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High Seropositivity Rate of Neutralizing Antibodies to Astrovirus VA1 in Human Populations

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ABSTRACT Astroviruses are common pathogens of the human gastrointestinal tract, but they have been recently identified from cases of fatal meningoencephalitis. Astrovirus VA1 is the most frequently detected astrovirus genotype from cases of human encephalitis, but the prevalence of neutralizing antibodies to VA1 in human sera is unknown. We developed a focus reduction neutralization assay (FRNT) for VA1 and measured the seroprevalence of neutralizing antibodies from two cohorts of adult and pediatric serum samples: (i) an age-stratified cohort from St. Louis, MO, collected from 2007 to 2008 and (ii) a cohort from the Peruvian Amazonian River Basin collected in the late 1990s. In the St. Louis cohort, the lowest seropositivity rate was in children 1 year of age (6.9%), rising to 63.3% by ages 9 to 12, and 76.3% of adults ≥20 years were positive. The Peruvian Amazon cohort showed similar seropositivity rates across all ages, with individuals under age 20 having a rate of 75%, while 78.2% of adults ≥20 years were seropositive. In addition, we also identified the presence neutralizing antibodies to VA1 from commercial lots of intravenous immunoglobulin (IVIG). Our results demonstrate that a majority of humans are exposed to VA1 by adulthood, with the majority of infections occurring between 2 and 9 years of age. In addition, our results indicate that VA1 has been circulating in two geographically and socioeconomically divergent study cohorts over the past 20 years. Nonetheless, a significant proportion of the human population lacks neutralizing immunity and remains at risk for acute infection.

IMPORTANCE Astroviruses are human pathogens with emerging disease associations, including the recent recognition of their capacity to cause meningoencephalitis. Astrovirus VA1 is the most commonly identified astrovirus genotype from cases of human encephalitis, but it is unknown what percentage of the human population has neutralizing antibodies to VA1. We found that 76.3 to 78.2% of adult humans ≥20 years of age in two geographically and socioeconomically distinct cohorts are seropositive for VA1, with the majority of infections occurring between 2 and 9 years of age. These results demonstrate that VA1 has been circulating in human populations over the past 2 decades and that most humans develop neutralizing antibodies against this virus by adulthood. However, a subset of humans lack evidence of neutralizing antibodies and are at risk for diseases caused by VA1, including encephalitis.

KEYWORDS astrovirus, epidemiology, humoral immunity, neutralizing antibodies, plus-strand RNA virus, serology, virology, virus-host interactions

Astroviruses are single-stranded, positive-sense RNA viruses that are the 3rd to 5th most common cause of gastroenteritis in humans (1–5). In addition, astroviruses are an emerging cause of fatal central nervous system (CNS) infections in humans and other mammals (6). Astroviruses may have additional extraintestinal tropisms, including the capacity to infect the liver and respiratory tract and to cause systemic disease (7). Currently, there are four species of astroviruses that are known to frequently infect humans, mamastrovirus 1 (classic human astrovirus 1-8), mamastrovirus 6 (astrovirus MLB1-MLB3), mamastrovirus 8 (astrovirus VA2/HMO-A and VA4), and mamastrovirus 9 (astrovirus VA1/HMO-C [VA1], VA3/HMO-B, and BF34/VA5) (1, 8–17). Three of the four species of human astroviruses have been associated with neuroinvasive disease, and VA1 is the most frequently identified neurotropic astrovirus genotype (18–27). The host factors involved with invasive astrovirus infection remain poorly described, but among cases of meningoencephalitis, a majority of patients had an underlying immunodeficiency due to X-linked agammaglobulinemia or hematopoietic stem cell transplantation (18–25). This association suggests a role for the adaptive immune response in controlling neuroinvasive astrovirus infection. Less frequently, astroviruses can cause encephalitis in immunocompetent children and adults, as three cases have been described (25–27).

One factor that may contribute to development of astrovirus infection in immunocompetent patients is the absence of preexisting humoral immunity. There is considerable variability in the seropositivity rates of antibodies to astroviruses in adults (28–32). For classic human astroviruses, the neutralizing antibody seropositivity rate for different strains range from 10 to 91% in a cohort from the Netherlands (29). For VA1, analysis of one cohort using a VA1 capsid binding assay demonstrated that 65% were seropositive, suggesting that a proportion of the population remains unexposed (33). However, this result is based on a single study from the United States, and most importantly, the assay used could not measure whether the seropositive subjects also had neutralizing antibodies (33).

It is unknown what proportion of the human population has neutralizing immunity for VA1. To address this gap, we developed a focus reduction neutralization test (FRNT) using a VA1 strain that has been propagated in cell culture (34). We quantified the prevalence of neutralizing antibodies to VA1 in two preexisting serum cohorts and determined if intravenous immunoglobulin (IVIG) lots contain neutralizing antibodies to VA1.

RESULTS

We first used a previously described luciferase immunoprecipitation assay (LIPS) assay to identify samples in the St. Louis, MO, cohort that contain antibodies that bind to VA1 capsid protein (33). A total of 14 out of 20 samples had luminescent scores greater than 22,000 light units, including 7 samples that were strongly positive with values greater than 100,000 light units (Fig. 1). We then tested the same seropositive serum samples using an FRNT that we developed. The same 14 samples had greater than 50% neutralization across all 2-fold dilutions from 1:8 to 1:128 (Fig. 1). Of the six samples scoring the lowest with the LIPS assay, three did not neutralize at any of the tested dilutions (Fig. 1). The other three samples were neutralizing at 1:8, 1:16, and 1:32 but lost their capacity to neutralize at higher dilutions (Fig. 1). Taking the results of the FRNT and the LIPS assay, we selected to test sera at a dilution of 1:128 to assess for the presence of neutralizing antibodies.

Using the St. Louis serum cohort, serum samples from 36.7% of pediatric and 76.3% of adults ≥20 years of age demonstrated neutralizing activity by FRNT. When the samples were stratified by age, children 0 to 6 months of age had a positivity rate of 52%. The seropositivity rate dropped to 6.9% for children at 1 year of age and increased to 63.3% by ages 9 to 12 (Fig. 2A). For adults older than age 20, the positivity rate binned across different ages ranged between 70.8 and 79.6% (Fig. 2A). Using logistic regression, age ($P < 0.001$), but not gender ($P = 0.34$), was associated with the presence of
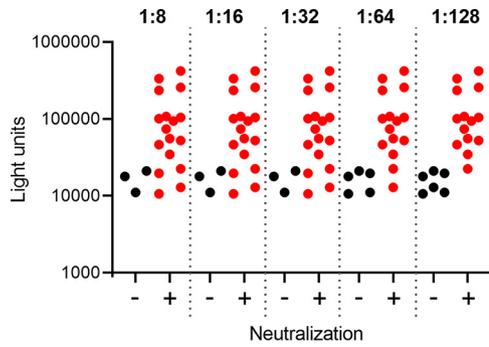
FIG 1 Comparison of light units quantified by the luciferase immunoprecipitation system (LIPS) assay and presence (+) or absence (−) of neutralizing antibodies as measured by focus reduction neutralization test (FRNT). For the FRNT, it was determined whether each sample neutralized >50% of VA1 infectious particles at each 2-fold serial dilution, from 1:8 to 1:128.

neutralizing antibodies. In analysis of the age groups, groups from 0.5 to 5.99 years of age had lower seropositivity rates than adults aged 20 to 49 (all *P* values < 0.006).

A second cohort from the Peruvian Amazon was analyzed for VA1 antibodies. Using FRNT, 77.2% of serum samples contained neutralizing antibodies. Unlike the St. Louis cohort, the seropositivity rates in the Peruvian Amazonian cohort were similar across all age bins (Fig. 2B). For adults age 20 to 49 years, the positivity rate was 76.6% (Fig. 2B). Moreover, the seropositivity rate was higher for children aged 0 to 8.99 years in the Peruvian Amazonian cohort (83.3%) than in the St. Louis cohort (29.1%; Fisher's exact test, *P* = 0.011).

We also obtained residual commercially available IVIG lots and tested them for neutralizing antibodies to assess for their potential usage as a therapeutic. For the three lots of IVIG, the anti-VA1 neutralizing antibody activity ranged from a titer of 1:1,000 to a titer of 1:10,000 (Fig. 3).

DISCUSSION

Our results demonstrate that human populations are commonly exposed to astrovirus VA1 and, most importantly, develop a neutralizing antibody response. In the age-
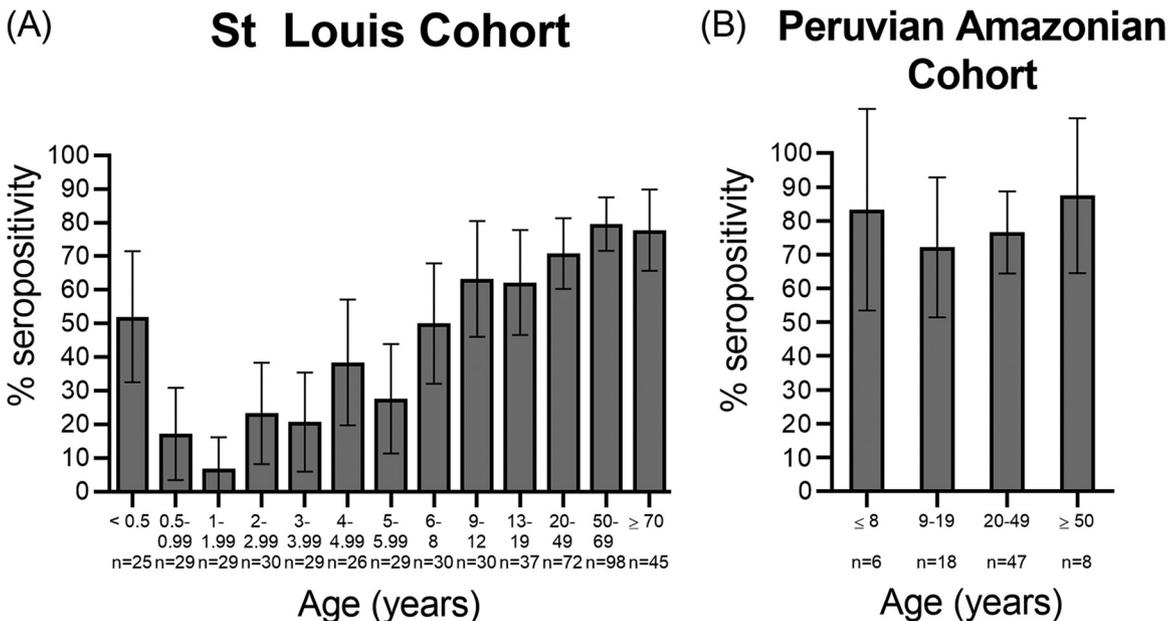

FIG 2 Seropositivity rate of VA1 neutralizing antibodies in the St. Louis, MO, cohort (A) and the Peruvian Amazonian River Basin cohort (B). Error bars represent 95% confidence intervals of the means.
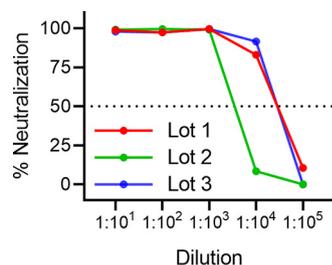
FIG 3 Quantification of neutralizing titers of three different commercial IVIG lots by serial dilution.

stratified cohort, the seropositivity rate declined after birth, increased in childhood, and plateaued in adulthood at 76.3%. Our results further corroborate the previous finding that 65% adults were seropositive for VA1 antibodies when tested by LIPS (33). These findings demonstrate that most humans have neutralizing antibodies, and future testing will quantify how protective these antibody titers are upon reexposure to VA1. Nonetheless, there are children and adults without any evidence of neutralizing antibodies to VA1, with most children under age 9 being seronegative, and these populations would be at risk for acute infection and subsequent development of invasive disease.

Our study also demonstrates that VA1 infection is common in two geographically distinct sites: an urban setting from the United States and a remote rural setting in the Amazonian Basin in Peru. The serum samples from Peru were collected in the late 1990s, suggesting that this virus has been circulating in the human population for at least the past 2 decades. Given the geographical locations of the published cases of VA1 encephalitis and other samples in which VA1 has been detected, this virus is known to circulate on at least five different continents, with no reports to date from Australia and Antarctica (17–22, 35–37).

The age-stratified cohort demonstrated two expected dynamics of the seroprevalence during childhood. First, there was significant transplacental immunity in infants under the age of 1, as the seropositivity rate was similar to the rate identified in adults. At age 1, almost all children lack evidence of neutralizing immunity, consistent with the expected loss of maternal antibodies. Once maternal passive immunity is lost, the cohort also demonstrated that a majority of VA1 infections occur from ages 2 to 9, as the seropositivity rate rose from 6.9% to 63.3%. These findings are consistent with previous hypotheses that most astrovirus exposures occur during childhood (1, 33). The Peruvian cohort demonstrated a higher seropositivity rate than the St. Louis cohort in children 0 to 8.99 years of age. While this result could simply be due to the small number of children studied from Peru, this could also be due to different risks of exposure based on socioeconomic status. Transmission dynamics could also be different based on geography. For example, VA1 has been detected in 11 out of 12 monthly sewage samples in Eastern China, suggesting that the virus can circulate in some locations throughout the year (37). It is currently unknown if the circulation dynamics differ between locations, leading to different seropositivity rates.

The detection of neutralizing antibodies for VA1 has clinical implications. Serological assays could be used to identify patients at risk for VA1 infection. The observation that commercially available IVIG lots contain neutralizing antibodies to VA1 suggests that IVIG could be used for passive immunization of patients at risk for infection and as a treatment for acute infections with VA1. Interferon, nitazoxanide, ribavirin, and favipiravir have been previously identified to have antiviral activity against VA1 in cell culture (34, 38, 39), but it is unclear if these therapies are effective *in vivo*.

There is currently no *in vivo* model of VA1 infection. Development of a small-animal model would facilitate *in vivo* testing of small-molecule drug efficacy and IVIG and quantifying protective neutralizing titers. It would also enable interrogation of the viral

epitopes important for neutralizing immunity to VA1. Knowledge of the antigens important for neutralization will also aid in rational development of a potential vaccine for VA1 and possibly other astroviruses. While the host receptor(s) required for infection of VA1 and all other astroviruses is currently unknown, the neutralizing antibodies could interact with essential viral epitopes necessary for host receptor binding.

In summary, our results demonstrate that the majority of humans have neutralizing antibodies to VA1 and that most exposures occur in childhood. However, a proportion of the population is seronegative and remains at risk for VA1 infection, including complications like encephalitis.

MATERIALS AND METHODS

Serum cohorts. Two previously studied serum cohorts of viral exposure were analyzed.

(i) St. Louis, MO, serum cohort. We first analyzed a previously published cohort of deidentified serum samples collected from Barnes-Jewish and St. Louis Children's Hospital, St. Louis, MO, between 2007 and 2008 (40, 41). A total of 509 samples were available for analysis that included 294 age-stratified pediatric specimens with 25 to 37 samples per age bin (bin ages of <0.5, 0.5 to 0.99, 1 to 1.99, 2 to 2.99, 3 to 3.99, 4 to 4.99, 5 to 5.99, 6 to 8.99, 9 to 12.99, and 13 to 19.99 years) and 215 adult specimens (age range, 20 to 85 years). Metadata regarding gender were also available.

(ii) Peruvian Amazon hepatitis cohort. We also tested a previously described cohort of serum samples from subjects living on four river systems in the Peruvian Upper Amazonian Basin collected in a 5-year period in the late 1990s (42). These deidentified serum samples were previously tested for the presence of hepatitis B and delta virus infections. A total of 79 serum samples were available for analysis, with available metadata including age (range, 1 to 62 years) and gender.

Luciferase immunoprecipitation assay. A previously published luciferase immunoprecipitation system (LIPS) assay for antibodies to the VA1 capsid (33) was used to test 20 randomly selected adult samples from the St. Louis residual serum cohort. The samples were measured in duplicate, and the average light units were compared to the results from the FRNT.

Cell culture, virus, and reagents. Caco-2 cells were maintained in growth medium consisting of Dulbecco's modified Eagle medium (DMEM) containing L-glutamine (Gibco) with 10% fetal bovine serum (FBS; Gibco) and 1% 10,000 U/ml of penicillin/streptomycin (Gibco) at 37°C with 5% CO_2. We used a 0.2-μm sterile-filtered VA1 viral stock that was passaged in Caco-2 cells (C-P8) and contains no mutations compared to a previously described C-P7 stock (34, 43). We obtained residual commercial IVIG (Gammagard) samples from three different lots and tested them in the FRNT for the presence of neutralizing antibodies.

Generation of MAb 2A2 to VA1. For production of monoclonal antibody (MAb) 2A2, a previously described protocol used to isolate neutralizing antibodies to classic human astroviruses was adapted for VA1 (44). Eight-week-old BALB/c mice were immunized with 20 μg of cesium chloride density gradient-purified VA1 at 1:1 with Freund's complete adjuvant. Three additional immunizations were performed every 2 weeks using a mixture containing 20 μg of virus with a ratio of 1:1 in Freund's incomplete adjuvant. Mice were sacrificed 4 days after the last immunization, and splenocytes were isolated and fused with Fox myeloma cells using 50% polyethylene glycol. Next, the cells were suspended in adenine-aminopterin-thymidine medium and added to 96-well plates. Enzyme-linked immunosorbent assay (ELISA) was used to screen for hybridomas secreting antibodies specific to VA1, with 2 μg/ml of purified virus used to sensitize the microtiter plates (44). Hybridomas positive by the ELISA were then assayed in a neutralization assay. The selected hybridoma was cloned three times by limiting dilution via thymocyte feeder layers and amplified as mouse ascites fluid.

Focus reduction neutralization test. Caco-2 cells were plated in 96-well plates at a density of 30,000/well. Cells were incubated 72 h to allow them to grow to confluence. Human sera were heat shocked at 56°C for 30 min, then serially diluted in FBS, and added to 100 focus-forming units (FFU) of VA1. As a positive control, intravenous immunoglobulin was also incubated with the virus, and mock incubation of serum and virus was performed by incubating the virus mixture with FBS only. The serum-virus mixture was incubated for 1 h at 37°C. The mixture was then diluted in DMEM and added to Caco-2 cells. The cells were incubated with the serum-virus mixture for 1 h at 37°C, with periodic rocking of the plate. After 1 h, the medium was removed, cells were washed once with DMEM, and then growth medium was added. The cells were incubated for 48 h at 37°C with 5% CO_2.

After 48 h, the cells were washed once with phosphate-buffered saline (PBS) and then fixed using 4% formaldehyde in PBS for 10 min. The fixed cells were washed 3 times with PBS, and then they were permeabilized with 0.2% Triton X-100 for 15 min at room temperature. The fixed and permeabilized cells were washed three times with PBS and incubated overnight with 10% horse serum in PBS at 4°C. Next, MAb 2A2 was diluted 1:10,000 in 10% horse serum-PBS and added to the cells. The plate was incubated for 1 h at 4°C. Cells were washed three times with PBS and the secondary antibody was added, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) diluted to 1:2,000 to 1:5,000 in 10% horse serum. The plate was incubated for 1 h at room temperature, and then the cells were washed three times with PBS. Fluorescent cells were then visualized using a 20× lens on a fluorescence microscope.

We defined neutralization as reduction of more than 50% of fluorescent foci compared to the mock wells in which virus was incubated with only FBS. Using the results from the LIPS assay and the FRNT on

20 randomly selected serum samples from the St. Louis cohort, we selected a 1:128 dilution to test all other serums for the presence of neutralizing antibodies.

Statistical analysis. A logistic regression model was developed for each cohort to determine the association of the presence of neutralizing antibodies to VA1 in the St. Louis cohort compared to age and gender. Models were calculated using SPSS v27 (IBM). Odds ratios were calculated, and P values of ≤ 0.05 were considered significant.

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