The Nature of Immunity to the *Escherichia coli* Shiga Toxins (Verocytotoxins) and Options for Toxoid Immunization

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INTRODUCTION

Shiga-toxin (stx)-producing *Escherichia coli* (STEC), also referred to as Verocytotoxin (VT)-producing *E. coli* (VTEC) (25) are causes of a major zoonotic food-borne illness (12, 20) whose spectrum includes non-specific diarrhea, hemorrhagic colitis (42), and the hemolytic uremic syndrome (HUS) (21, 24) which is the leading cause of acute renal failure in children. Not only do VTEC rival non-typhoidal *Salmonella* spp. and *Campylobacter* spp. as the commonest agents of sporadic bacterial diarrhea (12, 37), they have also been responsible for massive outbreaks of illness with significant morbidity and mortality. Examples include the lunch-food-associated Japanese outbreak in Sakai in 1996 (9) which affected over 6,000 children with 102 cases of HUS and 3 deaths and the food-borne outbreak in the U.S. in 1993 (8) which affected 500 cases with 171 hospitalizations, 43 HUS cases, and 4 deaths.

HUS, the most serious complication of VTEC infection, is associated with significant long-term morbidity, with renal dysfunction in up to 40% of the patients, and a mortality rate of 3%-5% (27, 50). The syndrome occurs most frequently in young children with an annual incidence, in North America, of about 2-3 cases per 100,000 children under 5 years of age (12) in contrast to a roughly 10-fold higher incidence in this age group in Argentina (29). Given that no specific treatment is currently available for HUS, immunization against VTEC infection may be the only practical option for limiting the serious morbidity and mortality associated with this condition. Unfortunately knowledge about the nature of protective immunity associated with VTEC infection is still in its infancy.
The disease mechanisms of VTEC infection involve two major components: colonization of the bowel (19) and the production of powerful exotoxins (Verocytotoxins or Shiga toxins) (36) which are thought to enter the circulation and to cause injury to target endothelial cells in the renal glomeruli and the gastrointestinal tract as well as in other organs and tissues (20). Thus antibodies that prevent colonization or neutralize the VTs in vivo are most likely to have a role in protective immunity. Patients with VTEC infection have been reported to develop serum antibody responses to various bacterial components including VTs (2, 4, 6, 23, 24) and factors involved in bowel colonization, such as the secreted proteins, EspA and EspB (18).

The peak incidence of VTEC infection, and of its toxemic complications such as HUS, in children less than 5 yrs (44) suggests that susceptibility to it, like to other childhood infectious diseases, is related to the absence of specific antibodies possibly to colonization factors and/or to the VTs. HUS occurs rarely during the first six months of life (12). This may be due to circulating anti-VT maternal antibodies, to specific anti-VT antibodies in breast milk (10), or to other substances in breast milk, such as glycolipids (34), that are able to bind the VTs.

The objective of this paper is to review work by us and others on the nature of anti-toxic immunity in VTEC infection and to discuss options for toxoid immunization.

Toxin types

Human VTEC strains produce at least three related cytotoxins (5, 20): VT1 (stx1; type strain C600:H19J), VT2 (stx2; type strain C600;933w), and VT2c (stx2c; type strains E32511; B2F1; 7279) which may be present alone or in combination. VT1 is virtually identical to Shiga toxin, but is serologically distinct from VT2 (and VT2c), the toxins showing no cross-neutralization in tissue-culture assay by homologous antisera. VT2 is completely neutralized \textit{in-vitro} by antiserum to VT2c, but VT2c is only partially neutralized by anti-VT2 (16).

Human immune response to VT1

The characterization of the nature of the antibody response to VT1, VT2, and VT2c in patients with VTEC infection and in different control populations is incomplete. Most of the studies to date have been performed on neutralizing antibody (NAb) and immunoglobulin G (IgG) responses to VT1.

Only a minority (about one-third or less) of patients with VTEC infection develop antibodies to VT1 (2, 4, 11, 23, 35, 45) even though the
frequency of seropositivity has been greater in sera from cases compared to those from controls.

The reported frequency of VT1-NAb in urban control persons from Japan, Canada, U.S., the Netherlands, India, Argentina, Germany, and Italy has ranged from 0% to about 10% (2, 23).

The apparent lack of a vigorous antibody response to VT1 in patients with VTEC infection parallels the data of Levine et al. (26) who found that only 7 of 21 patients with Shiga dysentery exhibited IgG antibody to Shiga holotoxin.

Possible explanations suggested for this by Levine et al. (26) include (i) secretory IgA should be measured rather than serum IgG, (ii) antibody response to the toxin may be muted because it is a powerful suppressant of protein synthesis, (iii) because of its potency, Shiga toxin may destroy the antigen-processing cells that pinocytose it and thus diminish the immune response, (iv) the toxin may be cytotoxic to B lymphocytes, (v) the immune response to Shiga toxin may be genetically restricted, and (vi) that the minute amounts of Shiga toxin, which are sufficient to induce a biological effect, may, in a manner analogous to tetanus toxin (47), be insufficient to reliably generate antibody responses. The latter was considered the most plausible. An extension of that is the possibility that a primary antibody response occurs but is undetectable because of an insufficient antigenic stimulus. Thus it would be expected that populations with greater exposure to VTEC (e.g., dairy-farm residents and abattoir workers) would experience detectable booster antibody responses after repeated exposures to VTEC and would thus have a much higher frequency of VT1-NAb than urban populations with limited exposure. This is supported by findings by Reymond et al. (39) of a frequency of VT1-NAb of 42% in 216 healthy dairy farm residents compared to a frequency of about 7% in an urban Toronto population and by the observations of Luzzi et al. (31) who found cattle slaughterers in Italy to have about twice the frequency of VT1-Nab (15.8%) than the frequency of VT1-NAb in urban residents (7.0%).

Using a very sensitive western blot assay for detecting anti-VT1 IgG, Reymond et al. (40) have corroborated the hypothesis (23, 39, 48) that a primary exposure to VTEC is insufficient to stimulate a detectable antibody response in many HUS patients, and suggested that the lack of a primary response may be associated with susceptibility to reinfection. Reports of patients with a second VTEC O157-associated illness, several months after the first (38, 43, 46), provide evidence that a primary infection with E. coli O157:H7 does not evoke protective immunity.
Human immune response to VT2

Knowledge about the frequency of antibodies to VT2 and to VT2c in patients with VTEC infection is limited. In a survey on the distribution of different VT genotypes in 176 isolates of *E. coli* O157:H7 belonging to different phage types, Thomas et al. (49) found that 75% of isolates contained genes for VT2, 62% for VT2c, and 53% for VT1. Thus the frequency of VT2 in *E. coli* O157 is significantly higher than that of VT1. In contrast, non-O157 VTEC serotypes tend to be positive predominantly for VT1 (33).

Given that O157 is, by far, the most predominant VTEC serotype, and the most frequent toxins expressed by this serotype are VT2 and VT2c, it would be expected that the frequency of antibodies to VT2 and to VT2c would be substantially higher than the frequency of antibodies to VT1 in patients with VTEC infection and in controls. Early studies, however, showed that virtually all sera from cases and from controls are able to neutralize VT2 (4, 7). Caprioli et al. (7) showed that the ubiquitous VT2-neutralizing activity was due, not to specific antibodies, but rather to the non-specific activity of serum high density lipoprotein.

Barrett et al. (2) used an ELISA to measure serum antibodies to VT2 in 83 cases of *E. coli* O157 infection and in 66 controls, and Greatorex and Thorne (11) tested sera from 27 *E. coli* O157:H7-associated HUS patients and 48 controls by ELISA and immunoblotting, but none of the samples in either study were positive for anti-VT2 IgG, IgM, or IgA. Gunzer and Karch (13), on the other hand, tested sera from 260 controls and acute and convalescent sera from 7 HUS patients by immunoblotting for reactivity to recombinant VT2 B subunit and found that 14 (5.4%) sera from the healthy controls were reactive in contrast to only one of 7 convalescent samples from HUS patients. In contrast, Ludwig et al. (30), using a western blotting method, have demonstrated anti-VT2 IgG in 66 of 94 (70%) patients with HUS compared to a frequency of 11% in 100 controls.

Takeda and colleagues (48) reported higher mean values of anti-VT2 ELISA antibodies in convalescent sera from HUS patients than in their acute sera, but found a significantly higher mean values of anti-VT2 ELISA antibodies in adult control sera compared to convalescent sera from HUS patients. This led them to postulate that a single episode of VTEC infection is not enough for the acquisition of a sufficiently high titer of anti-VT2 antibodies. This is in accord with the observation of Reymond et al. (39) in relation to the development of anti-VT1 antibodies.
Relationship of serum anti-VT antibodies with protective immunity to VTEC disease

 Whereas it has been postulated that the absence of detectable anti-VT antibody after a primary VTEC infection might be associated with absence of immunity (39), evidence in humans that the presence of antibody is associated with immunity is still limited. The presence of anti-VT1-IgG was associated with protective immunity in a large family outbreak of VTEC infection, associated with a VT1-producing E. coli O111:H-strain, in which symptomatic persons were seronegative for anti-VT1-IgG, whereas infected seropositive individuals remained asymptomatic (23). A high frequency of seropositivity among asymptomatic family contacts has also been observed in Argentina (28). Evidence that the presence of anti-VT antibody is protective has also be demonstrated in experimental animals which are protected from the lethal action of the toxin following toxoid immunization (3, 14, 17).

Options for toxoid immunization

 We suggested that immunization with VT toxoid may be a valid approach for preventing E. coli VT-associated HUS (21, 22, 24). Forty years ago Howard reported that injection of Shiga toxoid into laboratory animals gives rise to neutralizing antibodies and protects them from the lethal effects of Shiga toxin (17). Similarly, MacLeod and Gyles (32) demonstrated that active toxoid immunization with the pig-edema disease toxin, Vte, protects pigs from experimental disease induced by VTe. Furthermore, Harari et al. (14, 15) showed that active immunization with synthetic peptides from Shiga toxin B subunit protect mice against the lethal effects of shiga toxin. Acheson et al. (1) immunized rabbits orally with a recombinant Vibrio cholerae vaccine strain (CVD 103-HgR) in which 94% of the cholera toxin A subunit has been deleted, and which contains a plasmid encoding genes for the VT-1 B subunit. Such a strain given orally to rabbits was able to induce serum neutralizing antibodies to VT1.

 The choice of a VT vaccine immunogen and route of delivery for optimal protection against human VT-associated disease remains unresolved. The lack of cross-neutralization between VT1 and VT2 in-vitro would suggest that immunization with multiple immunogens may be necessary to provide broad protection against exposure to all the VTs.

 Traditional methods for investigating immunity in experimental animal models have depended on LD50 experiments involving a large number of animals. However, in an experimental rabbit model of VT-induced disease we have shown that the uptake of $^{125}$VT1 by specific target tissues
correlates directly with characteristic pathology induced in the same tissues by unlabelled toxin, and, furthermore, that toxin uptake by target tissues is prevented in animals immunized with VT1 toxoid (41).

Using this approach we made the unexpected observation that rabbits immunized with VT1 toxoid are cross-protected against challenge by both VT1 and VT2, and rabbits immunized with VT2 toxoid are also cross-protected against challenge by the homologous and heterologous toxoids (3). We showed that the toxin A subunit was responsible for evoking the cross-immunity. We therefore proposed that immunization with a single VT toxoid (at an appropriate dosage) or the “A” subunit may be sufficient to induce immunity to all the VTs.

Given that HUS is an outcome of systemic VT-toxemia, we propose that systemic delivery of a VT immunogen, in a manner analogous to immunization with diphtheria and tetanus toxoids, is the most logical approach for immunization against VT-induced HUS. Furthermore the current schedule for diphtheria-pertussis-tetanus (DPT) immunization with a primary immunization during the first six months of age followed by two boosters two months apart, and at 5 years of age, would be ideally suited for VT-toxoid immunization, since it would provide protection during the years of peak HUS incidence. The formulation and nature of the immunogen and the dosage schedule required to evoke protective immunity need to be worked out in an experimental animal model as a prelude for future studies in human volunteers. The rabbit model, developed in our laboratory (41), is well suited for this purpose.

It should be noted that while toxoid immunization would be expected to prevent the systemic complications of VTEC infection, such as HUS, it would not prevent bowel colonization or the development of diarrhea associated with VTEC infection.

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