lesions observed in the brain of piglets and humans who do develop CNS symptoms as a consequence of STEC infection are very similar and vascular in nature (Fig. 2) and characterized mainly by damage to the vascular endothelium (37). While the cause of death in piglets experimentally infected with STEC is due primarily to vascular lesions in the brain and not in the kidney, as in children, apparent kidney damage characterized by thrombotic microangiopathy in the kidney of piglets infected with STEC has also recently been described in animals that survived several days after oral challenge (37). These lesions include diffuse glomerular endothelial swelling and glomerular congestion, with narrowing of the capillary lumens. Blood vessels show typical morphological criteria of thrombotic microangiopathy-like narrowing of the lumen with concentric intimal thickening, endothelial proliferation, and hyaline fibrin thrombi occluding the lumen. Fragments of red blood cells are often seen in blood vessels and in glomerular capillary loops, which appear occluded with thrombi and due to glomerular endothelial swelling and detachment from the underlying basement membrane. Tubular damage is also apparent (37). These renal observations bring the piglet model even closer to the effects of Stx2 in humans.

A swine edema disease model was used successfully to establish interplay between the dose of Stx protective antibody and time of administration after experimental infection. In this study, with a naturally occurring swine STEC which produces Stx2e but lack the locus of enterocyte effacement gene responsible for the attaching-effacing lesions, the investigators show that higher doses are required for protection with an increased time interval between infection and administration of the antibody (71).

Murine infection model. Mice have been used to study the effect of Stx and Stx-neutralizing antibodies in vivo (123, 135). Two murine models have been used successfully to examine the ability of Stx-specific monoclonal antibodies to neutralize Stx in vivo. The infection model requires treatment of mice with streptomycin before an oral challenge with streptomycin-resistant Stx2-producing *E. coli*. This is followed by the systemic administration of specific monoclonal antibody (21, 74, 135) at intervals thereafter. Mice, however, are not sensitive to infection with all Stx2-producing *E. coli* or indeed to STEC that produce Stx1 and Stx2, as are piglets and, of course, humans. They are therefore only useful for evaluating Stx2-specific antibodies produced by a limited number of STEC strains (21, 56, 74, 141, 113). Unlike piglets, mice do not develop the locus of enterocyte effacement-mediated attaching-effacing lesions which are a key virulence factor for bacterial gut colonization, mucosal damage, toxin uptake, and diarrhea.

Mouse toxicity model. Mice have also been used for toxicity protection assays. Purified Stx1 or Stx2 is injected intraperitoneally or intravenously prior to or following intraperitoneal administration of a monoclonal antibody. Mice which are not protected by the administered monoclonal antibody do not

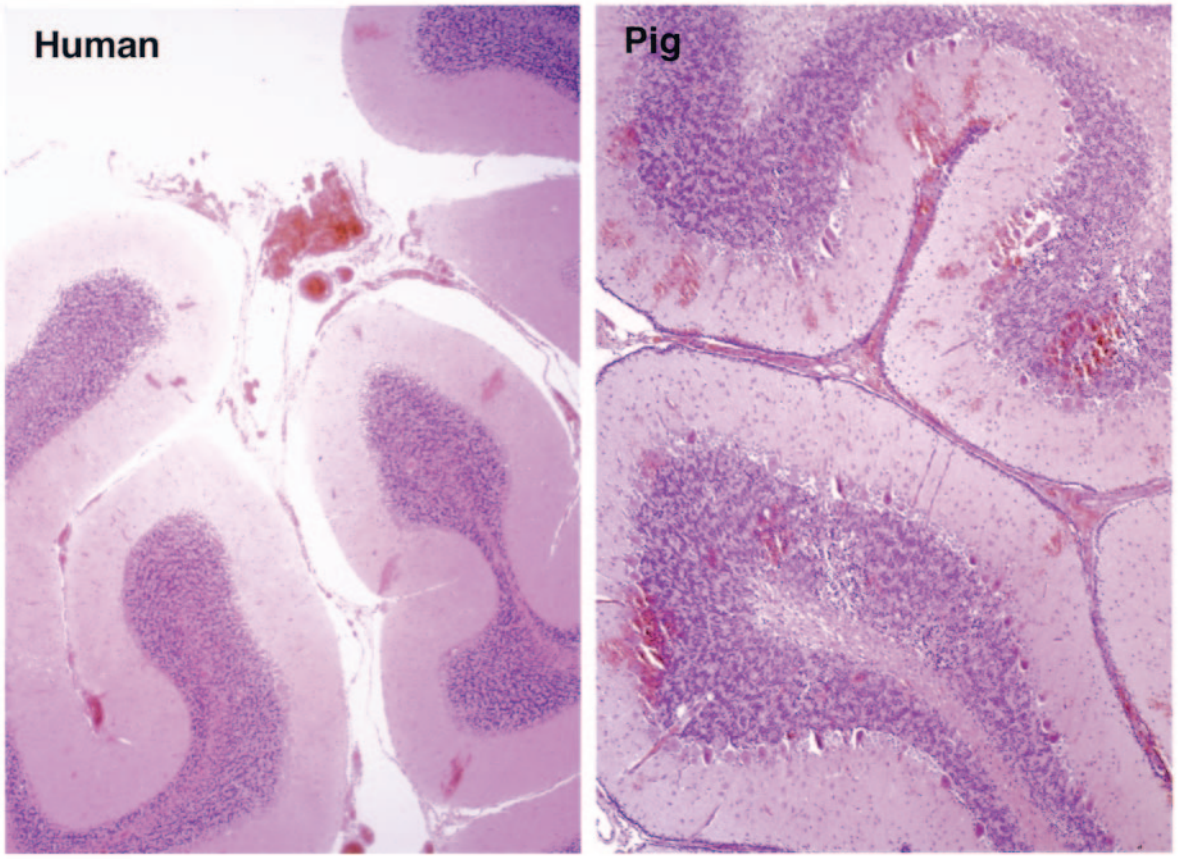

FIG. 2. Histological sections of the cerebellum of a 2-year-old patient who died of HUS and profound neurological complications (left) and a piglet challenged orally with the same Stx2-producing STEC isolated from the child (right). Both sections show various degrees of petechial hemorrhages in the molecular layer (human and piglet) and in the cortex layer (piglet). Extensive infarction in the granular layer with extremely shrunken nuclei can be seen under higher magnification (hematoxylin and eosin stain).

survive (46, 79, 80). Mice are extremely sensitive to the effects of systemically administered Stx2 but are less sensitive to Stx1 (123). Nonetheless, this model has been extremely useful for screening and evaluating the efficacy of monoclonal antibodies specific for either Stx1 or Stx2 (46, 79, 80, 90, 118).

Experimental Evaluation of Antibody Therapy against Stx

The potential for therapy with Stx-specific antibodies against HUS is currently being evaluated independently by three groups of investigators. The main differences among the three products are their relative protective efficacy, their spectrum of activity against Stx and Stx variants, whether they are chimeric or humanized (21, 56, 141) or completely human (79, 80, 113), and the benefit of having a large number of hybridomas available from which to select the most effective panel for therapy.

In the evaluation of human monoclonal antibodies, we utilized the piglet and the two mouse models. While less than ideal, the gnotobiotic piglet, which, as described above, develops diarrhea followed by CNS symptoms, is, in our view, the best available option for preclinical evaluation at this time.

Efficacy of human monoclonal antibodies against Stx2 and Stx2 variants. The monoclonal antibodies produced by all three investigators demonstrated effective protection when administered to mice infected with streptomycin-resistant Stx2-producing STEC and in the mouse toxicity assay. One humanized monoclonal antibody against the A subunit of Stx2 (46), given at a dose of 55.6 μg/kg, protected mice infected 24 h earlier with Stx2-producing STEC, but 180-fold more was required to protect against Stx2 variant-producing STEC, indicating a limited spectrum of activity against the Stx2 variant. The second monoclonal antibody (141), directed against the Stx2 B subunit of Stx2 (designated TMA-15), protected mice infected 24 h earlier with Stx2 variant-producing STEC at a dose of 1.0 mg/kg but failed to protect mice when a dose of 2.5 mg/kg was administered 48 h after challenge. In contrast, the human monoclonal antibody 5C12 which is directed against the A subunit of Stx2, significantly protect mice when administered at a dose of 2.1 mg/kg 48 h after challenge with B2F1 (113).

Dose-response studies in the mouse toxicity model have shown that a dose of 1.25 μg of 5C12 per mouse also protected at least 90% of mice against challenge with Stx2 and the Stx2 variant (113), indicating a broad spectrum of activity against several Stx2 variants. In contrast, human monoclonal antibodies directed against the B subunit, while highly effective against Stx2, displayed limited neutralizing activity against the Stx2 variant (113). These results are consistent with the fact that the sequence homology of the A subunit among STEC strains is much greater than that of the B subunit, indicating that a mono-

TABLE 2. Neutralization of HeLa cell cytotoxicity mediated by Stx of enterohemorrhagic *E. coli* strains by Stx2-specific antibodies

E. coli strain	Toxin(s) produced[a]	Neutralization by Stx2-specific antibodies (%)[b]						Reference
		5H8	6G3	2F10	3E9	5C12	Rabbit	
91-8000	Stx2	>100	>100	81	72	95	>100	
93-8059	Stx2	>100	>100	66	57	96	>100	113
91-8099	Stx2v	1	22	79	73	94	95	
92-9140	Stx2v	−4	20	66	54	80	92	
93-8021	Stx2v	2	37	66	53	65	65	
93-8053	Stx2v	−9	15	54	53	66	67	
95-8061	Stx2v	−4	10	62	63	77	77	
E32511	Stx2 + Stx2c	−7	13	52	43	57	64	113

[a] 16 strains producing Stx2 were tested, and data from two representative strains are shown because all the others showed similar neutralization patterns. Stx2v, Stx2 variant, the type we have not characterized yet.

[b] The extent of antibody neutralization was determined by comparison with wells to which no toxin was added, which represented 100% survival, and wells to which only toxin was added, which represented 100% cytotoxicity.

TABLE 3. Preliminary dose- and time-response relationships[a]

Treatment	Dose, mg (h post-infection)	No. of pigs	No. of surviving pigs (% survival)	Serum concn (μg/ml)
5C12	≤3 (24h)	16	13 (81)	4.0 ± 1.8
IgG-PBS	0	17	1 (0)	9.2 ± 2.5[b]
5C12	≤6 (48h)	8	8 (100)	16.9 ± 7.4
PBS	0	7	0 (0)	0

[a] Piglets were infected orally with Stx2-producing *E. coli* O157:H7 and subsequently treated by injection with different doses of 5C12. The antibody was injected either 24 h after infection, at the onset of diarrhea (bloody diarrhea in children), or 48 h after infection at the onset of CNS symptoms (HUS in children). The table also reflects the concentration of the antibody circulating in the blood stream of the protected piglets (<4.0 μg/ml). The same serum concentration of antibody is expected to protect children as well. The injectable dose of antibody required to achieve this level in the serum of sick children will be determined in phase I clinical trials in human volunteers. The isotype of 5C12 is human IgG1, and the serum concentration of human IgG1 is shown.

[b] Myeloma human IgG1 (Sigma) was given as a control to eight piglets.

clonal antibody against the A subunit will be expected to have a broader spectrum of neutralizing activity against STEC strains than would one against the B subunit. It appears from these investigations that human monoclonal antibodies directed against the A subunit of Stx2 are superior and are the choice for human therapy.

Table 2 illustrates the relative neutralizing abilities of a selected panel of human monoclonal antibodies against several Stx2- and Stx2 variant-producing STEC strains in the HeLa cell cytotoxicity assay. The table shows that all human monoclonal antibodies neutralized Stx2 but not Stx2 variants. Only the A subunit-specific human monoclonal antibodies (5C12, 2F10, and 3E9) strongly neutralized Stx2 variants. The B subunit-specific human monoclonal antibody 5H8 did not neutralize Stx2 variants, and 6G3 neutralized them only slightly. Strain E32511, which produces both Stx2 and Stx2c, was neutralized by A subunit-specific but not by B subunit-specific human monoclonal antibodies. These results further confirm the broad-spectrum reactivity of the selected A subunit-specific compared to the B subunit-specific human monoclonal antibodies.

Efficacy of monoclonal antibodies against Stx1. Since mice and piglets (and possibly humans) are not sensitive to infection with Stx1-producing *E. coli*, the mouse toxicity model has been used to evaluate the efficacy of Stx1-specific monoclonal antibodies. Only two groups of investigators have made Stx1-specific monoclonal antibodies. Both Edward et al. (21), with their humanized monoclonal antibody, and Mukherjee et al. (80), with their human monoclonal antibodies, have shown that mice can be protected against intraperitoneal administration of Stx1.

Time of administration after infection and protective dose. Earlier studies (79) have shown that Stx2-specific human monoclonal antibodies at a dose of 3 mg/kg of protect piglets against the development of CNS symptoms and death when administered systemically 12 h after oral challenge with Stx2-producing STEC. We have since performed dose- and time-response studies which have more accurately defined, although not completed, the time interval between bacterial challenge with 10^{10} CFU of bacteria and the amount of antibody needed for complete protection against systemic disease (Tables 3 and 4).

Piglets which normally develop diarrhea within 16 to 20 h after oral challenge with STEC were consistently protected against the development of systemic disease with 1.5 to 3.0 mg of Stx2-specific human monoclonal antibody/kg when administered well after the onset of diarrhea, at 24 h after infection (Table 3). The administration of this antibody to children after the onset of bloody diarrhea, we believe, will likewise protect them against development of HUS. Furthermore, piglets which developed CNS symptoms 48 to 72 h after bacterial challenge were consistently protected against such systemic complications when given 3 to 6 mg of Stx2 human monoclonal antibody/kg administered 48 h after infection (Table 3). This implies that children too may be protected with this antibody if treated at the onset of HUS. Further studies to define the exact interplay between time of antibody administration after infection and the optimal protective dose required after the onset of diarrhea (or bloody diarrhea in children) or at the onset of CNS (or HUS in children) are ongoing.

The serum level of human monoclonal antibody required to fully protect these animals under these circumstances is ≈4.0 μg/ml. A similar serum level will probably be required to protect children against the development of HUS, which can be determined in dose-response studies during phase I clinical trials in human volunteers. The observation that piglets can still be protected with a high antibody dose at or just before the onset of CNS symptoms gives heart that antibody-based therapy is not only more than likely to protect children presenting with bloody diarrhea but may well be helpful when given at the onset of HUS as well.

Antibody formulation for therapy. Because human monoclonal antibodies are directed against the toxins, they are likely to protect against HUS caused by all STEC strains regardless of serotype or whether they do or do not possess the diarrhea-

TABLE 4. Summary of the preliminary experiments comparing the total number of antibody- treated piglets who survived the infection with the total number of placebo-treated animals

Treatment	No. of pigs	No. of surviving pigs (% survival)
5C12	24	21 (87.5)
IgG-PBS[a]	24	1 (4)

[a] Myeloma human IgG1 (Sigma) was given as a control to eight piglets. The serum concentration of control human IgG1 was 9.2 ± 2.5 μg/ml.

TABLE 5. Frequency of detection of Stx2, Stx1 plus Stx2, and Stx1 in patients with HUS extracted from several reports

No. of STEC isolates	% of isolates[a] producing:			Reference
	Stx2	Stx1 and Stx2	Stx1	
53	83*	11	5	58
11	63.5	36.5	0	89
13	92	8	0	4
6	100	0	0	6
17	96*	6	0	9
32	37.5	62.5	0	65
50	85	15	0	44
194	84*	15	1	34

[a] *, strains reported to produce Stx2 and/or Stx2 variant.

genic locus of enterocyte effacement gene responsible for the bacterial attaching-effacing gut lesions. The formulation ideally will include the minimum number of antibodies, as the inclusion of several of them in a single formulation for clinical use is logistically complex and costly. Therefore, the decision of what should be included in a formulation has to be a balance between providing highly effective, broad-spectrum protection against the largest number of Stx molecules and their variants and the cost of treatment. The formulation clearly must include antibody against Stx2, preferably, as shown above, one which is directed against the A subunit.

While the exact contribution of Stx1 to HUS and other systemic complications remain uncertain at best, Stx1 is often liberated together with Stx2 by strains that are associated with HUS (50, 65, 119). With only a very few exceptions (see Table 5), Stx1 alone is not normally directly linked with HUS. While it may be assumed that Stx2 is the key virulence factor contributing to HUS in a patient infected with a double Stx producer, we and others have no preclinical evidence to demonstrate that antibody against Stx2 alone is sufficient to protect against infection with STEC that produce both toxins. Table 5 illustrates that strains associated with HUS have either Stx2 or an Stx2 variant, with or without Stx1.

It remains to be determined whether a combination of Stx2-specific antibodies targeting different epitopes—anti-A and anti-B subunit-specific monoclonal antibodies, for instance—might have a synergistic effect compared with each used alone. Our proposed strategy is to first use a single Stx2-specific human monoclonal antibody to establish the merit of this approach to the treatment and prevention of HUS. Future formulations consisting of two or more Stx-specific antibodies and perhaps other factors (e.g., anti-tumor necrosis factor alpha) will be justified after the administration of a single Stx2-specific monoclonal antibody is shown to be therapeutically effective.

STX-SPECIFIC ANTIBODIES AND ANTIBODY ENGINEERING

Significant developments in recent years in the areas of proteomics, genetic engineering, and antibody engineering have made antibody-targeted therapy of human diseases a reality. As outlined above, these human monoclonal antibodies have undergone extensive preclinical evaluation in animals in which their pharmacokinetic properties, including half-life, effector functions, dosage, and time and route of administration, have been determined. These will need to be repeated and confirmed for humans in phase I clinical trials. However, the production cost, and consequently the cost of therapy of hybridoma-generated antibodies, is likely to be high, which undoubtedly will limit their use. It is therefore essential to consider other, more cost-effective methods to produce these antibodies in large-scale quantities for clinical use. To accomplish this, these human antibodies must first be cloned, sequenced, and expressed in recombinant forms.

With this in mind, we have created a modular eukaryotic expression system to produce recombinant antibody molecules and fragments thereof. Our expression system is based on the Chinese hamster ovary cell-dihydrofolate reductase system (54, 91, 139). The expression vector has been designed to contain unique restriction enzyme sites, which permit the various heavy- and light-chain variable regions from our different Stx1- and Stx2-specific human monoclonal antibodies to be readily exchanged. Each antibody is expressed as either IgG1, IgG2, IgG3, or IgG4 molecules because the constant regions (Fc regions, Fig. 1) of each of these antibodies are modular as well. Using the same expression vector, we have also expressed the antibodies as Fab molecules (Fig. 3). Our expression vectors allow the expression of the original human monoclonal antibodies as different recombinant isotypic antibody molecules in order to evaluate the neutralization activity of each of these antibody molecules and investigate the importance of specific effector functions associated with each isotype.

Of particular interest are the Fab molecules, which, if efficacious, may be cost-effectively produced in bacteria, yeasts, or possibly transgenic domestic animals or plants (134). Our ability to produce these antibodies as recombinant molecules permits their manipulation at the molecular biology level. For example, since we know the sequences of the variable regions of the light and heavy chains of these antibodies, we plan to create designer Fab or $(Fab')_2$ molecules which possess the Fv regions from two different antibodies to either the same toxin subunit (but to different epitopes), to different subunits (A versus B) of the same toxin, or to the two Stx toxins (Stx1 and Stx2), as illustrated in Fig. 3. Smaller antibody molecules, such as Fabs, may be advantageous if the inhibitory activity is to block toxin attachment to the cell receptor, whereby the antibody need only be present for a short time to modulate a response. In other applications where the primary function was blocking, the absence of the Fc region and its effector functions was beneficial.

It is unclear if the rate of clearance, which is influenced by valency and size, of Fabs compared to full-length antibody molecules will affect their efficacy. In mammals, full-length immunoglobulins have a longer circulating half-life, in part, due to their large size. Smaller antibody molecules, including variable fragments, single-chain variable fragments, Fabs, and $(Fab')_2$s which are less than 60 to 70 kDa in size, below the threshold for renal uptake, and are therefore rapidly cleared in the kidneys. If Fabs are efficacious but require a longer serum half-life to be effective, this can be achieved by coupling them with polyethylene glycol (11, 97).

A number of strategies are frequently employed to attempt to improve the in vivo efficacy of a given antibody (72). One such strategy is to modulate the binding affinity of the antibody, usually to increase affinity and select a thermodynami-

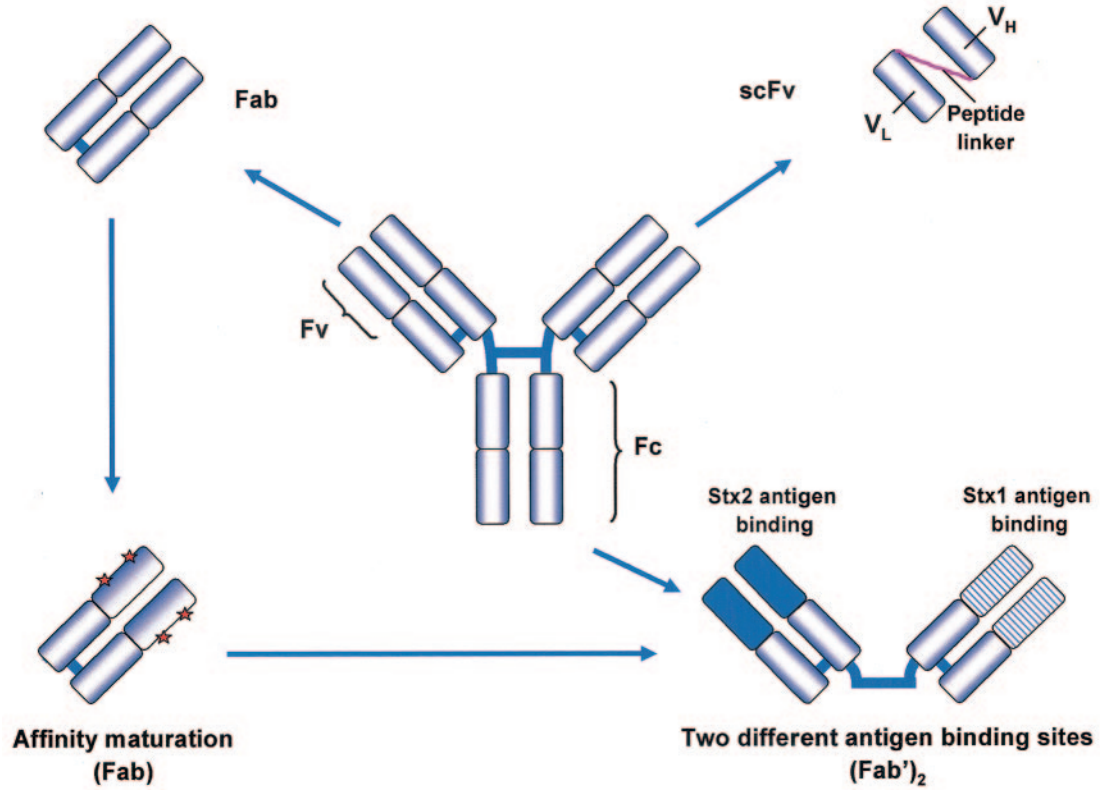

FIG. 3. Recombinant and engineered antibody molecules used in therapy. Antibodies of interest can be engineered in a number of ways to modify function and antigen specificity (lower right) or modulate binding affinity by affinity maturation (lower left; asterisks represent specific amino acid substitutions). Smaller Fab molecules (upper left), which lack the effector functions associated with the Fc regions, can be generated either by treatment with papain or by production of recombinant Fab molecules. Recombinant single-chain antibodies (scFv, upper right) can be produced once the nucleotide sequences of the variable regions of the light chain (V_L) and heavy chain (V_H) have been determined. The V_L and V_H regions are then cloned into an expression vector so that the V_L region is linked to the V_H region through a flexible glycine-serine peptide linker. This flexible linker permits the V_H and V_L regions to fold properly to form a functional antigen binding domain. Isotype variants of an IgG molecule can be created by replacement of the constant region (Fc) of one isotype with the constant region of one of the other isotypes. If the antibodies are recombinant, the constant regions can be readily swapped if a modular approach is taken in the design of the expression vector.

cally stable form of the molecule through affinity maturation experiments. Increasing the affinity of an antibody for its target will lower the dose required to protect patients and reduce the cost of treatment. Affinity maturation attempts to mimic the natural process of somatic mutation in vitro, accomplished through site-directed mutagenesis or chain-shuffling methodologies (12, 69, 72). Coupling affinity maturation with the structural information obtained by X-ray crystallography or nuclear magnetic resonance can identify critical amino acid residues within one or both toxin subunits and allow specific residues to be targeted for mutagenesis or left unaltered if they are absolutely required for activity. These engineered antibodies can be screened by flow cytometry to select antibodies with increased binding affinities (16). Surface plasmon resonance is another valuable tool to measure the rate of dissociation of the antibody-antigen complex of these antibodies (68).

CONCLUSIONS AND PROSPECTS

HUS is a serious disease which affects young children in the United States and other developed countries. STEC, the causative bacteria, are also high on the national list of food- and water-borne pathogens that can potentially be used as agents of bioterrorism. The lack of protective or therapeutic measures against HUS under these circumstances is a serious problem which must be addressed. Here we have reviewed the pertinent aspects of the disease and the toxins responsible and outlined an approach we have taken which we believe may provide a safe and effective therapy against HUS. In this context, we have developed and characterized a panel of human monoclonal antibodies which are highly effective against Stx2, Stx2 variants, and Stx1. The preclinical evaluation of several of them in the piglet model showed a great deal of promise as a potential therapy against HUS. A highly effective Stx2-specific human monoclonal antibody with a wide spectrum of activity against Stx2 and Stx2 variants was selected for phase I, II, and III clinical trials, which will hopefully begin shortly. Phase I clinical trials will determine the pharmacokinetics, including the half-life, safety, and antibody dose required to achieve a serum level of ≈5 μg/ml in human volunteers. Phases II and III will establish whether this antibody-based therapy protects children presenting with bloody diarrhea against developing HUS. Phases II and III will also determine whether the administration of higher doses of the antibody significantly modifies the clinical outcome of children presenting with HUS. An important issue which is yet to be addressed concerns the role

of Stx1 in HUS; since it is assumed that Stx2 is the toxin closely linked to HUS, will Stx2-specific antibody be sufficient to prevent the development of HUS in patients infected with STEC that produce Stx1 as well as Stx2?

Finally, the observation that piglets can still be successfully treated with a high dose of Stx-specific antibody at the onset of CNS symptoms gives heart that this therapy is not only more than likely to protect children presenting with bloody diarrhea but may do so even when given in high doses at the onset of HUS. The antibody will in particular help protect symptomatic and asymptomatic children exposed to known sources of infection.

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REFERENCES

1. **Andreoli, S. P., H. Trachtman, D. W. K. Acheson, R. L. Siegler, and T. G. Obrig.** 2002. Hemolytic uremic syndrome: epidemiology, pathophysiology, and therapy. Pediatr. Nephrol. **17:**293–298.
2. **Banatvala, N., P. M. Griffin, K. D. Greene, T. J. Barrett, W. F. Bibb, J. H. Green, and J. G. Wells.** 2001. The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical, and epidemiologic findings. J. Infect. Dis. **183:**1063–1070.
3. **Bell, B. P., P. M. Griffin, P. Lozano, D. L. Christie, J. M. Kobayashi, and P. I. Tarr.** 1997. Predictors of hemolytic uremic syndrome in children during a large outbreak of *Escherichia coli* O157:H7 infections. Pediatrics **100:**E12.
4. **Bielaszewska, M., H. Schmidt, M. A. Karmali, R. Khakhria, J. Janda, K. Blahova, and H. Karch.** 1998. Isolation and characterization of sorbitol-fermenting Shiga toxin (verocytotoxin)-producing *Escherichia coli* O157:H− strains in the Czech Republic. J. Clin. Microbiol. **36:**2135–2137.
5. **Bitzan, M., M. Klemt, R. Steffens, and D. E. Muller-Wiefel.** 1993. Differences in verotoxin neutralizing activity of therapeutic immunoglobulins and sera from healthy controls. Infection **21:**140–145.
6. **Bonnet, R., B. Souweine, G. Gauthier, C. Rich, V. Livrelli, J. Sirot, B. Joly, and C. Forestier.** 1998. Non-O157:H7 Stx2-producing *Escherichia coli* strains associated with sporadic cases of hemolytic-uremic syndrome in adults. J. Clin. Microbiol. **36:**1777–1780.
7. **Bruggemann, M., and M. J. Taussig.** 1997. Production of human antibody repertoires in transgenic mice. Curr. Opin. Biotechnol. **8:**455–458.
8. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin. J. Bacteriol. **169:**4759–4764.
9. **Chalmers, R. M., S. M. Parry, R. L. Salmon, R. M. Smith, G. A. Willshaw, and T. Cheasty.** 1999. The surveillance of vero cytotoxin-producing *Escherichia coli* O157 in Wales, 1990 to 1998. Emerg. Infect. Dis. **5:**566–569.
10. **Chandler, W. L., S. Jelacic, D. R. Boster, M. A. Ciol, G. D. Williams, S. L. Watkins, T. Igarashi, and P. I. Tarr.** 2002. Prothrombotic coagulation abnormalities preceding the hemolytic uremic syndrome. N. Engl. J. Med. **346:**23–32.
11. **Chapman, A. P., P. Antoniw, M. Spitali, S. West, S. Stephens, and D. J. King.** 1999. Therapeutic antibody fragments with prolonged in vivo half-lives. Nat. Biotechnol. **17:**780–783.
12. **Chowdhury, P. S., and I. Pastan.** 1999. Improving antibody affinity by mimicking somatic hypermutation in vitro. Nat. Biotechnol. **17:**568–572.
13. **Clark, C., D. Bast, A. Sharp, P. M. St. Hilaire, R. Agha, P. E. Stein, E. J. Toone, R. J. Read, and J. L. Brunton.** 1996. Phenylalanine 30 plays an important role in receptor binding of verotoxin-1. Mol. Microbiol. **19:**891–899.
14. **Cornick, N. A., I. Matise, J. E. Samuel, B. T. Bosworth, and H. W. Moon.** 2000. Shiga toxin-producing Escherichia coli infection: temporal and quantitative relationships among colonization, toxin production, and systemic disease. J. Infect. Dis. **181:**242–251.
15. **Cowan, L. A., D. M. Hertzke, B. W. Fenwick, and C. B. Andreasen.** 1997. Clinical and clinicopathologic abnormalities in greyhounds with cutaneous and renal glomerular vasculopathy: 18 cases (1992–1994). J. Am. Vet. Med. Assoc. **210:**789–793.
16. **Daugherty, P., B. Iverson, and G. Georgiou.** 2000. Flow cytometric screening of cell-based libraries. J. Immunol. Methods **243:**211–227.
17. **Dean-Nystrom, E. A., J. F. Pohlenz, H. W. Moon, and A. D. O'Brien.** 2000. *Escherichia coli* O157:H7 causes more-severe systemic disease in suckling piglets than in colostrum-deprived neonatal piglets. Infect. Immun. **68:**2356–2358.
18. **Donohue-Rolfe, A., I. Kondova, S. Oswald, D. Hutto, and S. Tzipori.** 2000. Escherichia coli O157:H7 strains that express Shiga toxin (Stx) 2 alone are more neurotropic for gnotobiotic piglets than are isotypes producing only Stx1 or both Stx1 and Stx2. J. Infect. Dis. **181:**1825–1829.
19. **Donohue-Rolfe, A., M. Jacewicz, and G. T. Keusch.** 1989. Isolation and characterization of functional shiga toxin subunits and renatured holotoxin. Mol. Microbiol. **3:**1231–1236.
20. **Donohue-Rolfe, A., I. Kondova, J. Mukherjee, K. Chios, D. Hutto, and S. Tzipori.** 1999. Antibody-based protection of gnotobiotic piglets infected with *Escherichia coli* O157:H7 against systemic complications associated with Shiga toxin 2. Infect. Immun. **67:**3645–3648.
21. **Edwards, A. C., A. R. Melton-Celsa, K. Arbuthnott, S. J. R., C. K. Schmitt, H. C. Wong, and A. O'Brien.** 1998. Vero cell neutralization and mouse protective efficacy of humanized monoclonal antibodies against *Escherichia coli* toxins Stx1 and Stx2, p. 388–392. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D.C.
22. **Essher, Z.** 1985. Monoclonal antibody strategy and techniques. Plenum Press, New York, N.Y.
23. **Fenwich, R. W., and L. A. Cowan.** 1998. Canine model of hemolytic uremic syndrome. In *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains, p. 268–277. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D.C.
24. **Fishwild, D. M., S. L. O'Donnell, T. Bengoechea, D. V. Hudson, F. Harding, S. L. Bernhard, D. Jones, R. M. Kay, K. M. Higgins, S. R. Schramm, and N. Lonberg.** 1996. High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. Nat. Biotechnol. **14:**845–851.
25. **Francis, D. H., J. E. Collins, and J. R. Duimstra.** 1986. Infection of gnotobiotic pigs with an *Escherichia coli* O157:H7 strain associated with an outbreak of hemorrhagic colitis. Infect. Immun. **51:**953–956.
26. **Friedrich, A. W., M. Bielaszewska, W. L. Zhang, M. Pulz, T. Kuczius, A. Ammon, and H. Karch.** 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J. Infect. Dis. **185:**74–84.
27. **Fuchs, S., I. Muhldorfer, A. Donohue-Rolfe, M. Kerenyi, L. Emody, R. Alexiev, P. Nenkov, and J. Hacker.** 1999. Influence of RecA on in vivo virulence and Shiga toxin 2 production in *Escherichia coli* pathogens. Microb. Pathog. **27:**13–23.
28. **Furutani, M., K. Ito, Y. Oku, Y. Takeda, and K. Igarashi.** 1990. Demonstration of RNA *N*-glycosidase activity of a verotoxin (VT2 variant) produced by *Escherichia coli* 091:H21 from patient with the hemolytic uremic syndrome. Microbiol. Immunol. **34:**387–392.
29. **Gallo, G., and C. Gianantonio.** 1994. Extrarenal involvement in diarrhea-associated haemolytic-uremic syndrome. Pediatr. Nephrol. **9:**117–119.
30. **Gannon, V. P., C. L. Gyles, and B. P. Wilcock.** 1989. Effects of *Escherichia coli* Shiga-like toxins (verotoxins) in pigs. Can. J. Vet. Res. **53:**306–312.
31. **Gannon, V. P., C. Teerling, S. A. Masri, and C. L. Gyles.** 1990. Molecular cloning and nucleotide sequence of another variant of the *Escherichia coli* Shiga-like toxin II family. J. Gen. Microbiol. **136:**1125–1135.
32. **Garcia, A., R. P. Marini, Y. Feng, A. Vitsky, K. A. Knox, N. S. Taylor, D. B. Schauer, and J. G. Fox.** 2003. A naturally occurring rabbit model of enterohemorrhagic *Escherichia coli*-induced disease. J. Infect. Dis. **186:**1682–1686.
33. **Gasser, C., C. Gautier, R. Steck, et al.** 1955. Hamolytisch-uramische syndrome: bilaterle niernindennekrosen bei akuten erworbenen hamolytischen anamkien. Schweiz. Med. Weochenschr. **85:**905–909.
34. **Gerber, A., H. Karch, F. Allerberger, H. M. Verweyen, and L. B. Zimmerhackl.** 2002. Clinical course and the role of shiga toxin-producing *Escherichia coli* infection in the hemolytic-uremic syndrome in pediatric patients, 1997–2000, in Germany and Austria: a prospective study. J. Infect. Dis. **15;186:**493–500.
35. **Gomez, S. A., G. C. Fernandez, S. Vanzulli, G. Dran, C. Rubel, T. Berki, M. A. Isturiz, and M. S. Palermo.** 2003. Endogenous glucocorticoids attenuate Shiga toxin-2-induced toxicity in a mouse model of haemolytic uraemic syndrome. Clin. Exp. Immunol. **131:**217–224.
36. **Griffin, P. M., and R. V. Tauxe.** 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol. Rev. **13:**60–98.
37. **Gunzer, F., I. Hennig-Pauka, K. H. Waldmann, R. Sandhoff, H. J. Grone, H. H. Kreipe, A. Matussek, and M. Mengel.** 2002. Gnotobiotic piglets develop thrombotic microangiopathy after oral infection with enterohemorrhagic *Escherichia coli*. Am. J. Clin. Pathol. **118:**364–375.
38. **Halloran, P. F., and S. Prommool.** 1998. Humanized monoclonals and other biological initiatives. Clin. Biochem. **31:**353–357.
39. **Hamano, S., Y. Nakanishi, T. Nara, T. Seki, T. Ohtani, T. Oishi, K. Joh, T. Oikawa, Y. Muramatsu, Y. Ogawa, and S. Akashi.** 1993. Neurological manifestations of hemorrhagic colitis in the outbreak of *Escherichia coli* O157:H7 infection in Japan. Acta Paediatr. **82:**454–458.
40. **Harding, F. A., and N. Lonberg.** 1995. Class switching in human immunoglobulin transgenic mice. Ann. N. Y. Acad. Sci. **764:**536–546.

41. **Head, S. C., M. A. Karmali, and C. A. Lingwood.** 1991. Preparation of VT1 and VT2 hybrid toxins from their purified dissociated subunits. J. Biol. Chem. **266:**3617–3621.
42. **Head, S. C., M. Petric, S. Richardson, M. Roscoe, and M. A. Karmali.** 1988. Purification and characterization of verocytotoxin 2. FEMS Microbiol. Lett. **51:**211–216.
43. **Hertzke, D. M., L. A. Cowan, P. Schoning, and B. W. Fenwick.** 1995. Glomerular ultrastructural lesions of idiopathic cutaneous and renal glomerular vasculopathy of greyhounds. Vet. Pathol. **32:**451–459.
44. **Heuvelink, A. E., N. C. Van de Kar, T. J. Van Der Velden, H. Chart, and L. A. Monnens.** 1999. Verocytotoxin-producing *Escherichia coli* infection in household members of children with hemolytic-uremic syndrome in the Netherlands. Pediatr. Infect. Dis. J. **18:**709–714.
45. **Ikeda, K., O. Ida, K. Kimoto, T. Takatorige, N. Nakanishi, and K. Tatara.** 1999. Effect of early fosfomycin treatment on prevention of hemolytic uremic syndrome accompanying *Escherichia coli* O157:H7 infection. Clin. Nephrol. **52:**357–362.
46. **Islam, M. S., and W. H. Stimson.** 1990. Production and characterization of monoclonal antibodies with therapeutic potential against Shiga toxin. J. Clin. Lab. Immunol. **33:**11–16.
47. **Ito, H., A. Terai, H. Kurazono, Y. Takeda, and M. Nishibuchi.** 1990. Cloning and nucleotide sequencing of Vero toxin 2 variant genes from *Escherichia coli* O91:H21 isolated from a patient with the hemolytic uremic syndrome. Microb. Pathog. **8:**47–60.
48. **Jacewicz, M., H. Clausen, E. Nudelman, A. Donohue-Rolfe, and G. T. Keusch.** 1986. Pathogenesis of *Shigella* diarrhea. XI. Isolation of a *Shigella* toxin-binding glycolipid from rabbit jejunum and Hela cells and its identification as globotriosylceramide. J. Exp. Med. **163:**1391–1404.
49. **Jackson, M. P., R. J. Neill, A. D. O'Brien, R. K. Holmes, and J. W. Newland.** 1987. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin 2 encoded by bateriophages from *Escherichia coli* 933. FEMS Microbiol. Lett. **44:**109–114.
50. **Kaplan, B. A., K. E. Meyers, and S. L. Schulman.** 1998. The pathogenesis and treatment of hemolytic uremic syndrome. J. Am. Soc. Nephrol. **9:**1126–1133.
51. **Kar, N. C. van de, L. A. Monnens, M. A. Karmali, and V. W. van Hinsbergh.** 1992. TNF-α and IL-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial cells: Implications for the pathogenesis of HUS. Blood **80:**2755–2764.
52. **Karmali, M., B. Steele, M. Petric, and C. Lim.** 1983. Sporadic cases of haemolytic uremic syndrome associated with faecal cytotoxin and cytotoxin-producing *E. coli* in stools. Lancet **i:**619–620.
53. **Karpman, D., A. Andreasson, H. Thysell, B. S. Kaplan, and C. Svanborg.** 1995. Cytokines in childhood hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Pediatr. Nephrol. **9:**694–699.
54. **Kaufman, R. J., L. C. Wasley, A. J. Spiliotes, S. D. Gossels, S. A. Latt, G. R. Larsen, and R. M. Kay.** 1985. Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. Mol. Cell. Biol. **5:**1750–1759.
55. **Keusch, G. T., M. Jacewicz, D. W. Acheson, A. Donohue-Rolfe, A. V. Kane, and R. H. McCluer.** 1995. Globotriaosylceramide, Gb3, is an alternative functional receptor for Shiga-like toxin 2e. Infect. Immun. **63:**1138–1141.
56. **Kimura, T., M. S. Co, M. Vasquez, S. Wei, H. Xu, S. Tani, Y. Sakai, T. Kawamura, Y. Matsumoto, H. Nakao, and T. Takeda.** 2002. Development of humanized monoclonal antibody TMA-15 which neutralizes Shiga toxin 2. Hybrid Hybridomics **21:**161–168.
57. **Kitov, P. I., J. M. Sadowska, G. Mulvey, G. Armstrong, H. Ling, N. S. Pannu, R. J. Read, and D. R. Bundle.** 2000. Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. Nature **403:**669–672.
58. **Kleanthous, H., H. R. Smith, S. M. Scotland, R. J. Gross, B. Rowe, C. M. Taylor, and D. V. Milford.** 1990. Haemolytic uraemic syndromes in the British Isles, 1985–1988: association with verocytotoxin producing *Escherichia coli*. 2. Microbiological aspects. Arch. Dis. Child. **65:**722–727.
59. **Lingwood, C. A.** 1996. Role of verotoxin receptors in pathogenesis. Trends Microbiol. **4:**147–153.
60. **Lingwood, C. A.** 1994. Verotoxin-binding in human renal sections. Nephron **66:**21–28.
61. **Lonberg, N., and D. Huszar.** 1995. Human antibodies from transgenic mice. Int. Rev. Immunol. **13:**65–93.
62. **Lonberg, N., L. D. Taylor, F. A. Harding, M. Trounstine, K. M. Higgins, S. R. Schramm, C. C. Kuo, R. Mashayekh, K. Wymore, J. G. McCabe, et al.** 1994. Antigen-specific human antibodies from mice comprising four distinct genetic modifications. Nature **368:**856–859.
63. **Louise, C. B., and T. G. Obrig.** 1995. Specfiic interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. J. Infect. Dis. **172:**1397–1401.
64. **Ludwig, K., E. Grabhorn, M. Bitzan, C. Bobrowski, M. J. Kemper, I. Sobottka, R. Laufs, H. Karch, and D. E. Muller-Wiefel.** 2002. Saliva IgM and IgA are a sensitive indicator of the humoral immune response to *Escherichia coli* O157 lipopolysaccharide in children with enteropathic hemolytic uremic syndrome. Pediatr. Res. **52:**307–313.
65. **Mackenzie, A. M. R., P. Rowe, and P. McLaine.** 2000. Stx1 and Stx2 among STEC-producing strains isolated from sporadic cases of HUS in Canada (personal communication).
66. **MacLeod, D. L., C. L. Gyles, and B. P. Wilcock.** 1991. Reproduction of edema disease of swine with purified Shiga-like toxin-II variant. Vet. Pathol. **28:**66–73.
67. **Mainil, J.** 1999. Shiga/verocytotoxins and Shiga/verotoxigenic *Escherichia coli* in animals. Vet. Res. **30:**235–257.
68. **Malmborg, A. C., and C. A. Borrebaeck.** 1995. BIAcore as a tool in antibody engineering. J. Immunol. Methods **183:**7–13.
69. **Marks, J. D., A. D. Griffiths, M. Malmqvist, T. P. Clackson, J. M. Bye, and G. Winter.** 1992. By-passing immunization building high affinity human antibodies by chain shuffling. Biotechnology **10:**779–783.
70. **Marques, L. R. M., J. S. M. Peiris, S. J. Cryz, and A. D. O'Brien.** 1987. *Escherichia coli* strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II. FEMS Microbiol. Lett. **44:**281–283.
71. **Matise, I., N. A. Cornick, S. L. Booher, J. E. Samuel, B. T. Bosworth, and H. W. Moon.** 2001. Intervention with Shiga toxin (Stx) antibody after infection by Stx-producing *Escherichia coli*. J. Infect. Dis. **183:**347–350.
72. **Maynard, J., and G. Georgiou.** 2000. Antibody engineering. Annu. Rev. Biomed. Eng. **2:**339–376.
73. **Mead, S. P., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, G. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
74. **Melton-Celsa, A. R., J. E. Rogers, C. K. Schmitt, S. C. Darnell, and A. D. O'Brien.** 1998. Virulence of Shiga toxin-producing *Escherichia coli* (STEC) in orally-infected mice correlates with the type of toxin produced by the infecting strain. Jpn. J. Med. Sci. Biol. **51:**S108–114.
75. **Meyer, T., H. Karch, J. Hacker, H. Bocklage, and J. Heesemann.** 1992. Cloning and sequencing of a Shiga-like toxin II-related gene from *Escherichia coli* O157:H7 strain 7279. Zentralbl. Bakteriol. **276:**176–188.
76. **Milford, D. V., C. M. Taylor, B. Guttridge, S. M. Hall, B. Rowe, and H. Kleanthous.** 1990. Haemolytic uraemic syndromes in the British Isles 1985–8: association with verocytotoxin producing *Escherichia coli*. 1. Clinical and epidemiological aspects. Arch. Dis. Child. **65:**716–721.
77. **Moxley, R. A., and D. H. Francis.** 1998. Overview of animal models, p. 249–260. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D.C.
78. **Mühldorfer, I., J. Hacker, G. T. Keusch, D. W. Acheson, H. Tschäpe, A. V. Kane, A. Ritter, T. Ölschläger, and A. Donohue-Rolfe.** 1996. Regulation of the Shiga-like toxin operon in *Escherichia coli*. Infect. Immun. **64:**495–502.
79. **Mukherjee, J., K. Chios, D. M. Fishwild, D. Hudson, S. L. O'Donnell, S. Rich, A. Donohue-Rolfe, and S. Tzipori.** 2002. Human Stx2-specific monoclonal antibodies prevent systemic complications of *Escherichia coli* O157:H7 infection. Infect. Immun. **70:**612–619.
80. **Mukherjee, J., K. Chios, D. M. Fishwild, D. Hudson, S. L. O'Donnell, S. Rich, A. Donohue-Rolfe, and S. Tzipori.** 2002. Production and characterization of protective human antibodies against Shiga toxin 1 (Stx1). Infect. Immun. **70:**5896–5899.
81. **Nakajima, H., N. Kiyokawa, Y. U. Katagiri, T. Taguchi, T. Suzuki, T. Sekino, K. Mimori, T. Ebata, M. Saito, H. Nakao, T. Takeda, and J. Fujimoto.** 2001. Kinetic analysis of binding between Shiga toxin and receptor glycolipid Gb3Cer by surface plasmon resonance. J. Biol. Chem. **276:**42915–42922.
82. **Neely, M. N., and D. I. Friedman.** 1998. Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. Mol. Microbiol. **28:**1255–1267.
83. **Nicholson, I. C., X. Zou, A. V. Popov, G. P. Cook, E. M. Corps, S. Humphries, C. Ayling, B. Goyenechea, J. Xian, M. J. Taussig, M. S. Neuberger, and M. Bruggemann.** 1999. Antibody repertoires of four- and five-feature translocus mice carrying human immunoglobulin heavy chain and kappa and lambda light chain yeast artificial chromosomes. J. Immunol. **163:**6898–6906.
84. **Nishikawa, K., K. Matsuoka, E. Kita, N. Ojkabe, M. Mizuguchi, K. Hino, S. Miyazawa, C. Yamasaki, J. Aoki, S. Takashima, Y. Yamakawa, M. Nishijima, D. Terunuma, H. Kuzuhara, and Y. Natori.** 2002. A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin producing *Escherichia coli* O157:H7. Proc. Natl. Acad. Sci. USA **99:**7669–7674.
85. **Nowakowski, A., C. Wang, D. B. Powers, P. Amersdorfer, T. J. Smith, V. A. Montgomery, R. Sheridan, R. Blake, L. A. Smith, and J. D. Marks.** 2002. Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. Proc. Natl. Acad. Sci. USA **99:**11346–11350.
86. **O'Brien, A. D., J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith, and S. B. Formal.** 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis of infantile diarrhea. Science **226:**694–696.
87. **O'Brien, A. D., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. T. Keusch.** 1992. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. Curr. Top. Microbiol. Immunol. **180:**65–94.

88. **Obrig, T. G., P. J. Del Vecchio, J. E. Brown, T. P. Moran, B. M. Rowland, T. K. Judge, and S. W. Rothman.** 1988. Direct cytotoxic action of Shiga toxin in human vascular endothelial cells. Infect. Immun. **56:**2373–2378.
89. **Ostroff, S. M., P. I. Tarr, M. A. Neill, J. H. Lewis, N. Hargrett-Bean, and J. M. Kobayashi.** 1989. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J. Infect. Dis. **160:**994–998.
90. **Padhye, V. V., T. Zhao, and M. P. Doyle.** 1989. Production and characterisation of monoclonal antibodies to Verotoxins 1 and 2 from Escherichia coli of serotype O 157:H7. J. Med. Microbiol. **30:**219–226.
91. **Page, M. J., and M. A. Sydenham.** 1991. High level expression of the humanized monoclonal antibody Campath-1H in Chinese hamster ovary cells. Biotechnology (N.Y.) **9:**64–68.
92. **Paton, A. W., R. Morona, and J. C. Paton.** 2000. A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. Nat. Med. **6:**265–270.
93. **Paton, A. W., J. C. Paton, M. W. Heuzenroeder, P. N. Goldwater, and P. A. Manning.** 1992. Cloning and nucleotide sequence of a variant Shiga-like toxin II gene from *Escherichia coli* OX3:H21 isolated from a case of sudden infant death syndrome. Microb. Pathog. **13:**225–236.
94. **Paton, A. W., J. C. Paton, P. N. Goldwater, M. W. Heuzenroeder, and P. A. Manning.** 1993. Sequence of a variant Shiga-like toxin type-I operon of *Escherichia coli* O111:H. Gene **129:**87–92.
95. **Paton, J. C., and A. W. Paton.** 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. **11:**450–479.
96. **Pavia, A., C. R. Nichols, D. P. Green, R. V. Tauxe, S. Mottice, K. D. Greene, J. G. Wells, R. L. Siegler, E. D. Brewer, D. Hannon, et al.** 1990. Hemolytic uremic syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiological observations. J. Pediatr. **116:**544–551.
97. **Pedley, R. B., J. A. Boden, R. Boden, R. H. J. Begent, A. Turner, A. M. R. Haines, and D. J. King.** 1994. The potential for enhanced tumour localisation by poly(ethylene glycol) modification of anti-CEA antibody. Br. J. Cancer **70:**1126–1130.
98. **Perera, L. P., L. R. Marques, and A. D. O'Brien.** 1988. Isolation and characterization of monoclonal antibodies to Shiga-like toxin II of enterohemorrhagic *Escherichia coli* and use of the monoclonal antibodies in a colony enzyme-linked immunosorbent assay. J. Clin. Microbiol. **26:**2127–2131.
99. **Perez, N., F. Spizziri, R. Rahman, A. Suarez, C. Larrubia, and P. Lasarte.** 1998. Steroids in hemolytic uremic syndrome. Pediatr. Nephrol. **12:**101–104.
100. **Pierard, D., G. Muyldermans, L. Moriau, D. Stevens, and S. Lauwers.** 1998. Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. J. Clin. Microbiol. **36:**3317–3322.
101. **Proulx, F., J. Turgeon, G. Delage, L. Lafleur, and L. Chicoine.** 1992. Randomized controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis. J. Pediatr. **121:**299–303.
102. **Raife, T., and R. Montgomery.** 2001. New aspects in the pathogenesis and treatment of thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. Rev. Clin. Exp. Hematol. **5:**236–261.
103. **Ramachandran, V., M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic.** 2001. The common ovine Shiga toxin 2-containing *Escherichia coli* serotypes and human isolates of the same serotypes possess an Stx2d toxin type. J. Clin. Microbiol. **39:**1932–1937.
104. **Ray, P. E., and X. H. Liu.** 2001. Pathogenesis of Shiga toxin-induced hemolytic uremic syndrome. Pediatr. Nephrol. **16:**823–839.
105. **Reichert, J. M.** 2002. Therapeutic monoclonal antibodies: trends in development and approval in the U.S. Curr. Opin. Mol. Ther. **4:**110–118.
106. **Reisbig, R., S. Olsnes, and K. Eiklid.** 1981. The cytotoxic activity of Shigella toxin. Evidence for catalytic inactivation of the 60S ribosomal subunit. J. Biol. Chem. **256:**8739–8744.
107. **Robson, W. L. M., G. H. Fick, T. Jadavji, and A. K. Leung.** 1991. The use of intravenous gammaglobulin in the treatment of atypical hemolytic uremic syndrome. Pediatr. Nephrol. **5:**289–292.
108. **Russmann, H., H. Schmidt, J. Heesemann, A. Caprioli, and H. Karch.** 1994. Variants of Shiga-like toxin II constitute a major toxin component in *Escherichia coli* O157 strains from patients with haemolytic uraemic syndrome. J. Med. Microbiol. **40:**338–343.
109. **Safdar, N., A. Said, R. E. Gangnon, and D. G. Maki.** 2002. Risk of hemolytic uremic syndrome after antibiotic therapy of *Escherichia coli* O157:H7 enteritis. JAMA **288:**996–1001.
110. **Schmidt, H., J. Scheef, S. Morabito, A. Caprioli, L. H. Wieler, and H. Karch.** 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl. Environ. Microbiol. **66:**1205–1208.
111. **Schmitt, C. K., M. L. McKee, and A. D. O'Brien.** 1991. Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H- strain E32511. Infect. Immun. **59:**1065–1073.
112. **Scotland, S. M., G. A. Willshaw, H. R. Smith, and B. Rowe.** 1987. Properties of strains of *Escherichia coli* belonging to serogroup O157 with special reference to production of Vero cytotoxins VT1 and VT2. Epidemiol. Infect. **99:**613–624.
113. **Sheoran, A. S., S. Chapman, P. Singh, A. Donohue-Rolfe, and S. Tzipori.** 2003. Stx2-spcific human monoclonal antibodies protect mice against lethal infection with *Escherichia coli* expressing Stx2 variants. Infect. Immun. **71:**3125–3130.
114. **Shimizu, H., R. A. Field, S. W. Homans, and A. Donohue-Rolfe.** 1998. Solution structure of the complex between the B-subunit homopentamer of verotoxin VT-1 from *Escherichia coli* and the trisaccharide moiety of globotriaosylceramide. Biochemistry **37:**11078–11082.
115. **Siegler, R. L., T. J. Pysher, R. Lou, V. L. Tesh, and F. B. Taylor, Jr.** 2001. Response to Shiga toxin-1, with and without lipopolysaccharide, in a primate model of hemolytic uremic syndrome. Am. J. Nephrol. **21:**420–425.
116. **Siegler, R. L.** 1994. Spectrum of extrarenal involvement in postdiarrheal hemolytic-uremic syndrome. J. Pediatr. **125:**511–518.
117. **Slutsker, L., A. A. Ries, K. Maloney, J. G. Wells, K. D. Greene, and P. M. Griffin.** 1998. A nationwide case-control study of *Escherichia coli* O157:H7 infection in the United States. J. Infect. Dis. **177:**962–966.
118. **Strockbine, N. A., L. R. Marques, R. K. Holmes, and A. D. O'Brien.** 1985. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. Infect. Immun. **50:**695–700.
119. **Tarr, P. I., and M. A. Neill.** 2001. *Escherichia coli* O157:H7. Gastroenterol. Clin. North Am. **30:**735–751.
120. **Tarr, P. I.** 1995. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin. Infect. Dis. **20:**1–8.
121. **Taylor, L. D., C. E. Carmack, S. R. Schramm, R. Mashayekh, K. M. Higgins, C. C. Kuo, C. Woodhouse, R. M. Kay, and N. Lonberg.** 1992. A transgenic mouse that expresses a diversity of human sequence heavy and light chain immunoglobulins. Nucleic Acids Res. **20:**6287–6295.
122. **Te Loo, D. M. W. M., V. W. M. van Hinsbergh, L. P. W. J. van den Heuvel, and L. A. H. Monnens.** 2001. Detection of verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with hemolytic uremic syndrome. J. Am. Soc. Nephrol. **12:**800–806.
123. **Tesh, V. L., J. A. Burris, J. W. Owens, V. M. Gordon, E. A. Wadolkowski, A. D. O'Brien, and J. E. Samuel.** 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect. Immun. **61:**3392–3402.
124. **Thomas, A., T. Cheasty, H. Chart, and B. Rowe.** 1994. Isolation of Vero cytotoxin-producing *Escherichia coli* serotypes O9ab:H- and O101:H-carrying VT2 variant gene sequences from a patient with haemolytic uraemic syndrome. Eur. J. Clin. Microbiol. Infect. Dis. **13:**1074–1076.
125. **Trachtman, H., and C. Christen.** 1999. Hemolytic uremic syndrome: current understanding of the pathogenesis and therapeutic trials and interventions. Curr. Opin. Pediatr. **11:**162–168.
126. **Trachtman, H., A. Cnaan, E. Christen, K. Gibbs, S. Zhao, D. W. Acheson, R Weiss, F. J. Kaskel, A. Spitzer, and G. H. Hirschman.** 2003. Investigators of the HUS-SYNSORB Pk Multicenter Clinical Trial. Effect of an oral Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: a randomized controlled trial. JAMA **10:**1337–1344.
127. **Tuaillon, N., L. D. Taylor, N. Lonberg, P. W. Tucker, and J. D. Capra.** 1993. Human immunoglobulin heavy-chain minilocus recombination in transgenic mice: gene-segment use in mu and gamma transcripts. Proc. Natl. Acad. Sci. USA **90:**3720–3724.
128. **Tzipori, S., K. I. Wachsmuth, and C. Chapman.** 1986. Studies on the pathogenesis of haemorrhagic colitis by *Escherichia coli* O157:H7 in gnotobiotic piglets. J. Infect. Dis. **154:**712–716.
129. **Tzipori, S., F. Gunzer, L. de Montigny, M. Donnenberg, J. Kaper, and A. Donohue-Rolfe.** 1995. The role of the *eaeA* gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. Infect. Immun. **63:**3621–3627.
130. **Tzipori, S., C. W. Chow, and H. R. Powell.** 1988. Cerebral involvement with *Escherichia coli* O157:H7 in humans and gnotobiotic piglets. J. Clin. Pathol. **41:**1099–1103.
131. **Tzipori, S., H. Karch, K. I. Wachsmuth, R. M. Robins-Browne, A. D. O'Brien, H. Lior, M. L. Cohen, J. Smithers, and M. M. Levine.** 1987. Role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. Infect. Immun. **55:**3117–3125.
132. **Tzipori, S., R. Gibson, and J. Montanaro.** 1989. Nature and distribution of mucosal lesions associated with enteropathogenic and enterohemorrhagic *Escherichia coli* in piglets and the role of plasmid-mediated factors. Infect. Immun. **57:**1142–1150.
133. **Van Setten, P. A., V. W. M. Van Hinsbergh, L. P. W. J. Van Den Heuvel, F. Preyers, H. B. P. M. Dijkman, K. J. M. Assmann, T. J. A. M. Van Der Velden, and L. A. H. Monnens.** 1998. Monocyte chemoattractant protein-1 and interleukin-8 levels in urine and serum of patients with hemolytic uremic syndrome. Pediatr. Res. **43:**759–767.
134. **Verma, R., E. Boleti, and A. J. T. George.** 1998. Antibody engineering: Comparison of bacterial, yeast, insect and mammalian expression systems. J. Immunol. Methods **216:**165–181.
135. **Wadolkowski, E. A., J. A. Burris, and A. D. O'Brien.** 1990. Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **58:**2438–2445.

136. **Wang, H., E. Christen, K. J. Tracey, and H. Trachtman.** 2001. HMG-1 excretion is increased in children with diarrheal-associated hemolytic uremic syndrome. J. Am. Soc. Nephrol. **12:**185A (abstract).
137. **Weinstein, D. L., M. P. Jackson, J. E. Samuel, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of a Shiga-like toxin type II variant from *Escherichia coli* strain responsible for edema disease of swine. J. Bacteriol. **170:**4223–4230.
138. **Wong, C. S., S. Jelacic, R. L. Habeeb, S. L. Watkins, and P. I. Tarr.** 2000. The risk of hemolytic-uremic syndrome after antibiotic therapy of *Escherichia coli* O157:H7 infections. N. Engl. J. Med. **342:**1930–1936.
139. **Wood, C. R., A. J. Dorner, G. E. Morris, E. M. Alderman, D. Wilson, R. M. O'Hara, Jr., and R. J. Kaufman.** 1990. High level synthesis of immunoglobulins in Chinese hamster ovary cells. J. Immunol. **145:**3011–3016.
140. **Woods, J. B., C. K. Schmitt, S. C. Darnell, K. C. Meysick, and A. D. O'Brien.** 2002. Ferrets as a model system for renal disease secondary to intestinal infection with Escherichia coli O157:H7 and other Shiga toxin-producing *E. coli*. J. Infect. Dis. **185:**550–554.
141. **Yamagami, S., M. Motoki, T. Kimura, H. Izumi, T. Takeda, Y. Katsuura, and Y. Matsumoto.** 2001. Efficacy of postinfection treatment with anti-Shiga toxin (Stx) 2 humanized monoclonal antibody TMA-15 in mice lethally challenged with Stx-producing *Escherichia coli*. J. Infect. Dis. **184:**738–742.
142. **Zhang, X., A. D. McDaniel, L. E. Wolf, G. T. Keusch, M. K. Waldor, and D. W. K. Acheson.** 2000. Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. J. Infect. Dis. **181:**664–670.