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Herd Immunity to GII.4 Noroviruses Is Supported by Outbreak Patient Sera[∇]

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Noroviruses (NoVs) of genogroup II, cluster 4 (GII.4), are the most common cause of outbreaks of acute gastroenteritis worldwide. During the past 13 years, GII.4 NoVs caused four seasons of widespread activity globally, each associated with the emergence of a new strain. In this report, we characterized the most recent epidemic strain, GII.4-2006 Minerva, by comparing virus-like particle (VLP) antigenic relationships and histo-blood group antigen (HBGA) binding profiles with strains isolated earlier. We also investigated the seroprevalence and specificity of GII.4 antibody in the years prior to, during, and following the GII.4 pandemic of 1995 and 1996 using a large collection of acute- and convalescent-phase serum pairs ($n = 298$) collected from 34 outbreaks. In a surrogate neutralization assay, we measured the blockade of HBGA binding using a panel of GII.4 VLPs representing strains isolated in 1987, 1997, 2002, and 2006 and a GII.3 VLP representing a strain isolated in the mid-1990s. Serum titers required for 50% HBGA blockade were compared between populations. In general, blockade of GII.4 VLP-HBGA binding was greater with convalescent-phase outbreak sera collected near the time of origin of the VLP strain. Heterotypic genotypes did not contribute to herd immunity against GII.4 NoVs based on their inability to block GII.4 VLP binding to HBGA. However, previous exposure to GII.4 NoV followed by infection by GII.3 NoV appeared to evoke an immune response to GII.4 NoV. These results support the hypothesis that herd immunity is a driving force for GII.4 evolution in the U.S. population. The data also suggest that complex patterns of cross-protection may exist across NoV genotypes in humans.

Human noroviruses (NoVs) of the family *Caliciviridae* are the most common cause of acute gastroenteritis worldwide and the leading cause of food-borne illnesses in the United States (16, 51). Although generally causing a short-lived but acute illness involving diarrhea and/or vomiting, more severe symptoms and fatalities have been reported among the elderly, infants, and immunocompromised persons (6, 17, 41, 53). Outbreaks in communal and institutional settings such as hospitals, nursing homes, cruise ships, university dormitories, and military barracks are frequently reported; furthermore, these institutions suffer the most significant economic damages during NoV outbreaks due to direct health care costs, decontamination efforts, and indirect losses (35).

Currently, there are no vaccines or antiviral therapies approved for the treatment of NoV infections, and such efforts have been significantly hampered by the lack of a simple cell culture or small-animal model for human NoVs. All information on host-pathogen interactions has come from human challenge studies and epidemiological investigations (1, 14, 16, 23, 27–29, 39, 40). As a result, the correlates of immune protection are poorly understood. Recently, the use of recombinant virus-like particles (VLPs) and replicon constructs has proven promising lines of research toward vaccine and antiviral development (7, 12, 31, 32, 46). Additionally, computer-generated

structural modelings of NoV capsid proteins combined with epidemiological and immunological investigations are proving to be valuable new tools for the more specific and rational design of vaccines and antivirals (30, 43).

NoVs have a 7.5- to 7.7-kb, single-stranded, positive-sense RNA genome consisting of three open reading frames (ORFs) packaged into a nonenveloped icosahedral capsid. Recombinant expression of the ORF2 major capsid protein (VP1) in a baculovirus or Venezuelan equine encephalitis (VEE) virus expression vector has been successful for the production of VLPs that are physically and antigenically similar to native virus particles (2, 18). The surface-exposed P2 subdomain of VP1 is the most hypervariable region of the genome and is responsible for carbohydrate ligand binding (5, 8).

Histo-blood group antigen (HBGA) expression on the gut mucosa has been shown to be correlated with susceptibility to infection with the prototype Norwalk virus (23, 29). Several but not all NoVs specifically bind to HBGAs, which likely function in some capacity as a receptor for docking and entry into the cell during infection (20–22, 24, 29). Recent epidemiological studies indicate that there is an association between HBGA expression and genetic susceptibility to NoV strains of genogroup II, cluster 4 (GII.4), although the association is not as clear as was shown previously with Norwalk virus (27, 47).

More than 25 genotypes of human NoVs have been described (52), but GII.4 strains are the most prevalent globally. Over the last two decades, NoV epidemic peaks associated with GII.4 have been reported worldwide in 1996, 2002, 2004, and 2006, and at least one novel GII.4 variant strain could be

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identified during each of these years (4, 11, 33, 38, 42). Epidemic seasons were followed by periods of moderate to low NoV activity, where GII.4 strains cocirculated indiscriminately along with NoVs of several other genotypes. During the GII.4 epidemic peaks of 1996 and 2002, the proportion of GII.4 strains causing outbreaks in the United States was greater than 50%, but between these years, the GII.4s were responsible for just 10 to 29% of all NoV outbreaks (Centers for Disease Control and Prevention [CDC], unpublished data). One year after the GII.4 pandemic of 1995 and 1996, GII.3 NoV circulation increased from about 4% in 1996 to about 25% in 1997 (38). After 2002, variant GII.4 strains emerged much more quickly, causing epidemics every 1 to 2 years. This type of epochal evolution has been described previously by our group and others (9, 30, 43).

Recently, our group has proposed a mechanism for the persistence of GII.4 in the human population based on the epidemic patterns observed for GII.4 strains during the past 20 years (30). Clear evidence of structural changes in the P2 region of the GII.4 capsid protein leading to alternative HBGA receptor usage and immune evasion could be observed for GII.4 strains isolated in 1987, 1997, 2002, 2004, and 2005. HBGA binding with representative GII.4 VLPs supported the structural models, and receptor blockade data provided by hyperimmune mouse antisera unambiguously demonstrated antigenic variation between GII.4 strains and phenotypic support for immune evasion. Furthermore, blockade patterns of human sera from a GII.4 outbreak supported herd immunity as the driving force for antigenic change (30).

Here, we expand upon our previous work by examining receptor blockade patterns of human sera collected during NoV outbreaks over a 21-year period from 1985 to 2006. Our results support a general pattern of receptor blockade expected when herd immunity is the driving force of antigenic change. Furthermore, we demonstrate that the receptor blockade assay has the specificity to discriminate minute differences between heterotypic genogroups important for understanding cross-protective immunity. Based upon these findings, we conclude that in addition to NoV strain surveillance, analyzing human sera collected during routine outbreak surveillance may be valuable for assisting in the choice of VLPs included in vaccine candidates in an approach similar to that for influenza virus.

MATERIALS AND METHODS

Serum and stool specimens. A total of 298 acute- and convalescent-phase serum pairs from 34 outbreaks that occurred between 1985 and 2006 which had been collected and archived at the National Calicivirus Laboratory at the CDC (Atlanta, GA) were selected for this study. Acute-phase serum samples had been collected between 0 and 7 days after the onset of illness, and convalescent-phase serum samples had been taken 13 to 47 days after the acute-phase sample. For outbreaks occurring from 1988 to 2006, corresponding stool specimens tested positive for GII NoV and negative for GI NoV by reverse transcription (RT)-PCR using primers targeting a small region of the capsid gene followed by sequence analysis (26). Since stool samples were no longer available for 10 of the 11 outbreaks from 1985 to 1988, archival reports from the original CDC outbreak investigation were studied for classifying outbreaks as being suspected of being caused by NoV based on (i) fulfillment of Kaplan criteria (25) or (ii) positive test for NoV by immune electron microscopy or enzyme immunoassay using convalescent-phase sera from patients infected with Snow Mountain virus or Toronto virus. In addition, outbreaks with serum samples demonstrating seroconversion to Norwalk virus were excluded.

Stool sample processing. Viral RNA was extracted from 10% stool suspensions (phosphate-buffered saline [PBS]) with the Ambion MagMax (Applied Biosystems) kit and the KingFisher automated extractor (Thermofisher) and tested for GII NoV by TaqMan real-time RT-PCR (48). Positive samples were genotyped by amplifying region C (26) using a Qiagen (Valencia, CA) one-step RT-PCR kit. Cycling conditions included an RT step for 1 h at 42°C and activation of *Taq* polymerase at 95°C for 15 min followed by 35 cycles of 94°C, 50°C, and 72°C for 30 s each, followed by a final extension step for 7 min at 72°C. Amplicons were sequenced using a Big Dye Terminator cycle sequencing kit, BigDye X-Terminator purification kit, and the ABI (Foster City, CA) 3130 genetic analyzer.

VLPs. Complete ORF2 genes of NoV strains from outbreak stool specimens collected in 1997, 2002, 2006, and 1999 and representing clusters GII.4-1997, GII.4-2002, GII.4-2006, and GII.3-TV (Fig. 1), respectively, were amplified and cloned into VEE virus replicons as previously reported (2, 30, 31). The GII.4-1987 ORF2 gene was commercially synthesized by BioBasic and inserted into the VEE virus replicon (30). VEE virus replicon particles were generated, and VLPs were expressed in baby hamster kidney (BHK) cells, purified, and visualized by negative-staining electron microscopy as described previously (2).

Serology. Between 1 and 35 serum pairs from each outbreak were tested for a fourfold increase (seroconversion) in anti-GII.4 VLP immunoglobulin G (IgG) titers between acute- and convalescent-phase sera by enzyme-linked immunosorbent assay (ELISA) as previously described (29). Briefly, 1 μg/ml of VLPs diluted in PBS was bound to 96-well plates for 4 h before plates were blocked overnight in 5% Blotto. Twofold serial dilutions of acute- and convalescent-phase outbreak patient sera were added to the plates, and human IgG specifically reacting with the VLPs was detected using mouse anti-human IgG-alkaline phosphatase (Sigma, St. Louis, MO) and *para*-nitrophenol phosphate (Sigma). Either GII.4-1987 or GII.4-1997 VLPs were used, as previous results indicated that they are antigenically very similar (30). NoV-specific IgG serum concentrations were calculated by comparison to a standard curve of purified IgG and used to calculate geometric mean titers (GMTs) of serum (dynamic range for IgG standard of 0.008 to 1 μg/ml IgG). Comparisons between groups of convalescent-phase serum specimens were done using Mann-Whitney analysis (http://faculty.vassar.edu/lowry/VassarStats.html).

HBGA binding and blockade assays. HBGA binding studies were performed under conditions identical to those for the HBGA blockade studies but without the addition of human sera (see below). Briefly, 116 convalescent-phase sera from patients from 28 outbreaks demonstrating seroconversion to GII.4 VLPs were subjected to a surrogate neutralization assay to measure blockade antibody titers as described previously (30, 31). Briefly, GII.4-1987, GII.4-1997, GII.4-2002, GII.4-2006, and GII.3-TV VLPs (1 μg/ml) were incubated with twofold serial dilutions of sera at 37°C for 1 h. After washing plates with PBS–0.05% Tween 20, the serum-VLP sample mixture was added to preblocked Neutravidin plates (Pierce) that had been coated with 10 to 20 μg biotinylated HBGA (Glycotech) in 5% Blotto and incubated for 1.5 h at 37°C. Binding or VLP blockade was detected using matched mouse anti-GII.4-VLP sera followed by goat anti-mouse IgG-alkaline phosphatase (Sigma) and detection by *para*-nitrophenol phosphate (Sigma). H type 3 HBGA was used for GII.4-1987, GII.4-1997, and GII.4-2006; Le^y was used for GII.4-2002; and A type or H type 3 HBGA was used for GII.3-TV based on the HBGA-VLP combination resulting in the highest optical density (OD) reading. For GII.3-TV experiments, the OD value for the 100% binding controls were lower for A type than for H type 3 HBGA, but blockade was linear and proportional, resulting in equivalent slopes.

By comparison to a control (VLP binding to HBGA in the absence of human sera), 50% antibody blockade titers (BT50s) were determined with twofold serum dilutions from 1:100 to 1:6,400. Geometric mean BT50s were calculated as log-transformed reciprocal blockade antibody titers and are reported as back-transformed titers. A BT50 was prescribed for a sample only when a linear relationship between blockade serum titer and percent control binding could be observed. To be considered acceptable, OD readings were between 0.1 and 3.0 above the background. Sera with a BT50 of less than 100 (a 1:100 dilution) or of greater than 6,400 (a 1:6400 dilution) were given values of 100 and 6,400, respectively. BT50 values were compared between groups of serum samples using Mann-Whitney analysis (http://faculty.vassar.edu/lowry/VassarStats.html). Blockade experiments using antisera collected from mice immunized with GII.4-1997 or GII.3-TV VLPs were done as previously reported (30).

Homology model of GII.3. A homology model of the GII.3 capsid P domain was generated using the X-ray crystal structure of the P domain of GII.4 virus VA387 in complex with B trisaccharide (5) (Protein Data Bank accession number 2OBT [http://www.rcsb.org/pdb/home/home.do]) as a template. Briefly, an alignment of the GII.4 and GII.3 P-domain regions was generated and demonstrated sequence identity greater than 60%, indicating that GII.4 was an appro-

Sera collections used in this study

Large triangles indicate epidemic peaks of GII.4 NoV activity
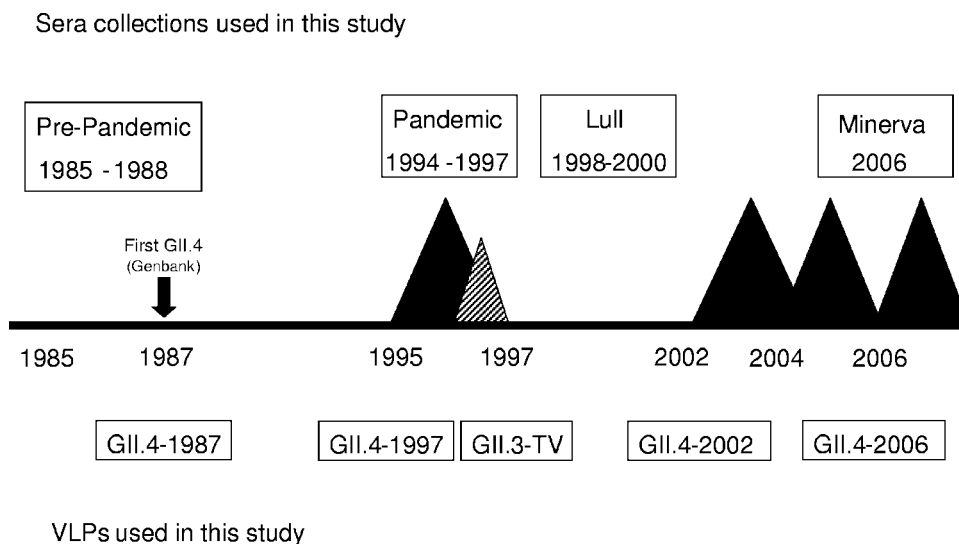
Small triangle indicates increased activity of GII.3 NoV

FIG. 1. Timeline from 1985 to 2006 indicating the classifications for the populations from which outbreak serum specimens were collected and the VLPs used in this study.

priate template for generating the GII.3 homology model. The GII.3 homology model was then generated using the program Modeler, version 8.2, using the automodel class (10, 36). The Protein Data Bank file generated for the GII.3 P domain was visualized on the molecular modeling tool MacPyMol (DeLano Scientific).

RESULTS

Characteristics of outbreaks from 1985 to 2006 and representative VLPs. Since first being described in 1987, at least seven GII.4 NoV variants have been circulating within the U.S. population. Figure 1 indicates epidemic peaks of NoV activity in the United States from 1985 to 2006, centered on the first documented GII.4 pandemic (1995 to 1996). Specific time periods, dubbed here "prepandemic," "pandemic," "lull," and "Minerva," were chosen to explore serological relationships of homotypic and heterotypic patient outbreak sera with VLPs representative of chronological GII.4 NoVs. Because the NoV pandemic of the mid-1990s was followed by a sharp rise in GII.3 activity in the United States and a subsequent period of very little GII.4 or GII.3 activity, serological relationships among GII.4 and GII.3 outbreak sera and their representative VLPs were investigated. We have previously reported the characteristics of VLPs representing GII.4-1987, GII.4-1997, and GII.4-2002 (30). For this study, we designed two additional novel VLPs. The first represents the epidemic GII.4-2006 Minerva strain that emerged in 2006, reaching epidemic proportions in the United States and Europe. The second, GII.3-TV, represents the GII.3-Toronto virus that circulated in the United States in 1999.

Variation within the P2 subdomain resulting in differential patterns of HBGA binding, and, thus, host susceptibility, has been suggested to be one possible mechanism for GII.4 NoV persistence (30). Figure 2 shows the HBGA binding patterns of

the VLPs used in this study. As reported previously, GII.4-1987 bound to H type 3; GII.4-1997 bound to H type 3, Le^y, Le^b, and A and B trisaccharides; and GII.4-2002 bound to H type 3 and Le^y (30). The HBGA binding pattern for GII.4-2006 VLP is nearly identical to that of GII.4-1997 binding to H type 3, Le^y, Le^b, and A and B trisaccharides. GII.3-TV VLPs bound to H type 3 and A trimer.

Serological relatedness among outbreak sera from 1985 to 2006. To study the antigenic relationship between the GII NoVs circulating between 1985 and 2006, we analyzed anti-GII.4 VLP IgG from serum pairs collected during 34 GII NoV outbreaks (Table 1). In all, 140 (47%) of the 298 GII outbreak serum pairs had at least a fourfold increase in levels of reactive IgG to GII.4-1987 or GII.4-1997 between acute- and convalescent-phase serum collection (seroconverted), including at least one serum pair from 32 of the 34 outbreaks. Within the four chronological periods, 56 (42%), 51 (49%), 27 (51%), and 6 (100%) serum pairs seroconverted to GII.4 VLPs during the prepandemic, pandemic, lull, and Minerva periods, respectively. To assess serological relationships among homotypic and heterotypic outbreak sera, the data were further stratified by genotype. Figure 3 indicates the GMTs (µg/ml) of IgG reactive with GII.4-1987 or GII.4-1997 VLPs in acute- and convalescent-phase sera from patients seroconverting to GII.4 VLP.

The GII.4 sera from the pandemic period had the greatest geometric mean convalescent IgG titers (mean GMT of 365 µg/ml) (Fig. 3A). Compared to this group, GMTs for convalescent-phase sera did not differ significantly between the GII.4 outbreaks during the prepandemic, lull, or Minerva periods ($P \geq 0.91$) (mean GMTs of 331, 306, and 297 µg/ml, respectively). Consonant with previous reports by our group (30), this suggests antigenic similarities among GII.4 NoVs circulating from 1985 to 2006. In addition, cross-reactivity was observed
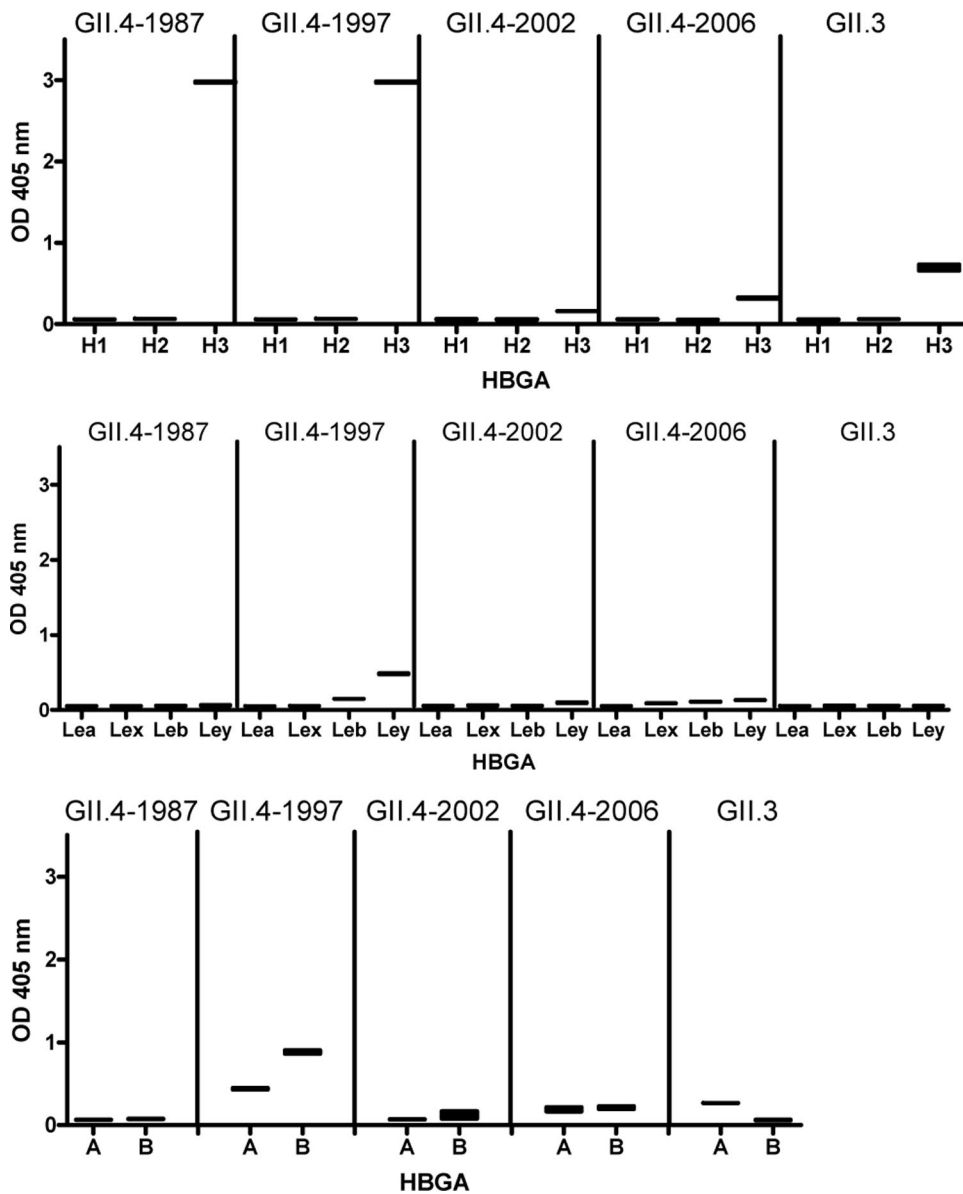
FIG. 2. HBGA binding of GII.4-1987, GII.4-1997, GII.4-2002 (30), GII.4-2006, and GII.3-TV VLPs. Boxes indicated averages, and bars indicate maximum OD values at 405 nm.

for the GII.4 VLPs and sera from the GII-unspecified outbreaks from the prepandemic period (mean GMT of 219 μg/ml) (Fig. 3B). In contrast, convalescent-phase sera from non-GII.4 outbreaks from the lull period had a mean GMT equal to 165 μg/ml, which was significantly lower than that of the GII.4 sera from the pandemic period ($P = 0.02$), and prepandemic and lull-period GII.3 sera were not very cross-reactive with GII.4 VLPs (mean GMTs of 46 μg/ml and 65 μg/ml, respectively), demonstrating antigenic dissimilarities between heterotypic genotypes. Interestingly, though, GMTs for GII.3 outbreak sera from the pandemic period were high (mean GMT of 282 μg/ml) and did not differ from that of the pandemic GII.4 sera ($P = 0.28$) (Fig. 3C), indicating antigenic similarity or evidence of an immunological memory response from a previous GII.4 infection.

Blockade of GII.4 VLP binding to HBGA by homotypic and GII.4 outbreak sera. Regardless of the GII outbreak strain, many of the serum pairs tested by ELISA demonstrated cross-reactivity to GII.4 VLPs. To further determine the specificity of serum antibodies, convalescent-phase sera from the 116 serum pairs that seroconverted to GII.4 VLPs were further tested for their ability to block GII.4 VLP binding to HBGAs. First, we analyzed blockade by homotypic antisera. The GII.4-2006 VLP was cloned from a stool specimen collected from a GII.4 Minerva outbreak in 2006. The GII.4-2006 outbreak sera discussed above were collected from this same outbreak. Acute-phase sera collected from this outbreak did not block the binding of GII.4-2006 to HBGA at any concentration tested, but binding could be efficiently blocked by the homotypic convalescent-phase sera from all six patients involved in

TABLE 1. Epidemiological characteristics of outbreaks described in this study

Period	Outbreak	Date (mo and yr)[a]	Genotype	No. of seroconverters	Total no. of samples	% Seroconversion	Avg age (yr)	Age range (yr)	% Female subjects	Setting	Location	Source
Prepandemic	1	Oct 1985	GII.un	2	4	50	86	82–92	75	Nursing home	FL	Person
	2	Oct 1986	GII.un	1	7	14	21	18–25	0	College	TN	Food
	3	Feb 1987	GII.un	4	11	36	Not available	Not available	0	Nursing home	SC	Person
	4	1987	GII.un	5	12	42	26	18–66	50	College	HI	Person
	5	Sep 1987	GII.un	5	19	58	19	17–20	0	College	OK	Food
	6	Dec 1987	GII.un	2	8	25	24	5–39	50	Party	AK	Food
	7	Jan 1988	GII.un	1	5	20	Not available	Not available	50	Hospital	Canada	Unknown
	8	Jan 1988	GII.un	1	11	9	Not available	Not available	Not available	Cruise ship	Cruise ship	Unknown
	9	Mar 1988	GII.un	5	8	63	41	37–48	50	Cruise ship	WA	Food
	10	Apr 1988	GII.un	5	6	83	22	15–30	50	Banquet	MI	Food
	11	Apr 1988	GII.4	17	35	49	66	24–93	16	School	SD	Unknown
	12	Feb 1989	GII.3	0	1	0	Not available	Not available	Not available	Nursing home	WI	Unknown
	13	Apr 1992	GII.3	2	7	29	72.24	Not available	Not available	Nursing home	ID	Unknown
Pandemic	14	Apr 1994	GII.4	2	5	40	85.3	70–101	100	Nursing home	VA	Unknown
	15	Nov 1994	GII.3	3	8	38	47	35–56	0	Banquet	MD	Unknown
	16	Feb 1995	GII.3	4	8	50	Not available	Not available	100	Nursing home	VT	Unknown
	17	May 1995	GII.3	3	9	33	81.3	78–87	100	Nursing home	PA	Ice
	18	Sep 1995	GII.4	6	14	43	90.2	83–100	86	Nursing home	HI	Unknown
	19	Jan 1996	GII.4	2	5	40	Not available	Not available	100	Medical facility	SC	Person
	20	Jan 1996	GII.4	4	5	80	81.33	85–92	75	Nursing home	MA	Unknown
	21	Mar 1996	GII.3	14	21	67	Not available	85–87	54	Nursing home	WA	Unknown
	22	Dec 1996	GII.3	5	7	71	69	78–87	83	Nursing home	NH	Unknown
	23	Dec 1996	GII.3	6	19	32	Not available	78–87	83	Banquet	TN	Food
	24	Mar 1997	GII.4	2	4	50	84.3	79–89	25	Nursing home	PA	Unknown
Lull	25	Mar 1998	GII.4	4	5	80	24.4	19–22	80	College	TX	Food
	26	June 1998	GII.2	1	7	14	36.3	22–47	100	Day care (workers)	FL	Unknown
	27	Nov 1998	GII.3	3	9	33	Not available	Not available	0	College	FL	Ice
	28	Dec 1998	GII.2	0	4	0	20.4	16–35	0	School	NH	Food
	29	Dec 1998	GII.1	4	6	67	Not available	Not available	100	Long-term care	WV	Unknown
	30	Dec 1998	GII.3	2	3	67	Not available	Not available	0	Country club	NM	Water/ice
	31	May 1999	GII.14	4	5	80	56.4	44–86	40	Wedding	ND	Food
	32	Dec 1999	GII.4	6	7	86	Not available	Not available	83	Nursing home	WV	Person
	33	May 2000	GII.10	3	7	43	82.1	57–87	57	Nursing home	HI	Person
Minerva	34	Oct 2006	GII.4	6	6	100	Not available	Not available	50	Cruise ship	MS	Person

[a] Jan, January; Feb, February; Mar, March; Apr, April; Sep, September; Oct, October; Nov, November.
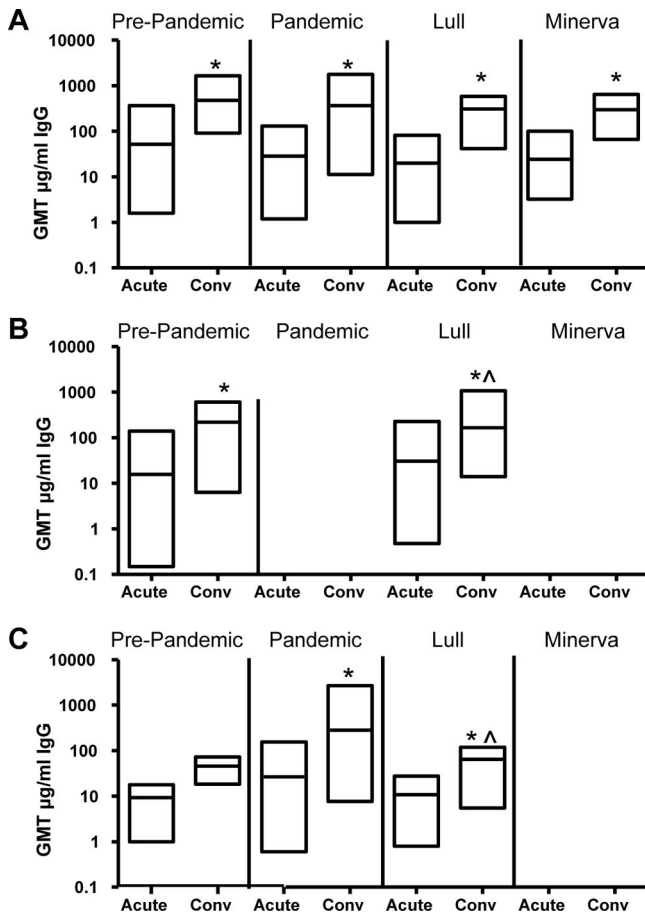
FIG. 3. Geometric mean IgG titers in acute- and convalescent-phase sera from outbreak patients seroconverting to GII.4-1997 or GII.4-1987 VLPs. Data are stratified by period and by genotype of the outbreak, including only GII.4 outbreaks (A), GII-unspecified (pre-1988 sera) and non-GII.4 outbreaks (non-GII.4 represents sera from outbreaks caused by GII.1, GII.2, GII.10, and GII.14 NoVs) (B), and only GII.3 outbreaks (C). Boxes represent maximum, mean, and minimum values for geometric mean IgG titers in each category. ∗ indicates a significant difference in mean IgG titers between acute- and convalescent (Conv)-phase sera, and ˆ indicates a significant difference between the mean convalescent IgG titers compared to those of GII.4 outbreaks of the pandemic period.

the 2006 outbreak, as the geometric mean BT50 of VLP-HBGA binding was 2,016 (Fig. 4).

To expand upon the HBGA blockade data reported above and to investigate if blockade patterns indicative of herd immunity could be revealed using outbreak sera from patients infected with GII NoVs, 65 convalescent-phase serum specimens collected from 15 outbreaks ranging from 1988 to 2000 were tested in the surrogate neutralization assay, in addition to the 2006 outbreak noted above. Sera from outbreaks that were confirmed to be caused by GII.4 NoV (45 specimens from eight outbreaks) were examined for the ability to block GII.4 VLP binding to HBGA (Fig. 5). In the prepandemic period, geometric mean BT50 values were similar for GII.4-1987 (BT50 of 680) and GII.4-1997 (BT50 of 737) VLPs ($P = 0.83$), and these values were significantly greater than the geometric mean BT50 value for GII.4-2002 VLP (BT50 of 301) ($P = 0.03$
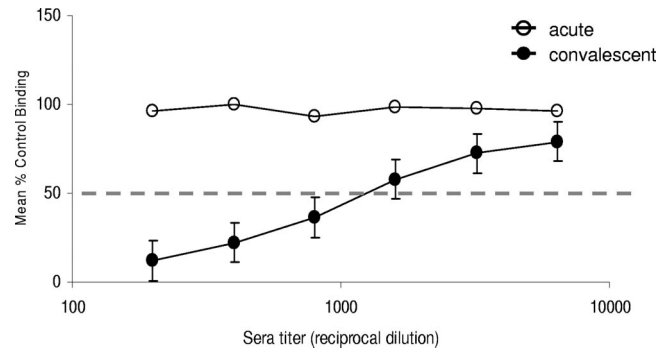

FIG. 4. Blockade of GII.4-2006 VLP by homotypic outbreak sera. Acute-phase ($n = 6$) and convalescent-phase ($n = 6$) serum pairs collected during the GII.4-2006 outbreak from which the GII.4-2006 VLP was cloned were assayed for the ability to block homotypic VLP interactions with H type 3. Error bars indicate the standard errors. The dashed line indicates the corresponding BT50 values on the x axis.

for 1987 versus 2002; $P = 0.02$ for 1997 versus 2002). Similarly, in the pandemic period, the geometric mean BT50 values for GII.4-1987 (BT50 of 1,467) and GII.4-1997 (BT50 of 2,363) were similar ($P = 0.14$) but were significantly greater than that of GII.4-2002 (BT50 of 336) ($P = 0.01$ for 1987 versus 2002; $P = 0.01$ for 1997 versus 2002). Alternatively, during the lull period, geometric mean BT50 values did not differ among the three VLPs (BT50 values of 1,213, 1,716, and 1,056 for GII.4-1987, GII.4-1997, and GII.4-2002, respectively) ($P = 0.51$ for 1987 versus 1997; $P = 1$ for 1987 versus 2002; $P = 0.47$ for 1997 versus 2002). Together, the higher BT50 values over time demonstrated with the GII.4-2002 VLP using GII.4 outbreak sera indicate that the circulation of strains similar to GII.4-2002 appeared only in the few years leading up to the GII.4-2002 epidemic that emerged in the United States in 2002. Geometric BT50 titers for the six convalescent-phase serum samples isolated from the 2006 GII.4-Minerva outbreak were all high and did not differ significantly between the different GII.4 VLPs, GII.4-1987 (BT50 of 800), GII.4-1997 (BT50 of 1,131), and GII.4-2002 (BT50 of 1,796) VLPs ($P = 0.58$ for 1987 versus 1997; $P = 0.21$ for 1987 versus 2002; $P = 0.42$ for
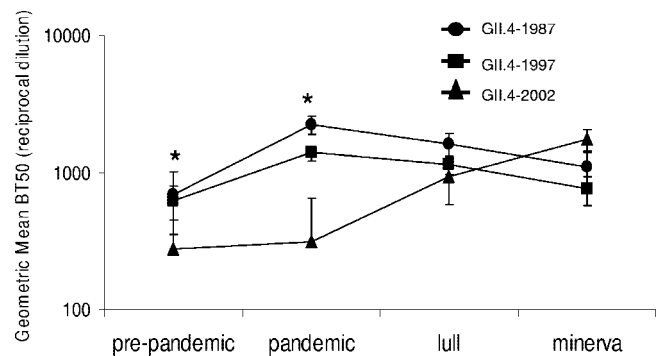

FIG. 5. Blockade of GII.4 VLP binding to HBGA by convalescent-phase GII.4 outbreak sera collected during 1988 to 2006. Geometric mean BT50 values for each VLP represent the geometric means of individual BT50 values for each serum sample within the indicated time period. Error bars indicate the standard errors. ∗ indicates significant differences between GII.4-1987, GII.4-1997, or GII.4-2002 VLPs within each period.

1997 versus 2002) (Fig. 5), suggesting an immunological memory response to earlier GII.4 strains stimulated upon infection with GII.4-Minerva.

Blockade of GII.4 VLP binding to HBGA by heterotypic outbreak sera. To investigate the influence of heterotypic GII NoVs on the GII.4-VLP HBGA blockade, we examined the heterotypic sera from each period, individually. During the prepandemic period, the geometric mean GII.4-specific IgG titer was high for the 37 serum pairs collected from patients from GII-unspecified outbreaks, but the geometric mean BT50 was very low for all VLPs tested (mean BT50 values of 364 for GII.4-1987, 604 for GII.4-1997, and 291 for GII.4-2002) (Fig. 6A). Similarly, convalescent-phase sera from GII.1, GII.2, GII.10, and GII.14 outbreaks in the lull period did not block GII.4 VLP binding, as geometric mean BT50 values were 381, 442, and 244 for GII.4-1987, GII.4-1997, and GII.4-2002, respectively, demonstrating antigenic dissimilarities between GII.4 and the heterotypic genotypes (Fig. 6B).

Convalescent-phase sera from GII.3 outbreaks in the pandemic period were less capable of blocking GII.4-1987 and GII.4-2002 VLP binding to HBGA (geometric mean BT50 values of 566 for GII.4-1987, 651 for GII.4-1997, and 519 for GII.4-2002) than for blocking the binding of GII.3-TV VLP (geometric mean BT50 of 2,691) ($P = 0.01$ for 1987 versus TV; $P = 0.05$ for 1997 versus TV; $P = 0.01$ for 2002 versus TV) (Fig. 6C), and GII.4 outbreak sera from the pandemic period were even less capable of blocking GII.3-TV binding to HBGA (geometric mean BT50 of 453; $P = 0.01$) (Fig. 6D). However, the geometric mean BT50 for GII.3 pandemic-period serum blockade of GII.4-1997 (mean BT50 of 1,131) did not significantly differ from the geometric mean BT50 (mean BT50 of 2,363) for the GII.4 pandemic-period serum blockade of GII.4-1997 VLPs ($P = 0.13$). This indicates either antigenic similarities between GII.4 and GII.3 or a memory response from a previous GII.4 infection elicited by the current GII.3 infection. Using mouse hyperimmune antisera, we removed the possible influence of previous NoV exposure and examined homotypic and heterotypic cross-reactivity and blockade of GII.4 and GII.3 VLP binding to HBGA. Figure 7 clearly indicates that heterotypic blockade does not occur between GII.4-1997 and GII.3-TV. GII.3-TV antisera effectively blocked homotypic VLP-HBGA interactions but were unable to block the GII.4-1997 interaction with HBGA. Likewise, antisera to GII.4-1997 VLPs blocked homotypic VLP but not GII.3-TV VLP interactions with HBGA.

DISCUSSION

During the winter season of 1995 to 1996 (pandemic period), an acute increase in GII.4 NoV activity was reported worldwide (38, 49). This was followed by a 5-year period of relative quiescence (lull period) for GII.4s until an antigenic variant GII.4 strain (Farmington Hills) emerged in 2002, causing the second reported NoV causing a global spike in GII.4 activity (33, 50). Since this time, antigenic variants of GII.4 NoVs have emerged every 1 to 2 years (30). Our group recently proposed antigenic drift as being one mechanism for GII.4 NoV evolution and described its similarities to influenza A virus evolution, for which a variant H3N2 virus emerges every 3 years on average (30, 44). Antigenic variation between current and con-

temporary GII.4 strains was demonstrated with hyperimmune mouse antisera and with convalescent-phase sera from a 1988 GII.4 NoV outbreak (30). Here we examined a larger panel GII NoV outbreak sera from 1985 to 2006 to estimate herd immunity to GII.4 NoV in the U.S. population over time, focusing on periods between the 1995 and 1996 pandemic and the emergence of the next GII.4 antigenic variant in 2002, the period prior to the first GII.4 strain to be sequenced, and during domination of a contemporary GII.4 (Minerva) strain in 2006.

The GII.4-MD145 strain detected in a 1987 NoV outbreak was the first characterized GII.4 strain (17), and to date, no prior GII.4 sequences have been reported. The GII outbreak sera collected during 1985 to 1987 required high concentrations of convalescent-phase sera to effectively block HBGA binding of GII.4-1987, GII.4-1997, and GII.4-2002 VLPs. This finding as well as the low numbers of serum specimens that seroconverted are evidence for either low circulation levels of GII.4 strains prior to 1987 or circulation of GII.4 antigenic variants having some cross-reactivity with recognized GII.4s but being unable to prevent GII.4 VLP binding to HBGA in the surrogate neutralization assay.

The GII.4 sera collected during the pandemic period showed a blockade pattern expected for a population recently exposed to GII.4-1997 but unexposed to the GII.4-2002 virus. All sera blocked the binding of GII.4-1997 VLPs (and the antigenically similar GII.4-1987 VLPs) to HBGA but were less capable of blocking binding of GII.4-2002 VLPs. In the years leading up to the next GII.4 epidemic with widespread activity in 2002, GII.4 strains circulated at relatively low levels, causing only about half the total number of NoV outbreaks annually (hence, the "lull-period" nomenclature) (34). By 2000 to 2001, GII.4 NoVs were detected in only 16% of outbreaks but increased to 61% during the following winter season (3). About 50% of our lull-period serum specimens were from GII.4 outbreaks, and the others were from other GII genotypes. Sera from non-GII.4 outbreaks during the lull period had low anti-GII.4 IgG titers and were unable to block GII.4 VLP binding to HBGA, indicating that these viruses likely share some common capsid epitopes with GII.4 VLPs but likely lack specific neutralizing epitopes. Therefore, infection by the heterotypic GII strains examined in this study (GII.1, GII.2, GII.10, and GII.14) would not likely be protective against GII.4 infection and likely did not contribute to herd immunity against GII.4 NoVs.

The lull period which we describe for GII.4 NoVs is also very similar to a period in the early 1980s when there was a stagnation in influenza A virus (H3N2) antigenic diversity for 8 years before a new antigenic variant emerged in 1987 (44). This period where mutants are unable to overcome a fitness barrier has been described as a "strain lock," which may occur in both naturally infected and vaccinated populations between periods of antigenic drift (13). Low-level circulation of GII.4 NoVs between 1997 and 2001, and the inability of non-GII.4 strains to cross-block HBGA binding of GII.4 VLPs, suggests a contribution to the suppression of GII.4-1997 epidemics by immunological memory.

Interestingly, sera from the GII.4 outbreak from the lull period blocked the binding of GII.4-1987, GII.4-1997, and GII.4-2002 VLPs, even though sera were collected before GII.4-Farmington Hills strains dominated globally. New vari-
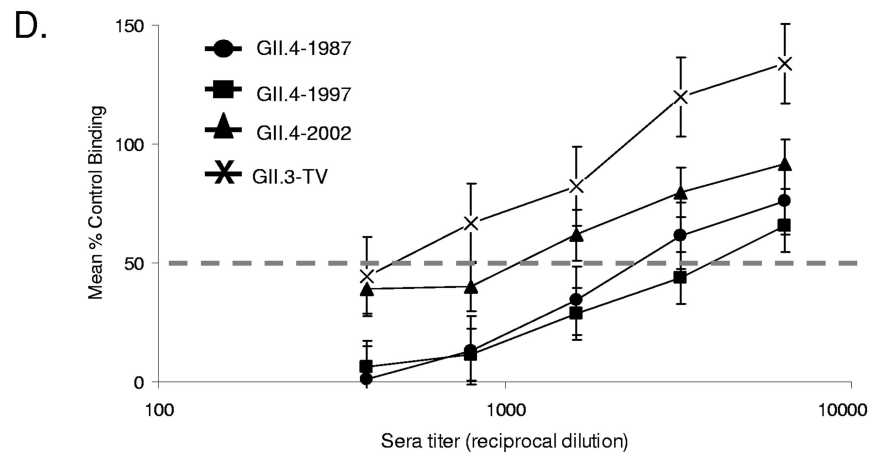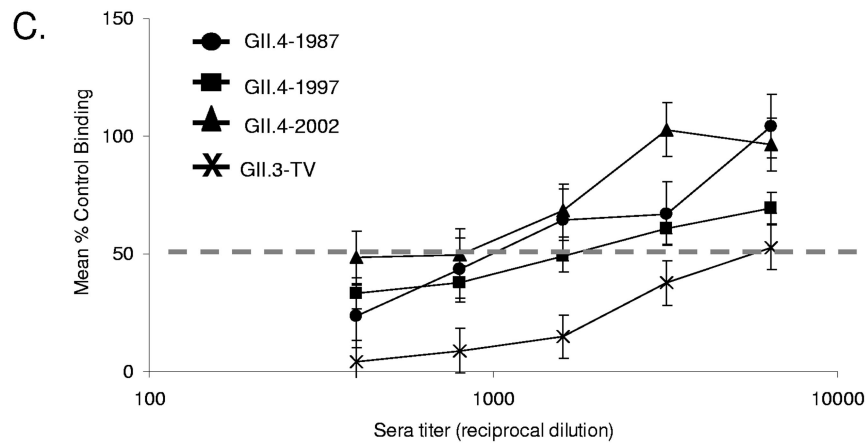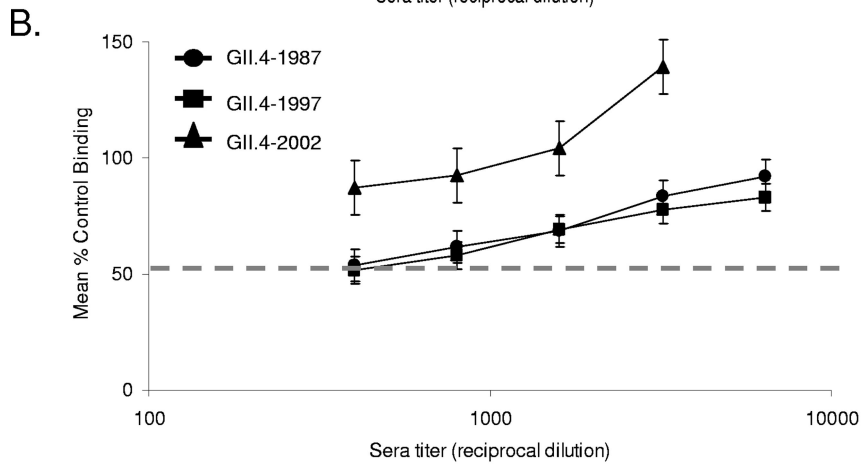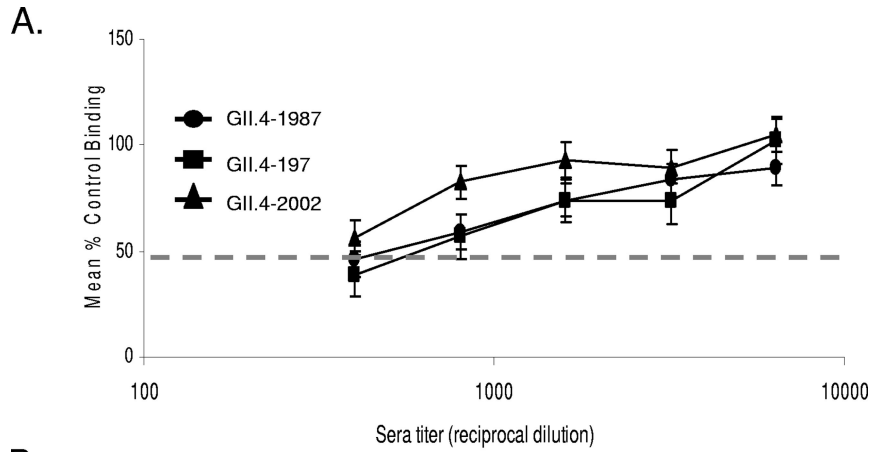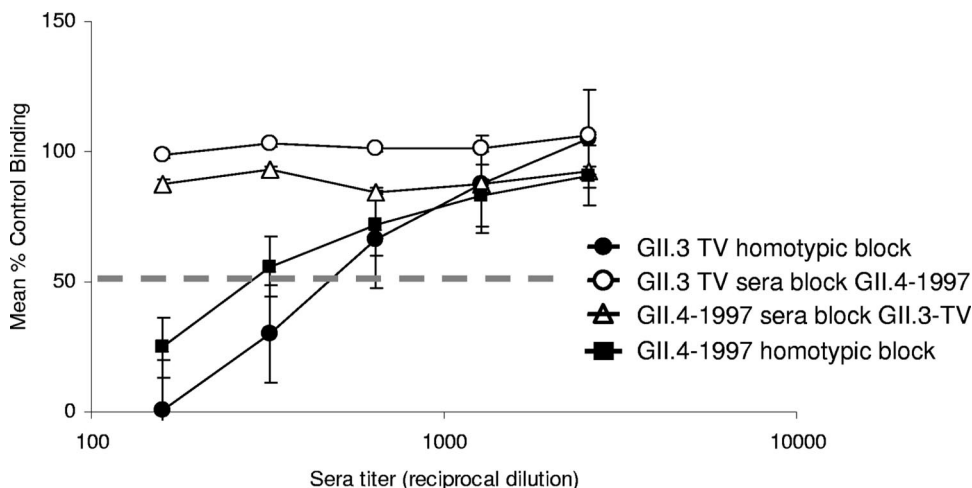
FIG. 7. Murine antiserum blockade of GII.4-1997 and GII.3 binding to HBGA. Antisera collected from mice immunized with GII.4-1997 ($n = 3$) or GII.3-TV ($n = 3$) were assayed for the ability to block homotypic VLP (solid symbols) or heterotypic VLP (open symbols) interactions with H type 3. The dashed line indicates the corresponding BT50 values on the x axis.

ants of influenza A virus begin to circulate 2 years prior to the period for which they were the dominant cluster (44). Several minor GII.4 strains in circulation during the lull period have genetic similarities to GII.4-2002 (CDC, unpublished) and may be antigenically similar as well. P2 region sequencing of the lull-period GII.4 strains revealed 100% amino acid identity with GII.4-1997, indicating that a blockade of GII.4-2002 by the patient sera was likely not the result of the current GII.4 infection but possibly due to a memory response to a previous exposure to a GII.4-2002-like strain. However, if immunity to GII.4-2002-like strains began prior to the years of its domination, the proportion must have still been too low to generate sufficient herd immunity to prevent the 2002 epidemic. It is therefore likely that the persons infected with GII.4 during the lull period in this study are not representative of the "herd" population.

Alternatively, we know that two strains of GII.4-Farmington Hills cocirculated in 2002. The representative VLPs, GII.4-2002 and GII.4-2002a, differ in the P domain by only 2 amino acids but are antigenically very distinct (30). While the proportion of each strain in circulation in the United States during this time is not known, it is possible that GII.4-2002-like strains circulated in the late 1990s but that GII.4-2002a-like strains (or other antigenically distinct GII.4-Farmington Hills strains) were primarily responsible for the U.S. epidemic.

During the pandemic period, 69% of the NoV outbreaks were caused by GII.4 viruses (38). The following year, the number of GII.4 outbreaks dropped to 31%, but the number of GII.3 outbreaks increased from about 4% in 1995 to 1996 to about 25% in 1996 to 1997 (38). We and others (37) have shown that convalescent-phase GII.3 outbreak sera are relatively cross-reactive with GII.4 VLPs. However, the pandemic-period GII.3 sera used in the current study had very high levels

of anti-GII.4 IgG that also effectively blocked GII.4-1997 VLP binding to HBGAs to about the same degree as GII.4 pandemic-period sera blocked GII.4-1997. GII.4 pandemic sera did not, however, block HBGA binding of the GII.3-TV VLPs, suggesting that the sharp rise in the number of GII.4 outbreaks in 1996 could not prevent the rise in numbers of GII.3 outbreaks the following year. Because no cross-blockade of HBGA binding could be observed between GII.4-1997 and GII.3-TV with hyperimmune mouse antisera, these data suggest that patients infected with GII.3 NoV during the pandemic period were previously infected with a GII.4 NoV and that an immunological memory response was evoked for GII.4 following the GII.3 infection. Not only could this have helped in generating sufficient herd immunity to quell the pandemic, but it also suggests that GII.3 and GII.4 NoVs share some structural similarities.

Comparison of the structural models of the dimer of the GII.4 and GII.3 P domains demonstrated that the two P domains share significant identity, which could allow cross-reactivity of GII.4 antibodies with the GII.3 VLPs (Fig. 8a). However, the major difference between these two P domains is that the P2 subdomain of the GII.3 strain probably contains a large loop that extends away from the surface and away from the region that contains the reported receptor binding domain (RBD) in the GII.4 P domain (Fig. 8b). Further comparison of the structural models of the GII.3 and GII.4 P domains revealed nearly identical residues in interaction site 1 and similar residues in interaction site 2, two regions which have been shown to be important for interacting with the HBGAs to which the GII.4 virus binds (5). This observation suggests that a similar RBD exists on the GII.3 surface, suggesting that the GII.3 viruses may use the same receptor binding site as GII.4-1997 (Fig. 8b). However, in site 2 of GII.3, there is a deletion, exemplifying that although there are many similarities between

FIG. 6. Blockade of GII.4 or GII.3 VLP binding to HBGA by convalescent GII-unspecified outbreaks from the prepandemic period (A), non-GII.4 sera from the lull period (B), GII.3 sera from the pandemic period (C), and GII.4 sera from the pandemic period (D). The dashed line indicates the corresponding BT50 values on the x axis. Error bars indicate the standard errors.
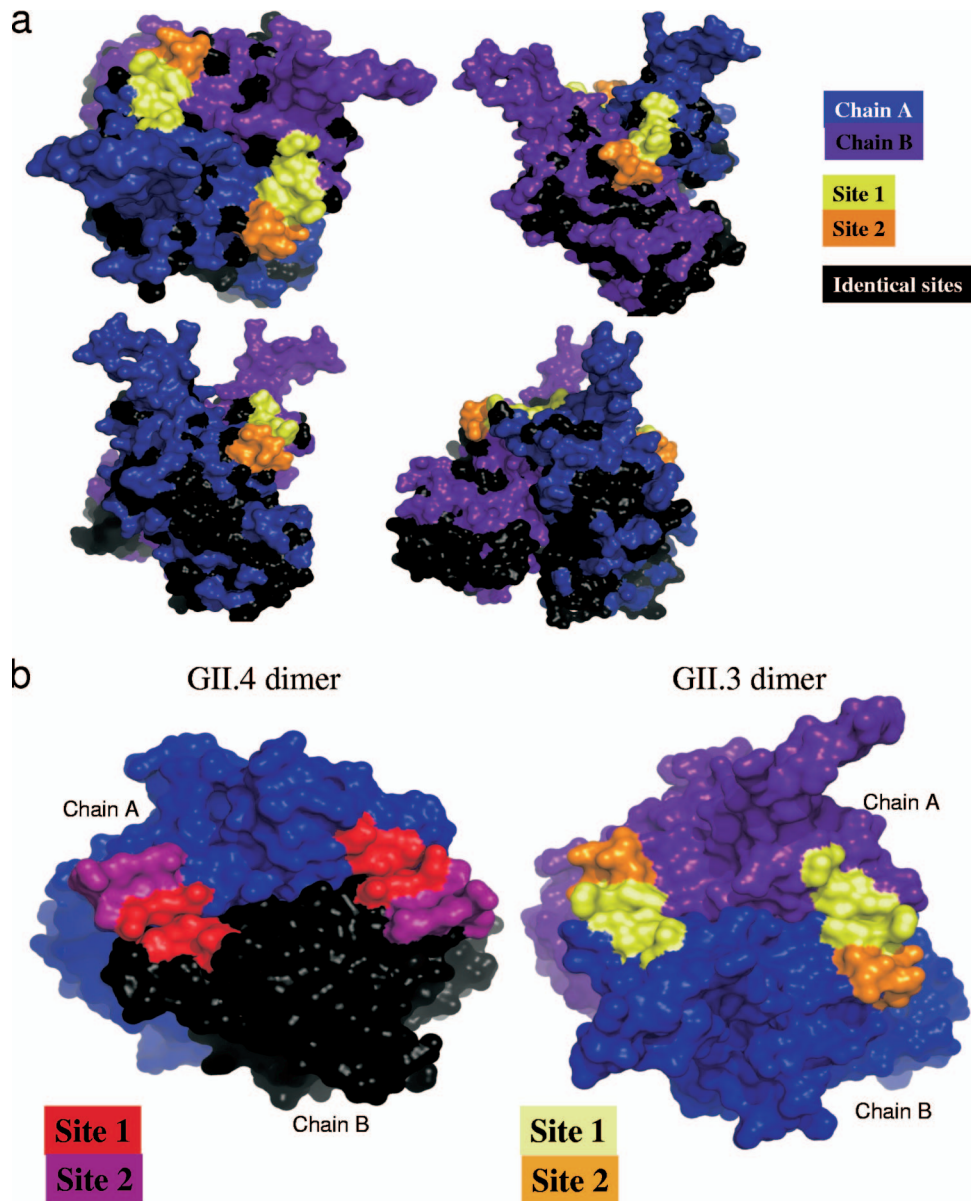
FIG. 8. Structural models of the GII.3 and GII.4 P-domain dimers. (a) Structural similarities between the GII.3 and GII.4 P-domain dimers suggest that similar structural features would appear on the capsid structure of each VLP or virus. The GII.3 P-domain dimer model is shown in various rotations, and sites that are identical between GII.3 and GII.4 P dimers are shown in black. Blue, chain A; purple, chain B; yellow, site 1 of the putative RBD; orange, site 2 of the putative RBD. (b) Direct comparison of the GII.4 P-domain dimer with the GII.3 P-domain dimer model indicates that a unique extended loop occurs on the GII.3 structure distal to the dimer interface, which would likely provide a unique target for antibody neutralization specific to the GII.3 virus/VLP.

GII.4-1997 and GII.3-TV, there are also several differences that could make them antigenically distinct.

We also describe GII.4-2006 HBGA binding and found it to be very similar to that of GII.4-1997 although appearing to be somewhat weaker in affinity. Previously, we demonstrated two contemporary GII.4 strains (GII.4-2004 and GII.4-2005) that did not bind to any of the HBGAs tested, but the reversion to HBGA binding further demonstrates the plasticity of the virus RBD. The sera from the 2006 GII.4 outbreak blocked HBGA binding for all GII.4 VLPs tested, suggesting a memory response to chronological GII.4 strains elicited by infection with an extant GII.4 strain. Alternatively, epitopes of chronological strains may be recycled in extant strains, allowing cross-reactivity. Mouse antisera raised against GII.4-2004 and GII.4-2005 strains block both GII.4-2002a and GII.4-1987 VLPs (30). In both scenarios, infection by the emergent strain will likely confer protection from strains of recent years and may provide some level of protection to strains of previous decades.

This study has many limitations because it was designed retrospectively, and the specimens used in our analysis were drawn from a subset of the population that may not be representative of the U.S. population. Because NoV outbreak sur-

veillance is passive, the samples received by the CDC may be biased toward large-scale outbreaks or outbreaks with more severe clinical symptoms such as those involving nursing home residents. Although serological responses to NoV infection by elderly patients were robust and did not appear to differ from those of younger persons, the sample size was not sufficient for statistical comparisons. Therefore, the large proportion of elderly patients sampled during the pandemic period may have biased our results. Also, by excluding sera from persons that did not serocovert to GII.4 NoV, we do introduce bias in our interpretation of the data. Figure 3 does not model population-level cross-reactive seroresponses to GII.4 NoV upon infection with GII NoV. However, by combining the data from Fig. 3 with the proportion of seroconvertants for each outbreak, together with surveillance data estimating the number of persons exposed to GII.4 NoV during the periods surrounding the pandemic period, we are better able to generalize data for the U.S. population. Additionally, we do not have exposure history information for the outbreak patients in our study, and thus, we do not know how much of the measured antibody reactivity is a reflection of a memory immune response from previous NoV infections. The seroconvertants included in this study had generally low levels of GII.4 NoV cross-reactive antibodies in their acute-phase sera, and none significantly blocked GII.4 VLPs when a subset was tested. This provides evidence for the apparent lack of a recent GII.4 NoV infection but does not exclude any possible contributions from a memory response.

In addition, only serum samples were available for our analyses, as no salivary or mucosal specimens were available, and fecal immunoglobulins are not sufficient for the detection limit of the surrogate neutralization assay (31). While an early salivary IgA response is correlated with protection from Norwalk virus but not Snow Mountain virus (28, 29), the contribution of serum IgG to the immune response against NoV infection remains unclear. However, at lower levels, mucosal antibodies are produced after NoV vaccination and natural infection. In addition, IgG in serum can transfer to the gut via transudation, and IgG from the gut and spleen is capable of blocking 100% of VLP binding to HBGA (31).

The application of the HBGA surrogate neutralization assay used in this study is comparable to the hemagglutinin inhibition (HI) assay for human influenza virus in that both measure blockade virus or VLP binding to a receptor. While an HI titer of 1:40 (40) is generally accepted as the titer needed for a 50% reduction in susceptibility to infection, all BT50 values exceeded 40 using the HBGA assay for NoV. Stephenson et al. (45) previously reported that while virus neutralization assays may be more sensitive and perhaps more strain specific than HI assays, no correlates of 50% protection have been established. The surrogate neutralization assay for human NoV may also overestimate true neutralization, as regions of the capsid distant from the receptor binding region may also be important for infection.

Currently, there is no vaccine or antiviral therapy for controlling NoVs. Efforts have been significantly hampered by the lack of an in vitro culture system or small-animal infectivity model. However, with our current knowledge of NoV evolution, such a vaccine will likely have to be updated every 1 to 2 years in a fashion similar to that of the influenza virus vaccine in order to protect against emergent strains (30).

This study has provided us with serological data to support long-term herd immunity against GII.4 NoVs. Using a surrogate neutralization assay, we were able to uncover minute antigenic differences between NoVs that cannot easily be distinguished by phylogenetic analysis or basic serological assays (ELISA). This study suggests a need for continued serological surveillance for human NoVs. Surrogate neutralization data along with outbreak sera can help us identify antigenically distinct, emerging GII.4 strains that may begin circulation toward the end of an epidemic, as neutralization assays help identify influenza virus vaccine candidates. Well-designed prospective studies including serological and HBGA blockade studies will become increasingly important as VLP vaccines for human NoVs become a reality.

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