Antibody Responses to Norovirus Genogroup GI.1 and GII.4 Proteases from two NoV strains (the GI.1 [NV] and GII.4 [Houston virus (HOV)]) were expressed, purified as previously reported (20) and used to measure antibody responses by ELISA. NV and HOV protease purity and integrity were confirmed by Coomassie staining and Western blot analysis. The concentrations of the expressed proteases were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). These proteases share 66% amino acid identity (see Fig. S1 in the supplemental material).

To measure the antiprotease IgG response, 96-well plates were coated overnight at 4°C with 200 ng of NV or HOV protease per well. All washing steps were performed in triplicate with 0.01 M phosphate-buffered saline (PBS) containing 0.05% Tween 20. After blocking the plate with 20% Blotto in 0.01 M PBS, serum samples (days 0, 7, 14, 28, and 180) were serially 2-fold diluted (1:50 to 1:3,200) in 0.01 M PBS, and 100 μl was added in duplicate to each plate, followed by 1 h of incubation at 37°C. Antiprotease antibodies were detected with an anti-human IgG-horseradish peroxidase-conjugated secondary antibody raised in goat (1:5,000; Sigma). The reaction was developed by the addition of 3,3′,5,5′-tetramethylbenzidine substrate (KPL) for 10 min at room temperature and then stopped by the addition of 1 M H₃PO₄. Reactions were read using a spectrophotometer at a wavelength of 450 nm. Serum from a rabbit immunized with NV protease served as a positive control, and a pool of NV-negative human sera was used as a negative control. The mean optical density (OD) value of the NV-negative human sera and uncoated wells plus 5 standard deviations was used as the cutoff value for each assay. Sera from study participants that did not have any detectable protease-specific antibodies at a 1:50 dilution were assigned a titer of 25.

In the experimental challenge study, 48 persons were enrolled, and 41 received different doses of the virus (0.48, 4.8, 48, and 4,800 RT-PCR units) while 7 received a placebo, as reported elsewhere (2, 11, 15). First, we determined the prevalence of antibodies against the proteases among all enrolled participants by testing serum samples collected at day 0 (prechallenge). Of the 48 study participants that did not have any detectable protease-specific antibodies at a 1:50 dilution were assigned a titer of 25.
jects, 8 (16.6%) had detectable prechallenge IgG against NV protease and 31 (64.6%) had IgG against HOV protease. The 8 volunteers with baseline antibodies against NV protease also had antibodies against HOV protease. The baseline geometric mean titers (GMT) were higher against HOV protease than against NV protease (HOV GMT, 66.7; 95% confidence interval [CI], 50.4 to 88.4; NV GMT, 32.9; 95% CI, 26.2 to 41.3; P = 0.0001, Wilcoxon signed-rank test) (Fig. 1A). To evaluate the specificity of the assay, we tested samples collected at day 0 and day 28 from persons who were challenged with the virus inoculum but did not become infected, as well as those who received placebo (Fig. 1B). No change in antibody titer was observed between the paired samples in either group of subjects. One subject who was not infected had the highest baseline titer (1,600).

After challenge, 19 of the 41 virus-challenged volunteers met the definition of infection (all shed virus, and all had a serore-

FIG 1 (A) Comparison of baseline serum antibody titers to NV and HOV protease prior to challenge among the 48 study participants (P = 0.0001, two-tailed Wilcoxon signed-rank test). Open circles indicate individuals who had 4-fold or greater increases in antibody titers to NV protease (n = 10) and to HOV protease (n = 8) following challenge with NV. (B) Comparison of NV-protease-specific antibody titers at day 0 and day 28 for 22 individuals who were challenged but not infected (filled circles) and 7 individuals who received a placebo (open circles). Study participants who had no detectable protease-specific antibody were given a titer of 25. Bars indicate the geometric mean titer for each group.
amount of virus shed in stool correlated with a change in antibody titer (data not shown).

At day 28 days postinfection, 8 of 19 infected study participants showed a ≥4-fold increase in HOV protease antibodies (Fig. 2). Among the 8 persons whose anti-HOV protease titers increased, 4 also had a ≥4-fold increase in NV protease antibodies. This result shows that cross-reactive immune responses to HOV protease occur following challenge with NV. Altogether, using responses to both NV and HOV proteases, a total of 14 (74%) infected individuals exhibited ≥4-fold increases in antibody titers. Using larger amounts of protease (50 to 400 ng) in the ELISA did not increase the sensitivity of detecting antibody responses to protease.

These results indicate that the protease expressed by NoVs during infection is immunogenic. However, not all infected individuals developed antibodies against NV protease after challenge. In contrast to the observed response to NV protease, all the infected volunteers had a ≥4-fold rise in NV-VP1-specific antibody 28 days postchallenge. The 10 individuals who exhibited a ≥4-fold increase in antibody titer to NV protease also had a significantly higher fold rise in IgG antibody titers against VP1 (day 0 versus day 28) than did the 9 persons who did not respond to NV protease: 284-versus 33-fold change, respectively (P = 0.008, Wilcoxon signed rank test). This result indicates that individuals who had a greater response to NV-VP1 also responded to the NV protease. Of the 8 individuals who showed a ≥4-fold increase in antibody titer to HOV protease, 6 also had a ≥4-fold increase in HOV-VP1 (data not shown). Of these, 4 individuals did not respond to NV protease. These results suggest that the cross-reactive response to a GII.4 viral protein after challenge with NV is most likely due to immunological memory acquired after previous exposures to GII.4 viruses, which are the most prevalent noroviruses. However, the history of previous exposures of the study participants to other noroviruses is unknown, and the underlying mechanism for the differences in the magnitude in seroresponses remains to be understood.

Previously, Matsui et al. identified that sera from 6 out of 7 convalescent individuals reacted to what is now known to be an overlapping region of the polyprotein that includes both p41 and p22 (14), providing evidence of the ability of nonstructural proteins to elicit an immune response. Future studies will evaluate whether detection of antibody responses to nonstructural proteins can be enhanced by testing larger polyprotein intermediates. Such studies require new methods to express and purify soluble native polyproteins.

Other studies have found that children and adults naturally infected with hepatitis A virus (HAV) make antibodies to the viral protease and these are detected at least up to 15 months after infection (10, 17). This has been useful in distinguishing antibody acquired in response to HAV infection and antibody induced by immunization with an inactivated vaccine. Persons infected with HIV also make antibodies against the viral protease in a fashion similar to that observed in the present study. Specifically, only a fraction (<65%) of HIV-infected adults make antibodies against HIV protease, decreasing by a third after 2 years (3).

In conclusion, our new ELISA detects antibody against two norovirus proteases, which appears to differentiate the immune response mounted against the vaccine from that in natural infection. The NV protease is immunogenic in humans, but the anti-protease response is less sensitive than the anticapsid response for detection of NV infection. However, there is apparent cross-reactivity in the response against the protease of GI and GII viruses. Future studies also need to evaluate whether the observed cross-reactivity to HOV protease is due to “original antigenic sin” after previous exposures to a GII.4 virus. Because the majority of infected volunteers (74%) showed an increase in antibodies to either homologous or heterologous protease after infection, detection of such antibodies may be suitable for differentiation between infections and immunization with a nonreplicating vaccine. Future studies in other populations, such as children, will be needed to
determine if a similar or higher rate of seroconversion is elicited following primary infection.

**ACKNOWLEDGMENTS**

This work was conducted with support from the National Institutes of Health (P01 AI 57788, N01 AI 25465, P30 DK56336, and M01 RR-000188), the Robert Welch Foundation (Q1279), and the John S. Dunn Research Foundation.

We thank Wendy Keitel for her critical review of the manuscript.

**REFERENCES**

1. **Atmar RL, et al.** 2011. Norovirus vaccine against experimental human Norwalk virus illness. N. Engl. J. Med. 365:2178–2187.
2. **Atmar RL, et al.** 2008. Norwalk virus shedding after experimental human infection. Emerg. Infect. Dis. 14:1553–1557.
3. **Boucher CA, et al.** 1989. Antibody response to human immunodeficiency virus type 1 protease according to risk group and disease stage. J. Clin. Microbiol. 27:1577–1581.
4. **Bull RA, Eden JS, Rawlinson WD, White PA.** 2010. Rapid evolution of pandemic noroviruses of the GII.4 lineage. PLoS Pathog. 6:e1000831.doi: 10.1371/journal.ppat.1000831.
5. **Chen TH, et al.** 2011. Differentiation of foot-and-mouth disease-infected pigs from vaccinated pigs using antibody-detecting sandwich ELISA. J. Vet. Med. Sci. 73:977–984.
6. **Glass RI, Parashar UD, Estes MK.** 2009. Norovirus gastroenteritis. N. Engl. J. Med. 361:1776–1785.
7. **Graham DY, et al.** 1994. Norwalk virus infection of volunteers: new insights based on improved assays. J. Infect. Dis. 170:34–43.
8. **Jia XY, Summers DF, Ehrenfeld E.** 1992. Host antibody response to viral structural and nonstructural proteins after hepatitis A virus infection. J. Infect. Dis. 165:273–280.
9. **Jiang X, Wang M, Wang K, Estes MK.** 1993. Sequence and genomic organization of Norwalk virus. Virology 195:51–61.
10. **Kabrane-Lazizi Y, Emerson SU, Herzog C, Purcell RH.** 2001. Detection of antibodies to HAV 3C protease in experimentally infected chimpanzees and in naturally infected children. Vaccine 19:2878–2883.
11. **Kavanagh O, et al.** 2011. Serological responses to experimental Norwalk virus infection measured using a quantitative duplex time-resolved fluorescence immunoassay. Clin. Vaccine Immunol. 18:1187–1190.
12. **Lindesmith L, et al.** 2005. Cellular and humoral immunity following Snow Mountain virus challenge. J. Virol. 79:2900–2909.
13. **Lindesmith LC, et al.** 2010. Heterotypic humoral and cellular immune responses following Norwalk virus infection. J. Virol. 84:1800–1815.
14. **Matsui SM, et al.** 1991. The isolation and characterization of a Norwalk virus-specific cDNA. J. Clin. Invest. 87:1456–1461.
15. **Reeck A, et al.** 2010. Serological correlate of protection against norovirus-induced gastroenteritis. J. Infect. Dis. 202:1212–1218.
16. **Silberstein E, Kaplan G, Taboga O, Duffy S, Palma E.** 1997. Foot-and-mouth disease virus-infected but not vaccinated cattle develop antibodies against recombinant 3AB1 nonstructural protein. Arch. Virol. 142:795–805.
17. **Stewart DR, Morris TS, Purcell RH, Emerson SU.** 1997. Detection of antibodies to the nonstructural 3C protease of hepatitis A virus. J. Infect. Dis. 176:593–601.
18. **Tacket CO, Sztein MB, Losonsky GA, Wasserman SS, Estes MK.** 2003. Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers. Clin. Immunol. 108:241–247.
19. **Wong SJ, et al.** 2003. Immunoassay targeting nonstructural protein 5 to differentiate West Nile virus infection from dengue and St. Louis encephalitis virus infections and from flavivirus vaccination. J. Clin. Microbiol. 41:4217–4223.
20. **Zeitler CE, Estes MK, Prasad BVV.** 2006. X-ray crystallographic structure of the Norwalk virus protease at 1.5-Å resolution. J. Virol. 80:5050–5058.
21. **Zheng DP, et al.** 2006. Norovirus classification and proposed strain nomenclature. Virology 346:312–323.