Age-Specific Frequencies of Antibodies to *Escherichia coli* Verocytotoxins (Shiga Toxins) 1 and 2 among Urban and Rural Populations in Southern Ontario

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In 173 urban residents and 232 rural dairy-farm residents (age range, 0–70 years) who were stratified for age, the frequency of antiverocytotoxin 2 antibodies (VT2 Abs) (frequency in urban residents, 46%; frequency in rural residents, 65%) was significantly higher than that of antiverocytotoxin 1 antibodies (VT1 Abs) (frequency in urban residents, 12%; frequency in rural residents, 39%) (P < .001). The frequency of VT2 Abs (93%) was also significantly higher than that of VT1 Abs (50%) in 14 patients with hemolytic uremic syndrome (HUS) associated with verocytotoxin-producing *Escherichia coli* (VTEC) strains that expressed both toxins. In urban residents, the frequency of both antibodies tended to decrease between the first and the second decades of life, and it then increased until the fifth decade of life, before, in the case of VT2 Abs, decreasing again. This pattern, which inversely reflects the age-related incidence of HUS, is consistent with a role for antiverocytotoxin antibodies in protective immunity. In dairy-farm residents, peak frequencies of antibodies to both toxins occurred during the first decade of life and remained elevated for 3 decades before decreasing, a pattern consistent with frequent exposure to bovine VTEC from an early age.

Verocytotoxin (VT)–producing *Escherichia coli* (VTEC) [1], also referred to as Shiga toxin–producing *E. coli* [2], are causes of a potentially fatal foodborne illness, the clinical spectrum of which includes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) [3–5]. Although >200 different OH serotypes of VTEC have been associated with illness in humans, a majority of the reported outbreaks and sporadic cases of such illness have been associated with serotype O157:H7 [6].

HUS is the leading cause of acute renal failure in otherwise healthy children [5], and it is thought to be mediated by the action of VTs on the endothelial cells of the renal glomeruli and other organs and tissues [7]. VTEC isolates from humans elaborate 2 major serologically distinct, bacteriophage-mediated cytotoxins (VT1 and VT2) that may be present alone or in combination [6, 8]. A small proportion of VTEC isolates from humans also elaborate variants of VT2 (namely, VT2c and VT2d), either alone or in combination with VT1 or VT2 [9]. The pathogenic significance of these variants is not fully understood, but the risk of HUS occurring after infection with strains that express the VT2 variants is considered to be low [9].

The nature of protective immunity to VTEC infection is not well understood [10]. The 2 main viru-
lence strategies associated with VTEC are their ability to colonize the bowel and their ability to produce VTs. The antibodies that are most likely to be protective are those that inhibit colonization of the bowel by VTEC and/or those that neutralize toxins before they reach their target sites. In practice, protective immunity to VTEC infection is likely to result from the interplay between antitoxin immunity and immunity to bowel colonization.

The peak age-related incidence of HUS occurs in childhood [5, 11]. The incidence of HUS then decreases with age but appears to increase again during old age [12]; this pattern would be consistent with an age-related increase in antibodies associated with anti-VT immunity, followed by a decrease in such antibodies during old age. Patients with VTEC infection develop anti-VT antibodies [10], but these antibodies have not necessarily been demonstrated to exhibit an age distribution consistent with a role in protective immunity. Support for the concept that such antibodies are protective against the development of systemic illnesses, such as HUS, comes from experimental and clinical findings. Rabbits immunized with VT1 or VT2 toxoid develop antitoxin antibodies that can be detected by the VT neutralizing antibody test, ELISA, and Western immunoblotting [13], and they are protected from injected iodine-125–labeled VT binding to target tissues or disease when challenged intravenously by toxin [13–15]. A seroepidemiological study of 2 outbreaks of VTEC infection found that individuals who were seropositive for antibodies to VT did not develop disease, whereas those who were seronegative for such antibodies had symptomatic disease [16].

Cattle are a major reservoir for VTEC, and infection in humans may be acquired directly or indirectly from cattle. Accordingly, it would be expected that people with increased contact with such animals would have a greater likelihood of acquiring VTEC infections. A previous study has reported a comparison of the prevalence of antibodies to VT1 in rural (dairy-farm) residents with that in urban residents, using a toxin neutralization assay, and has demonstrated that antibody to VT1 occurs up to 6 times more frequently among rural residents than among urban residents [17]. In the present study, we extend these findings by investigating the age-specific frequency of antibodies to both VT1 and VT2 in these populations, using a more sensitive immunoblot method described elsewhere [18].

MATERIALS AND METHODS

Serum Samples

Samples from urban residents. The test sample consisted of serum samples obtained during 1987 from 173 urban residents of Toronto (93 males and 80 females; age range, 6 months to 70 years). The 7 age-distribution groups used in the stratification of serum samples from urban residents were as follows: 6 months to 10 years (43 samples), 11–20 years (27 samples), 21–30 years (21 samples), 31–40 years (24 samples), 41–50 years (24 samples), 51–60 years (23 samples), and 61–70 years (11 samples). The samples were obtained from 2 sources. The 58 serum samples from children (35 males and 23 females) up to 16 years of age were obtained from a serum bank at The Hospital for Sick Children (Toronto, Ontario, Canada) and comprised serum samples submitted to the microbiology laboratory of the hospital for the investigation of upper respiratory infection. The ethics committee of The Hospital for Sick Children approved the use of all banked pediatric serum samples obtained from case patients and controls who participated in the present study. The remaining 115 samples (obtained from 58 males and 57 females and supplied by R. Herst of the Ontario Red Cross, Toronto, Ontario, Canada) were from anonymous blood donors.

Samples from dairy-farm residents. From July 1992 through February 1993, serum samples from dairy-farm populations were obtained from 232 individuals (122 males, 109 females, and 1 individual whose sex was unknown) from 68 families living on 68 farms. The individuals all gave written permission for the samples to be obtained. Because the age of 8 of the individuals was not available, only 224 samples were used to determine the age-specific frequency of VT1 antibodies (VT1 Abs) and VT2 antibodies (VT2 Abs). The 7 age-distribution groups used in the stratification of serum samples from dairy-farm residents were as follows: 1–10 years (33 samples), 11–20 years (51 samples), 21–30 years (21 samples), 31–40 years (54 samples), 41–50 years (31 samples), 51–60 years (19 samples), and 61–70 years (15 samples).

Samples from patients with HUS. Acute-phase and follow-up specimens that were obtained from 14 patients with HUS (5 males and 9 females) who were culture positive for E. coli O157:H7 strains that expressed both VT1 and VT2 were investigated. The specimens had been obtained for routine testing, and any remaining serum samples were stored at −30°C at The Hospital for Sick Children. Acute-phase and follow-up serum samples were tested to follow the development of VT Abs. The ethics committee of The Hospital for Sick Children approved the use of these stored serum samples.

VT preparation and purification. VT1 was purified from recombinant E. coli strain JB 28, an E. coli strain transformed by recombinant plasmid pUC19B that contains the stx genes cloned from bacteriophage H19B (provided by J. Brunton, University of Toronto, Toronto, Ontario, Canada). VT2 was purified from recombinant E. coli strain R82pJES 120DH5 (provided by J. Samuel, Texas A & M University, College Station, Texas, United States). The toxins were purified from culture filtrates by use of sequential column chromatography (with
hydroxyapatite, chromatofocusing, and Cibachron Blue agarose), as described elsewhere [19, 20].

**Immunoblot assay for the detection of IgG antibody to VT1 and VT2.** This assay, adapted from the method of Towbin et al. [21], was performed as described elsewhere by use of highly purified VT1 [18] and VT2 [19]. In brief, a standard concentration of VT1 and VT2 (20 μg) was run on a sodium dodecyl sulphate polyacrylamide gel electrophoresis. The protein bands were transferred electrophoretically onto sequencing-grade polyvinylidene difluoride (PVDF) membranes (Sequiblot; Bio-Rad Laboratories) for 1 h by use of a current of 0.14–0.20 A. Each membrane was cut into longitudinal strips, and 1 strip was stained with Coomassie blue to confirm the protein transfer. After overnight blocking with 5% skimmed milk and 10% goat serum in Tris buffer (50 mmol/L of Tris [pH 7.4]), the strips were incubated for 1 h at room temperature in respective serum specimens diluted 1:100 in Tris buffer (50 mmol/L of Tris; pH 7.4) containing 5% skim milk and 10% goat Tris buffer. After further washing, the strips were incubated for 1 h at room temperature in a 1:10,000 dilution of goat anti-human IgG (H+L) peroxidase conjugate (Bio-Rad Laboratories) in Tris buffer with 1% skim milk and 2% goat serum. After undergoing further washing in Tris buffer, the strips were developed using a chemiluminescent detection system (ECL; Amersham Biosciences). The strips were exposed to Kodak AR film (Kodak) for 2-40 s, and the film was then developed.

Each serum sample was tested at least twice. A serum sample was considered to be reactive with anti-VT if a band that corresponded to one of the A, A₁, or B subunits was detected. Serum samples for which bands were detected that corresponded to the A and/or the A₁ subunit were designated as being reactive with the A subunit. Serum samples for which no bands could be detected were considered to be negative [18, 19]. With each batch of test serum samples, we included serum samples that were known to be negative or positive for VT1 Abs and VT2 Abs.

**Characterization of VTEC strains from patients with HUS.** The selection of serum samples from patients with HUS was based on evidence of infection by VTEC serotype O157:H7 strains that expressed VT1 and VT2. VTEC O157:H7 strains were isolated on sorbitol-MacConkey agar, by use of standard methods, and they were tested genotypically and phenotypically for VT1 and VT2, as described elsewhere [22].

**Statistical Methods**

The Pearson χ² test [23] was used to test whether the proportions of anti-VT1 and anti-VT2 were similar among the rural and urban populations. Within each rural and urban population, the 2-sided Z test [24] was applied to verify whether the proportion of anti-VT1 was similar to the proportion of anti-VT2. Because the expected frequency in the off-diagonal cells, under the assumption of no difference, was <5%, the binomial test [24] was applied to verify whether the proportion of anti-VT1 was similar to the proportion of anti-VT2 within the population with HUS. For all tests, the null hypothesis was rejected for α ≤ 0.05.

**RESULTS**

**Frequencies of VT Abs among urban residents.** The overall frequency of VT2 Abs (46%) was significantly higher (difference, 34%; 95% confidence interval [CI], 26%–43%) than that of VT1 Abs (12%), in the 173 serum samples obtained from urban residents aged 6 months to >60 years (P < .001, 2-sided Z test). The age-specific frequencies of both VT1 and VT2 Abs varied in a comparable manner with age (figure 1). There is a tendency toward a decrease in the frequency of antibody from the first to the second decade of life, followed by a progressive increase in antibody frequency from the second decade to the fifth (for VT1 Abs) or fourth (for VT2 Abs) decades of life and by a decline in frequency thereafter. On the immunoblot, all of the serum samples that were positive for VT1 Abs were reactive with both the A and B subunits. Of the serum samples that were positive for VT2 Abs, 70% were reactive with both subunits, 7.5% were reactive only with the A subunit, and 22.5% were reactive only with the B subunit.

**Frequencies of VT Abs among rural residents.** The overall frequency of VT2 Abs (65%) was significantly higher (difference, 26%; 95% CI, 18%–34%) than that of VT1 Abs (39%), in the 232 serum samples obtained from dairy-farm residents who were 1–70 years of age (, 2-sided Z test). In contrast to the age-specific patterns seen in urban residents, the peak frequencies of VT1 Abs and VT2 Abs in the samples from rural residents were evident during the first decade of life (figure 2). The levels remained roughly similar during the subsequent 3 decades and declined thereafter, with the decline being more pronounced for VT1 Abs than for VT2 Abs (figure 2). Of the serum samples that were antibody positive, 90% of the samples that were positive for VT1 Abs and 87.5% of those that were positive for VT2 Abs were reactive with both the A and B subunits. The proportions of samples reactive for VT1 Abs and VT2 Abs with only 1 of the subunits were 4.4% and 8.5%, respectively, against the A subunit, and 5.6% and 4%, respectively, against the B subunit.

**Comparison of frequencies of VT Abs among urban and rural residents.** The overall frequency of VT1 Abs in the rural population (39%) was ~3-fold higher than that in the urban population (12%) (P < .001, Pearson χ² test). The overall frequency of VT2 Abs in the rural population (65%) was approximately one and one-half times greater than that in the urban population (46%) (P < .001, Pearson χ² test).

**Frequencies of VT Abs in patients with HUS.** The age
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**DISCUSSION**

The present study has provided comprehensive data on the relative frequency of VT1 Abs and VT2 Abs in populations from both urban and rural settings. In the urban residents, an upward trend in the age-specific frequencies of these antibodies was generally evident from the second decade to the fourth-to-fifth decades of life (figure 1), in contrast with a downward trend in the age-specific incidence of HUS. This is consistent with the postulated role of these antibodies in conferring protective immunity against systemic toxin-mediated disease, such as HUS. Furthermore, the decrease in the age-specific frequency of anti-VT antibodies during the sixth decade of life, which reflects a postulated decrease in immunity to VT, is consistent with the apparent increase in systemic VTEC disease reported among elderly individuals [12]. The reason for the observed decrease in the frequency of VT1 Abs and VT2 Abs between the first and the second decades of life (figure 1) is not known.

Although there were significant differences between the urban and rural populations with regard to the prevalence and age-specific patterns of VT antibodies, these differences could be influenced by the fact that the samples from urban residents were obtained in 1987, whereas the samples from rural residents were obtained in 1992 and 1993. Nevertheless, the finding of an increased prevalence of anti-VT1 and anti-VT2 in the rural residents is in keeping with the postulated earlier and more-frequent exposure of these individuals to VTEC as a result of contact with farm animals. In our previous study, it was demonstrated that, as determined by serum neutralization, the frequency of anti-VT1 among rural residents (42%) was ∼5-fold higher than that among urban residents (7.7%) [17]. In the present study, which used the more-sensitive and more-specific immunoblot assay, a 3-fold higher frequency of anti-VT1 and a 1.5-fold higher frequency of anti-VT2 were observed among the rural residents, compared with those among the urban residents. Moreover, there also were differences in the age-specific frequencies of VT Abs in these 2 populations. In urban residents (figure 1), the frequencies of anti-VT1 and anti-VT2 increase after the first decade of life and during the following 4–5 decades, after which time they decrease. In contrast, among rural residents (figure 2), the frequencies of antibodies to both toxins were already at their peak during the first decade of life, and they remained at these peak levels until the fifth decade of life, after which time they tended to decrease. This is consistent with the postulated more-frequent exposure of individuals on dairy farms to VTEC, through contact with cattle, from a very...
Figure 2. Age-specific proportion of antiverocytotoxin 1 antibodies (VT1 Abs) and antiverocytotoxin 2 antibodies (VT2 Abs) in the rural population.

With respect to the first factor, Friedrich et al. [9] conducted the most comprehensive investigation to date of the distribution of VT genotypes in 626 VTEC isolates from humans—isolates that belonged to a wide range of serotypes and were associated with different disease categories, including diarrhea and HUS. Although the specific objective of the study of Friedrich et al. [9] was to correlate the distribution of VT2 variant toxin genotypes (VT2c, VT2d, and VT2e) with disease category, the findings of these investigators also provide unique information about the frequency of exposure of humans (with mild or severe disease) to VTEC producing different toxin types, including VT1 and VT2. From their findings, we calculated the prevalence of individual VT genotypes, either alone or in combination with other VT types, in the 626 VTEC isolates: prevalence of VT1, 44.1%; VT2, 52.4%; VT2c, 23.6%; VT2d, 9.9%; and VT2e, 2.6%. The prevalence of VT2 among these isolates is significantly higher (difference, 8%; 95% CI, 3%–14%) than that of VT1 ($P = .005$, 2-sided $Z$ test). This finding supports the hypothesis that the comparatively higher frequency of anti-VT2 in the population is related to greater exposure to VT2.

With respect to factor 2, Ludwig et al. [19] reported that the frequency of an anti-VT2 response after infection by a VT2-
producing strain (34 [71%] of 48 patients) was higher than that of an anti-VT1 response after infection by a VT1-producing strain (4 [40%] of 10 patients). It is possible that VT2 is more “immunogenic” than VT1, either by virtue of being produced in greater quantity in vivo or by virtue of being presented “more efficiently” to the immune system. To determine whether the difference in response to VT1 and VT2 was also evident in Canadian children with HUS, we investigated antibody responses in 14 patients with HUS who were infected with VTEC strains that produced both VT1 and VT2. Overall, 13 (93%) of 14 patients were positive for VT2 Abs, compared with 7 patients (50%) who were positive for VT1 Abs. These findings confirm the observations of Ludwig et al. [19] that patients with HUS respond more frequently to VT2 than to VT1. Possible differences between the persistence of anti-VT1 and anti-VT2 in the serum could also influence the difference in their overall incidence in populations.

In a cohort of 110 healthy control children (age range, 0.3–17 years) in Germany, Ludwig et al. [19] found that 11 (10%) had VT2 Abs and 2 (1.8%) had VT1 Abs. In contrast, in the pediatric subgroup (58 children; age range, 6 months to 16 years) of the Canadian urban residents in our study, 24 children (41.4%) were positive for VT2 Abs, and 5 (8.6%) were positive for VT1 Abs. As in the case of our comparative seroepidemiological analysis of urban and rural residents, this contrast in the frequencies of anti-VT1 and anti-VT2 in Canadian and German urban children suggests that Canadian children may have a substantially greater exposure to VTEC than do German children. This is consistent with the higher reported prevalence rates of HUS among Canadian children (1.44 cases/100,000 children <15 years of age) [11] than among German children (0.99 cases/100,000 children <16 years of age) [25].

Our findings indicate that comparative seroepidemiological studies of the immune response to VT may be useful, not only for assessing the relative levels of antitoxin immunity in populations, but also for indirectly assessing the degree and patterns of exposure to VTEC in different populations in the urban or rural setting or in different geographic locations.

References