Seroprevalence of Antibodies against Noroviruses among Students in a Chinese Military Medical University

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Received 16 January 2004/Returned for modification 7 May 2004/Accepted 1 June 2004

Noroviruses (NVs) are important causes of nonbacterial gastroenteritis in humans, but the role of NVs as a cause of diseases in the Chinese people, particularly in Chinese military personnel, remains unclear. This study investigated antibody prevalence and factors that associate with the prevalence of antibody to NVs among students attending a military medical university. Serum specimens were tested by an enzyme-linked immunosorbent assay for immunoglobulin G antibody to recombinant capsid antigens of three NVs (rNorwalk, rMxV, and rVA387). Of 588 serum samples tested, the antibody prevalence was 88.9, 54.1, or 90.0% for the three antigens, respectively. There were significant differences in the prevalence of antibody to rMxV between blood types (P < 0.05); the prevalence for type O was the highest (62.5%), and the prevalence for type B was the lowest (49.1%). The average optical density values for antibody to rNorwalk and rMxV were lowest among students with type B. The number of students who did not have antibody to any of the three antigens was the highest for blood type B (6.9%) compared to other blood types (0.8 to 3.4% [P < 0.006]). The antibody prevalence also varied with the hometown residencies of the students before joining the military, with the highest rates for students from rural areas, lower rates for students from small towns or villages, and the lowest rates for students from large cities. The numbers of students who did not have antibody to any of the three antigens were highest for students from the large cities, lower for students from small towns or villages, and lowest for students from rural areas. The distribution of ABO blood types did not differ among the three groups. These data suggest that NVs are prevalent in China and that both genetic and environmental factors play a role in NV infection.

Noroviruses (NVs), previously known as Norwalk-like viruses or small round structured viruses, belong to the Norovirus genus of the Caliciviridae. NVs have been recognized as the most important cause of nonbacterial epidemics of acute gastroenteritis since the application of molecular diagnostic methods in epidemiology studies of diarrhea diseases in different countries after the cloning of the prototype Norwalk virus in 1990 (17). In developed countries such as the United States and many European countries, NVs have been found to be the most important cause of nonbacterial outbreaks of acute gastroenteritis (6, 7, 27, 37). Such outbreaks can occur in a variety of settings, affecting all ages, including child care centers, schools, restaurants, summer camps, hospitals, nursing homes, cruise ships, and military settings. NV outbreaks have been a serious problem in nursing homes for the elderly (3, 10, 21), on board large commercial cruise ships (11, 30), and in military camps and battleships during deployment and wartime (1, 2, 15, 36).

NVs are also one of the most important causes of acute gastroenteritis in developing countries. Surveillance using reverse transcription-PCR by many laboratories has shown that NVs are the most important cause, after rotaviruses, of pediatric gastroenteritis (8, 29, 32, 35, 38). NVs are commonly detected in stool specimens of children with acute gastroenteritis at inpatient and outpatient clinics, indicating that NVs may cause severe gastroenteritis in children that requires hospitalization or a physician visit. Seroepidemiology studies also showed that children acquire antibodies to NVs at early ages, and the antibody prevalence continued to increase during the school years to adulthood (4, 5, 9, 25, 33). The antibody prevalence is generally higher in developing countries than in developed countries. Limited studies have also shown that NV infection is common among Chinese populations, including several reports of detection of NVs in patients with acute gastroenteritis in hospitalized children and one study of the prevalence of antibodies to two NV strains in a selected population (25, 34).

We investigated here the prevalence of antibodies to three NV strains in the sera of students attending a Chinese military medical university. Because the students at this university were selected from different provinces across China, we also analyzed the antibody prevalence according to the hometown residencies of the students before they joined the military. NVs recently have been found to recognize human histo-blood group antigens as receptors for the initiation of infection and different NVs recognize different receptors (12–14, 26, 28). To determine whether strain-specific host specificity occurs among students with different blood types, we also compared the an-
tobody prevalence according to the ABO blood types of the students. Thus, we provide information not only on the overall antibody prevalence but also on the environmental (socioeconomic status and living conditions) and genetic factors (blood types) that may affect the prevalence of antibodies to NVs.

MATERIALS AND METHODS

Recombinant capsid antigens. Three recombinant capsid antigens from three strains of NVs representing three genetic clusters—Norwalk virus (GI1), MxV (GI3), and VA387 (GI4) (19, 22, 24)—were used as the coating antigens to measure specific antibodies from serum specimens. These viral capsid antigens self-formed virus-like particles when expressed in baculovirus-infected insect cell cultures (19, 22, 24). The virus-like particle antigens used in the enzyme immunoassays were partially purified from the insect cell culture by sucrose and/or CsCl gradient centrifugations, lyophilized after purification, and stored at −70°C.

Study population. Blood samples were collected from students attending the First Military Medical University, Guangzhou, Guangdong, China. Serum samples were stored at −20°C. The study was approved by the Research Committee for the Key Program of Military Medical Science and Technique Foundation during the 10th-Five-Year-Plan period of China.

Enzyme-linked immunosorbent assay (ELISA) for measuring antibodies to NVs. The conditions for ELISAs to detect antibodies in serum specimens described in previous studies (16, 23) were used with minor modifications here. Partially purified NV capsid proteins (rNorwalk, rMxV, and rVA387) were used to coat 96-well microtiter plates at 0.25 μg/ml in phosphate-buffered saline (PBS), and the plates were incubated at 4°C overnight. The plates were washed once with 0.05% Tween-PBS (PBST) before they were blocked with 5% dried milk (Blotto)-PBST at 37°C for 1 h. The plates were washed twice with PBST, serum samples were added at a 1:200 dilution in 1% Blotto-PBS, and the plates were incubated at 37°C for 2 h. After the plates were washed six times with PBST, horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) antiseraum (Cappel Organon Teknika) was added at a 1:15,000 dilution in 1% Blotto-PBS. The plates were washed six times with PBST before the addition of the 3,3,5,5-tetramethylbenzidine (TMB) substrates, followed by incubation at room temperature for 10 min. The colors developed in the plates were then measured by an ELISA reader (318MC) at an optical density of 450 nm (OD450).

According to the average OD value of the negative controls, an OD450 of >0.25 was used as the cutoff point for a positive result.

Methods for determination of ABO blood types. Serum samples were coated onto microtiter plates overnight at 4°C. After being blocked with 5% Blotto, monoclonal antibodies specific to A and B antigens were added at a dilution of 1:100. After incubation for 1 h at 37°C, horseradish peroxidase-conjugated goat anti-mouse IgG antibodies were added. After each step, the plates were washed five times with PBST. The color reaction was developed and recorded as described above.

Statistical methods. The antibody detection data were recorded and checked by using an SPSS10.0/PC statistical package (SPSS, Chicago, Ill.). Proportions were compared by using the chi-square test combined with the Fisher exact test. The quantitative dependent variables were analyzed by one-way analysis of variance (ANOVA) with Games-Howell or Tukey’s honestly significant differences according to results of a variance homogeneity test. A two-tailed P value of 0.05 was considered statistically significant.

RESULTS

Demographics of the study population. The 588 students were randomly selected from ~2,000 students currently enrolled in the First Military Medical University in China; 298 were male, and 290 were female. The median age was 20.5 years (range, 17 to 24 years). The students came from 24 of the 28 provinces in China and were divided into three groups according to their hometown residencies prior to joining the military; 285 were from rural areas, 128 were from small towns or villages, and 175 were from large cities. Student blood types were 22.1, 36.7, 31.3, and 9.9% types A, B, O, and AB, respectively (Table 1), values comparable to the percentages of the national distribution (29.0, 28.3, 33.8, and 8.5%, respectively).

The distribution of ABO blood types of students was not significantly different according to hometown residency (Table 1).

Strain-specific prevalence of antibody to three NVs. Of the 588 serum samples tested, specific IgG antibodies were detected in 523 (88.9%), 318 (54.1%), and 529 (90.0%) serum samples to rNorwalk, rMxV, and rVA387, respectively; these values were significantly different (χ² = 282.9, df = 2, P < 0.01). There was no difference between genders in prevalence of antibody to any or all of the three antigens (data not shown). About half (49%) of the students had antibodies to all three antigens, 39% had antibodies to two antigens, 8% had antibodies to one antigen, and 4% did not have antibody to any of the three antigens (see Table 3).

Prevalence of antibodies to NVs among students with different ABO types. There were significant differences in the prevalence among students with different blood types of antibody to rMxV (P < 0.05); type O was the most prevalent (62.5%), and type B was the least prevalent (49.1%; Table 2). The prevalence of antibody to rNorwalk and rVA387 also was highest among type O students compared to the other blood types, although the data were not statistically significant (Table 2). The distribution of students who did not have antibody to any of the three antigens also was highest among type B individuals (6.9%) compared to other blood types (0.8 to 3.4%, P < 0.006) (Table 3). The mean titers of antibody (i.e., the ODs) to rNorwalk and rMxV were lowest among students with type B (P = 0.008 and P = 0.010, respectively), but there was no significant difference in the mean titers of antibody to strain VA387 (Table 4).

Prevalence of antibody to NVs among students with different hometown residency. The prevalence of antibody to all three antigens was significantly different among students with different hometown residencies. Students who came from rural areas had the highest antibody prevalences, students who came from...
from small towns or villages had lower antibody prevalences, and students who came from the large cities had the lowest prevalences of antibody to all three antigens (Table 5). The numbers of students who did not have an antibody to any of the three antigens were just the opposite, with the highest numbers found for students from large cities, lower numbers for students from small towns or villages, and the lowest numbers for students from rural areas (Table 5). The ABO blood type distributions of the students did not show significant differences among the three groups, indicating that environmental factors and living conditions are independent factors in NV infection.

**DISCUSSION**

We investigated here the prevalence of antibody to NVs among students at a military university as a first step in a long-term investigation of the role of NVs in diarrheal diseases in Chinese military populations. Acute gastroenteritis is an important disease in both military and civilian populations in China. In the military, acute gastroenteritis has been found to be the primary cause of non-battle-related dysfunction in most military units in both war and peacetime. It has been shown that the annual incidence of diarrhea varied from 49.5 to 64.0% in military units stationed in southern China and that ca. 30% of the diarrhea episodes had no known etiology. In civilian populations, according to a nationwide surveillance carried out in 21 provinces in 1988 in China, 863 million diarrheal episodes were estimated to occur annually, 290 million of which occur in children. NVs now have been recognized as one of the most important causes of nonbacterial diarrhea in many developed and developing countries; however, the role of NVs relative to other common enteric pathogens in causing acute gastroenteritis in Chinese populations remains unclear.

Since most of these students had been part of the military for only a few years, and since they had not been exposed to military training as much as average soldiers have been, these data are well suited to determining the baseline of NV infection for military populations. Furthermore, because the students from this university came from different parts of the country that cover more than 77.4% of the nation’s population, these data also should be valuable for assessing impact of NVs on a nationwide basis. Finally, we also compared the prevalence of antibody among students with different hometown residences and among students with different blood types, which provides additional information about the epidemiology and impact of NVs as causes of gastroenteritis.

In the present study, the overall prevalence of antibody to the three NVs among the study population was high, a finding that is comparable to most of the studies performed in other developing countries (5, 20, 31). However, the prevalence of antibody to MxV was lower in our study than the data reported by Jing et al. in a study on a population in the Beijing area, in which high prevalences of antibody to both Norwalk and MxV viruses were observed (25). We hypothesize that this difference could be due to different geographical locations of the two studies, because the Beijing study enrolled subjects from one metropolitan area, whereas our study included subjects from all over the country.

In fact, significant differences in the prevalence of antibody to the three strains were observed among students according to their hometown residencies before they joined the military. Students from rural areas showed the highest prevalences of antibody; students from small towns or villages had lower prevalences of antibody; and students living in large cities had the lowest prevalences of antibody to one or all of the three antigens. These differences apparently reflect the socioeconomic status and living conditions of the students in each group. In general, people living in rural areas have poorer health and hygiene conditions than people living in small towns or villages, and people living in large cities have the highest socioeconomic and living conditions.

The study of NV receptors and host range has progressed significantly in the past few years since the description of the prototype Norwalk virus-binding receptors of secretors (H antigen) but not of nonsecretors (28). Direct evidence of involvement of human histo-blood group antigens in host specificity to NVs has been demonstrated in volunteer studies that nonsecretor individuals are naturally resistant to challenges of the prototype Norwalk virus (13, 26). Extended studies have also

**TABLE 3. Numbers of students with antibody to all three, two, one, or no antigens according to blood type**

<table>
<thead>
<tr>
<th>Blood type</th>
<th>No. of subjects</th>
<th>No. (%) of subjects with antibody to:</th>
<th>No. (%) of subjects with antibody to:</th>
<th>No. (%) of subjects with antibody to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All three antigens</td>
<td>Two antigens</td>
<td>One antigen</td>
</tr>
<tr>
<td>A</td>
<td>130</td>
<td>61 (47)</td>
<td>56 (43)</td>
<td>12 (9.2)</td>
</tr>
<tr>
<td>B</td>
<td>216</td>
<td>100 (46)</td>
<td>81 (38)</td>
<td>20 (9.3)</td>
</tr>
<tr>
<td>AB</td>
<td>58</td>
<td>27 (46)</td>
<td>22 (38)</td>
<td>7 (12)</td>
</tr>
<tr>
<td>O</td>
<td>184</td>
<td>101 (54)</td>
<td>70 (38)</td>
<td>10 (5.4)</td>
</tr>
<tr>
<td>Total</td>
<td>588</td>
<td>289 (49)</td>
<td>229 (39)</td>
<td>49 (8.3)</td>
</tr>
</tbody>
</table>

*Tested by χ² test (P < 0.05).*

**TABLE 4. Mean antibody titers to rNorwalk, rMxV, and rVA387 in sera of Chinese military medical students according to blood type**

<table>
<thead>
<tr>
<th>Blood type</th>
<th>No. of subjects</th>
<th>Average OD versus:</th>
<th>A</th>
<th>B</th>
<th>AB</th>
<th>O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rNorwalk</td>
<td>rMxV</td>
<td>rVA387</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>130</td>
<td>0.83</td>
<td>0.42</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>216</td>
<td>0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.37&lt;sup&gt;v&lt;/sup&gt;</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>58</td>
<td>0.83</td>
<td>0.44</td>
<td>0.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>184</td>
<td>0.72</td>
<td>0.48</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Tested by one-way ANOVA test (P < 0.05).*
shown that different NVs recognize different receptors and six receptor-binding patterns have been described according to the host ABO, Lewis, and secretor types (12; X. Jiang, unpublished data). The prototype Norwalk virus represents one of six binding patterns (12, 26, 28). As analogs, the host specificity of the other five binding patterns has been predicted, although direct evidence of this prediction remains lacking.

Based on this prediction, we tested three strains of NVs representing distinct genetic clusters and receptor-binding patterns. VA387 belong to GII and binds to A, B, and O antigens; Norwalk virus belongs to GI and binds to A and O antigens; and MxV belongs to GII and binds to A antigen but does not bind or weakly binds to B antigen (12; Jiang, unpublished). Thus, we predicted that the prevalence of antibody to the three strains would vary among students with different blood types, and the following observations support this hypothesis. (i) The overall prevalence of antibody to MxV virus was lower than that against Norwalk and VA387, a finding that agrees with our prediction that MxV has a more restricted host range than the other two strains. (ii) The prevalence of antibody and the average titers of antibody to MxV and Norwalk viruses were lowest among students with B blood type compared to other blood types, a finding that agrees with the prediction of type B individuals being naturally resistant to MxV and Norwalk viruses. This difference was not observed against VA387 because individuals with all ABO blood types are predicted to be susceptible to VA387. (iii) The overall prevalence of antibody to Norwalk and VA387 among type O individuals was higher than for individuals of other blood types, a finding that agrees with the fact that these two strains recognize the H types that are the major antigenic epitopes among the type O individuals. (iv) Finally, the strain-specific antibody prevalences among subjects with different blood types were independent of the hometown residencies of the various students. In the present study we also observed data that do not completely agree with our hypothesis. For example, the MxV virus is predicted to be sensitive to type A only, but many individuals with types O and B had antibodies to MxV. Similarly, Norwalk virus is predicted to be susceptible to types A and O, but not type B; however, significant numbers of individuals with type B had antibody to Norwalk virus. One explanation for these controversial findings could be the shared antigenic epitopes between strains with different receptor-binding patterns (16, 18). To verify this, we compared the rates for subjects who did not have antibodies to any of the three strains, and the results still showed significant differences among subjects with different blood types and with different hometown residencies, with the highest values for students with type B and students living in the large cities.

This is the first study of NV association with human histo-blood group antigens in the Chinese population. We also have noticed that the distribution of ABO blood types of the Chinese populations is different from that of other nations; in particular, there is a higher rate of type B and a lower rate of type O individuals in China than in North American and European countries. This raises the question of whether there are differences in the epidemiology of NVs among different countries, such as differences in major circulating strains of NVs due to different ABO predispositions. Furthermore, it is known that histo-blood group antigens other than ABO antigens also are involved in NV-host specificity, such as the secretor and Lewis antigens (12). However, these data are not available in the present study since only blood samples were collected, which do not allow to testing for the Lewis and secretor phenotypes. In conclusion, the present study provides significant positive data regarding several aspects related to the prevalence of antibody to NVs in the Chinese populations. Future studies to further characterize NVs are needed.

ACKNOWLEDGMENTS

This study was supported by funding from the Key Program of Military Medical Science and Technique Foundation during the 10th-Five-Year-Plan period in China (01L050) and from the National Institute of Allergy and Infectious Diseases (AI37093).

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VOL. 42, 2004 SEROPREVALENCE OF ANTIBODIES AGAINST NOROVIRUSES 4619


