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Cellular and Humoral Immunity following Snow Mountain Virus Challenge

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Little is known about the immune response to noroviruses. To elucidate the immunobiology of norovirus infection in humans, 15 volunteers were challenged with Snow Mountain virus (SMV), a genogroup 2 norovirus. We assessed the cellular and humoral immune response and infection by analyzing stool, serum, saliva, and peripheral blood mononuclear cell (PBMC) responses pre- and postchallenge. In contrast to Norwalk virus (NV), SMV infection was not dependent upon blood group secretor status. Nine of 15 volunteers were infected and showed a ≥4-fold increase over the prechallenge anti-SMV serum immunoglobulin G (IgG) titer, mostly subclass IgG1. Although serum IgG elicited by SMV infection was cross-reactive with Hawaii virus (HV), another genogroup 2 norovirus, salivary IgA was less cross-reactive. Neither SMV-elicited serum IgG nor salivary IgA cross-reacted with NV, a genogroup 1 norovirus. Significant increases in serum gamma interferon (IFN-γ) and IL-2, but not IL-6 or IL-10, were noted on day 2 postchallenge. For the majority of volunteers, both infected and uninfected, PBMCs stimulated with norovirus virus-like particles secreted IFN-γ and other Th1 cytokines, suggesting previous norovirus exposure in most volunteers. Like the IgG antibodies, the SMV-activated T cells were cross-reactive with HV but not NV. IFN-γ production was dependent upon $CD4^+$ cells, consistent with a predominant, but not exclusive, Th1 response. To our knowledge, this is the first report characterizing T-cell and cytokine responses following live norovirus challenge.

Norovirus infection is common among individuals throughout the world, regardless of economic status, resulting in significant morbidity and loss of productivity. Recent reports of consecutive norovirus outbreaks on cruise ships, involving hundreds of passengers and crew, garnered substantial media attention and dramatically illustrate the potentially explosive community spread of these viruses (8). Although the severity of the disease is usually moderate and short term, an effective vaccine would be advantageous to specific segments of the population, such as food handlers, care providers, and military personnel. The major obstacles to norovirus vaccine development are the great degree of antigenic heterogeneity within the family, the possibility that immunity to noroviruses may be short-lived, and the lack of understanding of the correlates of protective immunity. An effective vaccine should also provide protective immunity against an ever-growing list of norovirus strains. Evidence from outbreak investigations and human challenge studies suggests that these viruses are highly infectious, yet the biology and immunology of norovirus infection remain poorly understood (32).

Noroviruses are members of the *Caliciviridae* family of single-stranded, positive-sense RNA viruses. The majority of the human noroviruses are classified into genogroup 1 (G1) and G2 (47) Each of these genogroups is further divided into genetic clusters. Norwalk virus (NV), Snow Mountain virus (SMV), and Hawaii virus (HV) are the prototype strains of genetic clusters G1.1, G2.2, and G2.1 and are the causative agents of an estimated 5, 8, and 7% of norovirus outbreaks, respectively (13). Strains within a genetic cluster typically show ≥80% amino acid identity in the major capsid protein sequence. Strains within the same genogroup show ≥60% identity, and strains in different genogroups show ≤50% identity. This high degree of genetic variability translates into a high degree of antigenic variability within the noroviruses.

Early human challenge studies demonstrated that some volunteers remained uninfected even when challenged with high doses of NV (30, 35), suggesting that some individuals may be genetically resistant to NV infection. Recently, we demonstrated that the *FUT2* gene that encodes an α1,2-fucosyltransferase responsible for the histo-blood group antigen (BGA) H type 1 is a susceptibility allele for NV infection (32). Volunteers who were homozygous for the inactive *FUT2* allele were resistant to infection at all dose levels studied. Other BGAs displayed weak modulating effects on NV interaction and pathogenesis. We and other groups have shown that NV virus-like particles (VLPs) bind to H type 1-related BGAs secreted in saliva (14, 20, 32) and membrane bound on mucosal cells (34) and also to synthetic H type 1-related BGA (20, 24). This H type 1-dependent in vitro binding pattern is so far restricted to NV, as other strains of noroviruses demonstrate different BGA binding affinities. Salivary binding assays have suggested that SMV VLP binding is associated with expression of the B BGA, while HV VLPs did not bind to any saliva sample (20). These data suggest that noroviruses use multiple methods for attachment to cells and hence for infection. It is unclear

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whether the *FUT2* allele determines susceptibility to all norovirus infections.

Short-term immunity to NV has been observed in previous human challenge studies (48). However, this immunity did not necessarily extend to heterologous virus challenge, as volunteers who became ill upon challenge with NV also became ill upon subsequent challenge with HV at the same rate as volunteers not previously infected with NV (48). Further rechallenge studies have shown that long-term immunity is not conferred by a single NV challenge, as some volunteers who were susceptible to an initial NV infection were susceptible to infection in subsequent challenges 27 to 42 months later (41).

Previously, in both volunteer studies and outbreak investigations, immunity to noroviruses has almost exclusively been studied by monitoring serum immunoglobulin M (IgM) and IgG responses to virus (35). IgM was shown to be a marker of recent NV infection (5). In many studies, a fourfold increase in anti-norovirus serum IgG titer has been considered indicative of norovirus infection (17). Attempts to study fecal IgA in NV infection have been unsuccessful in associating secretory IgA (sIgA) with infection outcome (40). However, our recent study utilizing saliva samples as a source of sIgA showed a correlation between protection from NV infection and the presence of a memory sIgA response in uninfected susceptible volunteers (32). Importantly, some susceptible volunteers who were not infected did not have strong salivary IgA responses, indicating that non-sIgA mechanisms are also involved in immunity to noroviruses.

In addition to antibody-producing B cells, T cells play an important role in the control of viral pathogens. The cytokines secreted by antigen-specific $CD4^+$ helper T cells guide the adaptive immune response. T helper 1 (Th1) and Th2 responses can be activated by both the same and different epitopes within the same pathogen, but one response type will often dominate over the other. A Th2-favored immune response is primarily characterized by interleukin 4 (IL-4) and IL-10 secretion by $CD4^+$ cells, resulting in production of IgG2 and other anti-inflammatory factors. A Th1-favored response is primarily characterized by secretion of gamma interferon (IFN-γ) by $CD4^+$ cells, resulting in macrophage activation, B-cell differentiation to IgG1 synthesis, and support for cytotoxic T lymphocytes (CTLs). CTLs are important in the control of human immunodeficiency virus (HIV) (4), Epstein-Barr virus (42), influenza virus (26), lymphocytic choriomeningitis virus (52), and numerous other viruses. To date, cellular immunity has not been studied following norovirus infection, but we expect effector T cells to play a fundamental role in viral clearance and immunity.

In this study, we demonstrated that SMV infection was independent of secretor status and BGA expression and that the serum IgG, salivary IgA, and effector T cells elicited following infection were cross-reactive between strains within a genogroup but not between strains in different genogroups. This is the first study to examine the association between mucosal expression of BGAs and infection with a G2 norovirus in humans and to study T-cell activation after challenge with any live norovirus.

MATERIALS AND METHODS

Study design and subjects. A human challenge study was conducted to examine the infectivity of SMV. Fifteen healthy adult volunteers were admitted to the University of North Carolina General Clinical Research Center (GCRC) for collection of prechallenge samples and dosing. SMV inoculum was prepared from stool filtrates and safety tested as reported for Norwalk virus (10), with additional testing for other noroviruses, culturable human enteric viruses, hepatitis A virus, HIV, coronavirus, astrovirus, adenovirus, rotavirus, cytopathic viruses, endotoxins, common enteric pathogens (bacteria and protozoa), mycobacteria, and *Clostridium difficile* toxin. Each volunteer received one dose of SMV (11) ranging from 10 to 10^5 PCR detectable units, as defined by endpoint titration reverse transcription (RT)-PCR (37). Dose levels were randomized, and no single dose level was administered in sufficient numbers to validate statistical analysis of clinical outcomes by dose. The study population was 47% male, 73% white, and 27% other races, with an average age of 31 years (range 21 to 54). Volunteers remained in the GCRC for the first 5 days postchallenge and returned for follow-up visits on days 8, 14, and 21 postchallenge. During the first 7 days, all volunteer stools and vomit were collected and stored at 4 or −20°C. Sera and saliva were collected daily during the first 5 days postchallenge and at each follow-up visit. Samples were stored at −20°C. PBMCs were harvested from whole blood collected prechallenge and on days 8 and 21 postchallenge using a Ficoll-Pacque (Pharmacia-Amersham) gradient. The cells were cryopreserved in 90% fetal calf serum–10% dimethyl sulfoxide until they were used. All study protocols and methods were approved by the University of North Carolina School of Medicine Committee for Protection of Human Subjects. All volunteers participated after providing informed consent.

Diagnosis of SMV infection. Volunteers were classified as infected if SMV RNA was detected in any postchallenge stool sample by RT-PCR. All fecal specimens were examined by RT-PCR using G2-specific primers located in the RNA-dependent RNA polymerase gene (SR33/SR48) (1).

Measurement of anti-norovirus serum IgG. All sera were tested for anti-norovirus IgG by ELISA using recombinant SMV, HV, or NV VLPs as the antigen (20) and anti-human IgG (whole molecule)-alkaline phosphatase (Sigma Chemicals) as the detector antibody. The VLPs used in this study were composed of the open reading frame 2 (ORF2) major capsid protein from each strain expressed in the VEE vector system and purified as reported previously (3, 20). Seroconversion was defined as a ≥4-fold increase over the prechallenge titer in postchallenge samples collected on either day 14 or 21, depending on patient sample availability. By day 14 postchallenge, all nine of the infected volunteers had a ≥4-fold increase over the prechallenge (day zero) anti-SMV serum IgG titer. Titers remained at ≥4-fold above day zero levels for the duration of the study. For IgG subclass determination, anti-IgG1-biotin or anti-IgG2-biotin antibodies, followed by avidin-alkaline phosphatase (all from Sigma Chemicals), were used as detector antibodies.

Histo-blood group antigen expression. Blood type was inferred from red blood cell (RBC) (Ortho Clinical Diagnostics) hemagglutination by serum samples. Secretor and Lewis phenotypes were determined from saliva by ELISA using anti-Lewis^a, anti-Lewis^b, anti-A, and anti-B antibodies (Ortho Clinical Diagnostics) (20). The secretor phenotype was confirmed by the *FUT2* genotype (32).

Measurement of anti-norovirus and total salivary IgA. Saliva samples were collected, processed, and assayed for anti-norovirus and total IgA by ELISA, as described previously (32). Of the 15 sample sets collected, 12 were included in the analysis. Three sample sets were eliminated from analysis because of poor sample quality (total IgA < 50 μg/ml) (32). Conversion was defined as a ≥4-fold increase over the prechallenge titer in postchallenge samples collected on either day 14 or 21, depending on patient sample availability.

VLP stimulation of PBMCs. Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed, washed with medium (RPMI 1640, 10% fetal calf serum, 1% L-glutamine, 100 U of penicillin-streptomycin/ml), and dispensed into 96-well plates at 5×10^5 cells/well with either 10 ng of phorbol myristate acetate/ml–0.5 μg of ionomycin/ml, medium only, or 1 μg of SMV, HV, or NV VLPs/ml. The optimum cell number, stimulation time, and VLP concentration were determined empirically. The cells were stimulated for 48 h at 37°C in a 5% CO_2 incubator in duplicate or triplicate wells, depending on the number of available cells. Cell culture supernatants were collected and stored at −20°C until they were used.

Measurement of cytokine secretion. Culture supernatants were simultaneously analyzed for secreted Th1 cytokines (IFN-γ, tumor necrosis factor alpha [TNF-α], and IL-2) and Th2 cytokines (IL-4, IL-5, and IL-10) using the Cytometric Bead Array (CBA) assay (BD Biosciences) as recommended by the manufacturer, with the exception that reagents were used at half the recommended volumes. This change did not affect performance ($P = 0.92$; Mann-Whitney two-tail test). Inclusion of duplicate assay tubes did not significantly affect the results ($P = 0.4065$; Mann-Whitney two-tail test). Fluorescent beads were counted on a BD Biosciences FacsCalibur flow cytometer, and data were analyzed using Cell Quest Software (BD Biosciences). In additional samples, IFN-γ secretion was measured in duplicate by ELISA (BD Biosciences). The lower limit of detection was 1 pg/ml for the CBA assay and 4 pg/ml for the IFN-γ ELISA. Samples below these limits were assigned a value equal to half the lower limit of detection for analysis purposes (43). For all samples, the medium-stimulated background was subtracted.

TABLE 1. SMV infection is not associated with blood type, secretor phenotype, or Lewis phenotype

Parameter	No. infected[a]	No. uninfected	% Infected
Blood type			
O	4	4	50
A	2	2	50
B	1	0	100
AB	2	0	100
Total	9	6	60
Secretor type			
Positive	8	4	67
Negative	1	2	33
Lewis type			
Positive	7	6	64
Negative	1	0	100

[a] Viral RNA detected in stool.

Depletion of CD4$^+$ or CD8$^+$ cells. PBMC samples were depleted of CD4$^+$ or CD8$^+$ cells using antibody-coupled magnetic beads (Dynabeads; Dynal Biotech) according to the manufacturer's instructions. Depletion (≥90%) was monitored by fluorescence-activated cell sorter analysis of the samples using anti-CD4–fluorescein isothiocyanate and anti-CD8–phycoerythrin (Sigma Chemicals).

Statistical analysis. The Mann-Whitney two-tail test was used to compare the median responses between groups. The Fisher exact test (Epi Info 2000; Centers for Disease Control and Prevention) was used to test the association among prechallenge IgG, blood type, sIgA, secretor and Lewis phenotypes, and risk of infection.

RESULTS

Infectivity of SMV. Fifteen volunteers were dosed with SMV and were classified as infected if viral RNA was detected in any postchallenge stool sample by RT-PCR (10). Nine (60%) of the volunteers became infected (Table 1). Of these, 7 (78%) experienced symptoms of illness. Since NV infection has been shown to be dependent upon expression of the BGA H type 1 (32), we examined the effect of BGA expression on SMV infection. Expression of BGA on RBCs (blood type) was determined by RBC hemagglutination. Mucosal-cell expression of BGA (secreted BGA) was determined by ELISA of saliva samples, as reported previously (32). In contrast to NV infection, volunteers from every blood type, secretor phenotype, and Lewis type became infected. SMV infection was not associated with blood type ($P = 0.608$), secretor phenotype ($P = 0.525$), or Lewis phenotype ($P = 0.505$) (Table 1). These data likely indicate that NV and SMV strains use different mechanisms for infection, independent of the secretor phenotype.

Serum IgG response to SMV challenge. Seroconversion, a ≥4-fold increase over the prechallenge serum IgG titer, has been used to define norovirus infection in challenge studies and outbreak investigations (18, 11). By day 14 postchallenge, all nine of the infected volunteers had a ≥4-fold increase over the prechallenge (day zero) anti-SMV serum IgG titer, verifying the association between virus excretion and seroconversion. There was no significant difference between the titers of prechallenge SMV-reactive IgG in infected and uninfected volunteers ($P = 0.215$) (Fig. 1), suggesting that the prechallenge anti-SMV IgG titer is not predictive of the risk of SMV infection. The infected volunteers had postchallenge (day 14 or 21) anti-SMV IgG titers that were significantly higher than prechallenge titers ($P = 0.0004$), with a median 23.8-fold increase (range, 4.9- to 91.8-fold). The uninfected volunteers did not have a significant rise from the prechallenge titer, with a median change in the anti-SMV IgG titer of 1.5-fold (range,

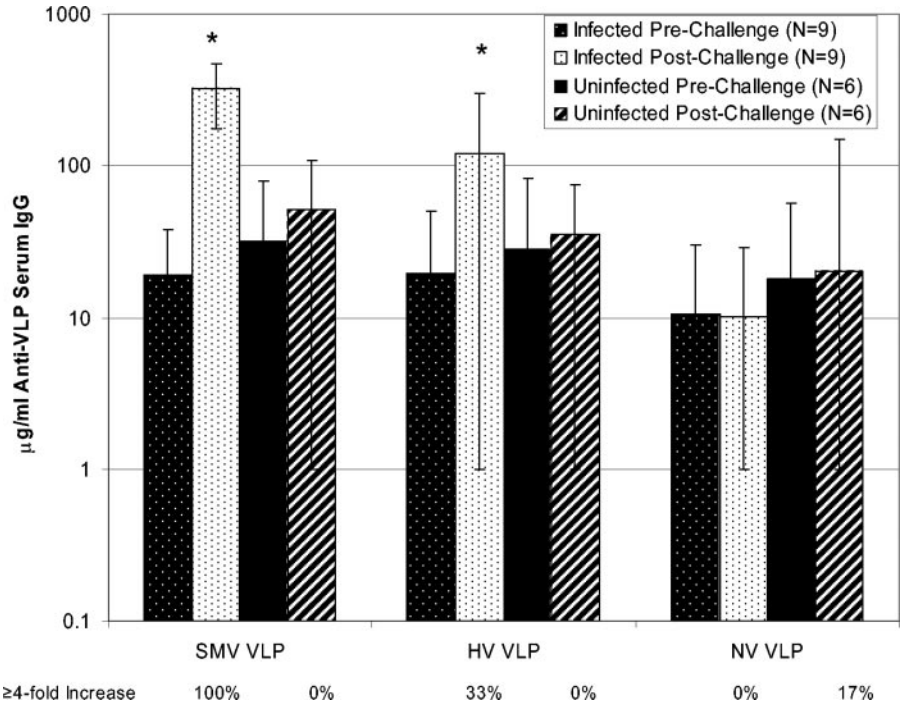

FIG. 1. SMV elicits a cross-reactive serum IgG response in challenged volunteers. Pre- and postchallenge sera from each volunteer were tested for anti-SMV, anti-HV, and anti-NV IgG by ELISA. *, $P < 0.05$. Also shown are the percentages of volunteers with a ≥4-fold increase over the day zero titer. The error bars indicate standard deviations.

0.8- to 2.0-fold) (Fig. 1 and data not shown). Seroconversion was highly predictive of infection ($P = 0.0002$).

Cross-reactivity of serum IgG after SMV challenge. Serum samples collected during outbreak investigations have shown that some norovirus strains share cross-reactive epitopes (18, 19). This observation may in part explain our results indicating that the prechallenge anti-SMV IgG titer was not predictive of SMV infection. To investigate the cross-reactivity of serum IgG generated after SMV challenge, prechallenge and postchallenge (day 14 or 21) serum samples from each volunteer were also tested for anti-HV and anti-NV IgG by ELISA (Fig. 1). As described above for anti-SMV IgG, there were no significant differences between prechallenge titers of anti-HV IgG ($P = 0.8572$) or anti-NV IgG ($P = 0.8572$) in infected and uninfected volunteers. After challenge, infected volunteers had postchallenge titers of anti-HV IgG ($P = 0.0173$) that were significantly higher than prechallenge titers (Fig. 1). Three (33%) of the SMV-infected volunteers had ≥4-fold increases, and an additional two volunteers had a ≥3-fold increase in anti-HV IgG. One volunteer produced a response with greater reactivity to HV than SMV. In contrast to the response to HV antigen, SMV infection did not result in a significant increase in anti-NV IgG ($P = 0.90$), suggesting that cross-reactive IgG responses are genogroup specific. Among the uninfected volunteers, significant increases over the prechallenge titers were not detected in anti-HV IgG ($P = 0.5755$) or anti-NV IgG ($P = 0.69$) titers. None (zero of six) of the uninfected volunteers seroconverted to HV. One (one of six) uninfected volunteer did produce antibody to NV (Fig. 1). The group difference between pre- and postchallenge NV IgG titers was not significant. These data suggest four important points: (i) SMV infection, not just exposure, is essential for activation of an anti-SMV serum IgG response, even in volunteers with prechallenge anti-SMV-reactive IgG; (ii) IgG elicited during an SMV infection is cross-reactive with VLPs from strains within the genogroup but not across genogroups; (iii) SMV and HV share some cross-reactive epitopes, and prechallenge IgG measurements reflect the sum of cross-reactive epitopes among the noroviruses each volunteer has previously encountered and should not be considered strain-specific IgG; and (iv) occasionally, a heterotypic IgG response may dominate following norovirus challenge, reminiscent of a response influenced by exposure history.

Salivary IgA response to SMV challenge. Norovirus infection is likely limited to the gut; thus, mucosal antibodies may be important in protection from infection (32). To measure the mucosal immune response to SMV challenge, salivary samples from 12 volunteers were assayed for anti-SMV and total IgA by ELISA, and the ratio of the two measurements was used for comparisons. As with serum IgG, there was no significant difference between the prechallenge salivary mucosal IgA ratios in infected and uninfected volunteers ($P = 0.4237$). However, infected volunteers had anti-SMV sIgA titers on day 14 or 21 postchallenge ($P = 0.0308$) that were significantly higher than prechallenge titers and had a median 6-fold increase (range, 1.5- to 36.8-fold) (Table 2). Four out of six (67%) infected volunteers had a ≥4-fold increase in anti-SMV sIgA titer. Uninfected volunteers did not have a significant rise in anti-SMV sIgA titer (median, 1.4-fold increase; range, 0.64- to 2.0-fold) (Table 2). As with serum IgG, a ≥4-fold increase in anti-SMV sIgA by day 14 or 21 postchallenge was associated with infection ($P = 0.0278$).

TABLE 2. Anti-norovirus salivary IgA response elicited by SMV challenge

Parameter	Value [median (range)]	
	Infected[a]	Uninfected
Anti-SHV IgA[b]		
Prechallenge[c]	0.12 (0.04–1.21)	0.24 (0.07–0.73)
Postchallenge[d]	0.85 (0.33–1.84)	0.22 (0.16–1.2)
Increase[e]	5.6[g] (1.5–36.8)	1.4 (0.64–2.0)
Volunteers converted (%)[f]	67	0
Anti-HV IgA[h]		
Prechallenge	0.06 (0.03–0.32)	0.15
Postchallenge	0.19 (0.13–0.28)	0.15
Increase	2.2 (0.8–9.3)	1.0
Volunteers converted (%)	20	0
Anti-NV IgA[h]		
Prechallenge	0.03 (0.008–0.06)	0.06
Postchallenge	0.07 (0.005–0.12)	0.07
Increase	2.3 (0.63–3.0)	1.2
Volunteers converted (%)	0	0

[a] Viral RNA detected in stool.
[b] (Microgram of VLP-reactive salivary IgA per milliliter/microgram of total salivary IgA per milliliter) × 100. $n = 6$.
[c] Day zero.
[d] Day 14 or 21.
[e] Median of individual increases (n-fold) above prechallenge titer.
[f] Percentage of volunteers with ≥4-fold increase over the prechallenge titer.
[g] $P \leq 0.05$.
[h] $n = 5$ (infected); $n = 1$ (uninfected).

Cross-reactivity of salivary IgA after SMV challenge. To investigate the cross-reactivity of salivary IgA generated after SMV challenge, prechallenge and convalescent-phase saliva samples from five infected volunteers and the one uninfected volunteer who seroconverted to NV were measured for anti-HV and anti-NV sIgA, and the ratio of anti-norovirus sIgA to total sIgA was determined (Table 2). One infected volunteer had a ≥4-fold increase in salivary IgA reactive to HV. None of the infected volunteers had a ≥4-fold increase in salivary IgA reactive to NV. A ≥3-fold increase in anti-HV and anti-NV sIgA was observed in another infected volunteer. Fewer volunteers had a ≥4-fold increase in HV-reactive sIgA compared to IgG. This result may reflect the higher titer of antibody found in serum versus saliva, as opposed to suggesting that sIgA is more strain specific than IgG.

SMV challenge elicits a Th1 immune response. The cellular immune response to norovirus challenge has not been studied previously. However, the role of T cells in some human viral infections has been described (27). To begin to characterize the cellular immune response elicited by SMV challenge, PBMCs were collected from whole blood prechallenge and on days 8 and 21 postchallenge and cryopreserved for in vitro VLP stimulation followed by analysis of secreted cytokines. Eleven volunteers had at least partial PBMC sample sets. Three infected and two uninfected volunteers had complete sample sets. PBMCs from these five volunteers were stimulated with 1 μg of SMV VLPs/ml for 48 h, and the clarified tissue culture supernatants were assayed for Th1 cytokines (IFN-γ, TNF-α, and IL-2) and Th2 cytokines (IL-10, IL-5, and IL-4) using the flow cytometry-based CBA assay (Fig. 2). Cytokine responses

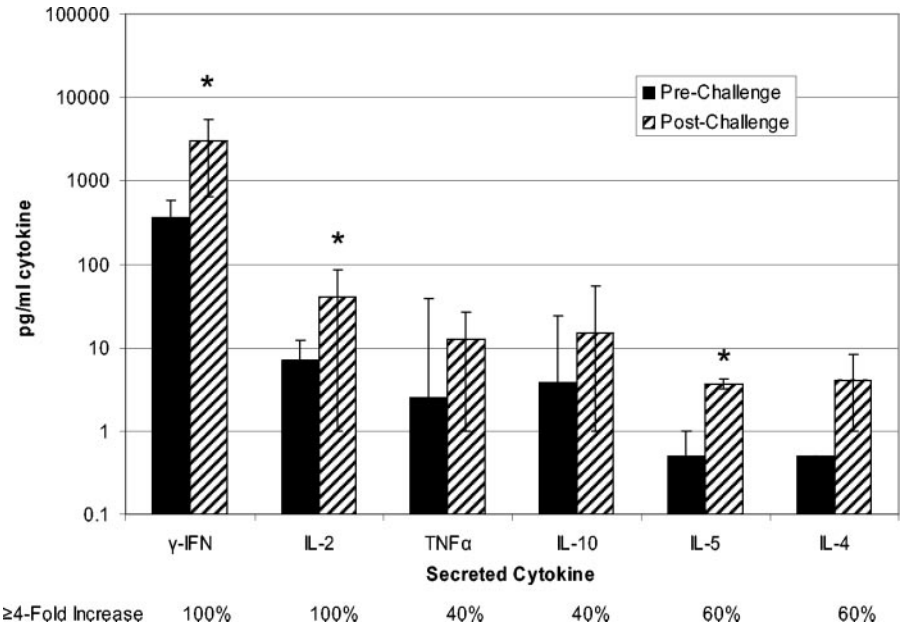

FIG. 2. SMV challenge elicits a predominately Th1 cytokine response. PBMCs from two uninfected and three infected SMV-challenged volunteers were collected prechallenge and on days 8 and 21 and stimulated with SMV VLPs, and the culture supernatants were analyzed for secreted Th1 cytokines (IFN-γ, TNF-α, and IL-2) and Th2 cytokines (IL-4, IL-5, and IL-10) using the CBA assay. The data shown represent day zero compared to the peak day of cytokine secretion for each volunteer (day 8 or 21). *, $P < 0.05$. Also shown are the percentages of volunteers with a ≥4-fold increase over the day zero titer. The error bars indicate standard deviations.

were compared by group using the Mann-Whitney two-tail test. In both the infected and uninfected volunteers tested, the greatest cytokine response to SMV VLP stimulation was increased IFN-γ secretion ($P = 0.0121$, comparing median concentrations of secreted IFN-γ from prechallenge PBMCs to those from peak [day 8 or 21] postchallenge PBMCs). Each volunteer demonstrated a rise in IFN-γ secretion (median increase, 20.0-fold [range, 6.1- to 6,003-fold]). Smaller, but significant, rises above the prechallenge titer were also detected for IL-2 ($P = 0.0121$), with a median increase of 5.5-fold (range, 4.2- to 276.8-fold), and IL-5 ($P = 0.0121$), with a median increase of 4.2-fold (range, 4.1- to 16-fold). The timing (day 8 or 21) of peak cytokine up-regulation varied between different volunteers regardless of their infection status. Significant changes were not detected between prechallenge and postchallenge PBMC secretion of TNF-α, IL-10, or IL-4, although some individual responses were elevated (Fig. 2). The lack of a TNF-α response was somewhat surprising, since TNF-α and IFN-γ are often correlated (44), and it may reflect the small sample size of our population. Increased IL-5 is a marker for a Th2 response. The response shown here is statistically significant, even though the absolute concentration of IL-5 detected was low (median, 3.7 pg/ml), especially in comparison to IFN-γ secretion (median, 3,002 pg/ml), as described for rhinovirus-stimulated PBMCs (6). These data indicate a predominant, but not exclusive, Th1 cellular immune response to SMV challenge.

The enhanced secretion of IFN-γ in SMV-stimulated PBMCs from both infected and uninfected volunteers suggested that measurement of IFN-γ levels could provide a quantitative indication of cellular immune response in all SMV-challenged volunteers. Thus, secreted IFN-γ was measured by ELISA for the 11 volunteers with at least two PBMC collections available for comparison (Fig. 3 and data not shown). Four out of six uninfected and all seven infected volunteers had PBMCs collected prechallenge. All four (100%) of the uninfected and six of the seven (86%) infected volunteers had prechallenge SMV-reactive cells that secreted IFN-γ in response to in vitro SMV stimulation, suggesting previous norovirus exposure and immunological memory in 91% of the challenged volunteers. There was no significant difference between prechallenge IFN-γ secretion levels in the infected (median, 73 pg/ml) and uninfected (median, 264 pg/ml) volunteers ($P = 0.1738$). Among the seven infected volunteers, a significant change from prechallenge IFN-γ secretion was detected in postchallenge PBMC samples ($P = 0.0105$), with a median increase of 33.2-fold (range, 0.61- to 3,025-fold) (Fig. 3). Five of the seven infected volunteers (71%) had a ≥4-fold increase over prechallenge IFN-γ secretion in postchallenge PBMC samples. Among the four uninfected volunteers, two (50%) had a ≥4-fold increase over prechallenge IFN-γ secretion, with a median increase of 3.0-fold (range, 0.02- to 14-fold) (Fig. 3). As noted for the five volunteer samples analyzed by CBA, peak up-regulation of cytokine secretion (day 8 or 21) between different volunteers varied regardless of their infection status.

To further address the nature of the SMV-induced immune response, prechallenge and postchallenge serum samples were tested for IFN-γ, IL-2, IL-6, and IL-10 by ELISA. Significant increases in both IFN-γ (median increase, 10.9-fold; range, 1.0- to 966-fold [$P = 0.0117$]) and IL-2 (median increase, 4.8-fold; range, 1.0- to 141.6-fold [$P = 0.0093$]) concentrations in serum were detected in acute-phase (day-2) serum samples collected from infected volunteers. Significant changes in cytokines were not detected in IL-6 or IL-10 in sera from infected volunteers or in any cytokine in serum samples from uninfected volunteers. Furthermore, prechallenge and postchallenge serum sam-

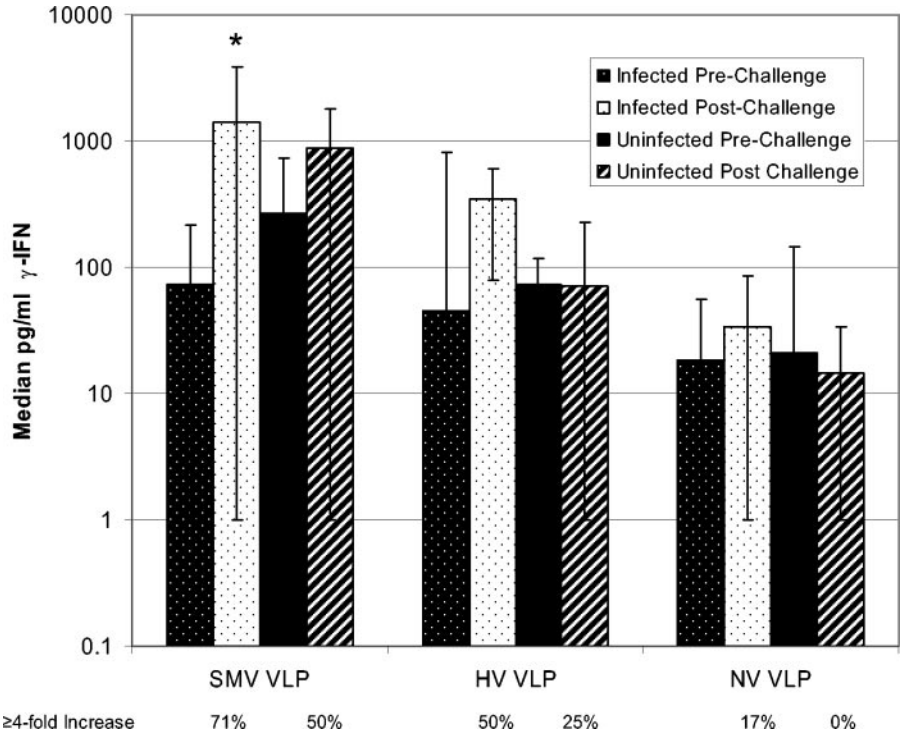

FIG. 3. PBMCs from both infected and uninfected volunteers secrete IFN-γ after VLP stimulation. PBMCs collected pre- and postchallenge from SMV-challenged volunteers were stimulated separately in vitro with SMV, HV, or NV VLPs, and the culture supernatants were analyzed for secreted IFN-γ by ELISA. The data shown represent day zero compared to the peak day of cytokine secretion for each volunteer (day 8 or 21). *, $P \leq 0.05$. Also shown are the percentages of volunteers with a ≥4-fold increase over the day zero titer. The error bars indicate standard deviations.

ples from infected volunteers were tested for anti-SMV IgG1 and IgG2. In agreement with previous studies of anti-norovirus IgG subclass distribution (2, 21), most of the detectable response was an increase in IgG1 (median increase, 3.99-fold; range, 0.16- to 19.5-fold). Little change in anti-SMV IgG2 titer was detected (median increase, 1.03-fold; range, 0.98- to 6.4-fold). These acute-phase serum cytokine and IgG subclass data support the PBMC-secreted cytokine data, indicating that the SMV-induced immune response is weighted toward a Th1 response.

Five of the seven infected volunteers (71%) had a ≥4-fold increase over prechallenge IFN-γ secretion in postchallenge PBMC samples. Suppression of IFN-γ responses by other cytokines has been reported in some HIV-infected individuals (16). To determine if additional cytokines could explain the lack of response in the two infected IFN-γ nonresponders (data not shown), culture supernatants from the infected nonresponders were assayed for additional cytokines using the CBA assay (data not shown). One volunteer was found to be producing low levels of the Th1 cytokines IFN-γ (4.9-fold increase over the prechallenge titer), TNF-α (4.2-fold increase), and IL-2 (2.2-fold increase). No changes in Th2 cytokines were detected, indicating that infection in this volunteer did elicit a weak Th1-type immune response. In the other infected nonresponder, IL-10 was the only cytokine response detected (4.9-fold increase), identifying this volunteer as the only infected IFN-γ nonresponder. Among the three uninfected nonresponders, no additional cytokines were detected by CBA analysis (data not shown), supporting previous results suggesting multiple mechanisms for resistance to norovirus infection (32).

SMV challenge elicits a cross-reactive T-cell response. Analogous to our studies of antibody epitopes cross-reactive between norovirus strains, the cross-reactivity of the Th1 cellular response elicited by SMV challenge was investigated. PBMCs from the two uninfected and three infected volunteers (as seen in Fig. 2) who responded to SMV challenge with increased IFN-γ secretion were stimulated separately with HV and NV VLPs, and the culture supernatants were assayed for IFN-γ, TNF-α, IL-2, IL-10, IL-5, and IL-4 using the CBA assay (Fig. 4). After stimulation with HV, postchallenge PBMCs from the infected and uninfected volunteers secreted significantly more IFN-γ than prechallenge PBMCs ($P = 0.0214$), with a median increase of 10.7-fold (range, 3.2- to 2,086-fold), and IL-2 ($P = 0.0121$), with a median 3.8-fold increase (range, 2.2- to 180.6-fold) (Fig. 4A). Significant differences were not detected in the other cytokines, indicating that the Th1 response mounted to SMV was cross-reactive with HV (Fig. 4A). In contrast, after NV VLP stimulation, no significant changes were detected between prechallenge and postchallenge PBMCs in the ability to secrete IFN-γ, IL-2, TNF-α, IL-10, or IL-4. There was significantly increased secretion of IL-5 in response to NV VLP stimulation ($P = 0.0477$), but the concentrations detected were low, as noted for SMV stimulation (Fig. 4B).

To further study the cross-reactivity of the Th1 response generated after SMV challenge, culture supernatants from additional PBMCs stimulated with HV or NV VLPs were assayed for secreted IFN-γ by ELISA (Fig. 3). Ten volunteers had PBMCs available for the cross-reactivity studies. Those volunteers who did not respond to SMV did not respond to HV or

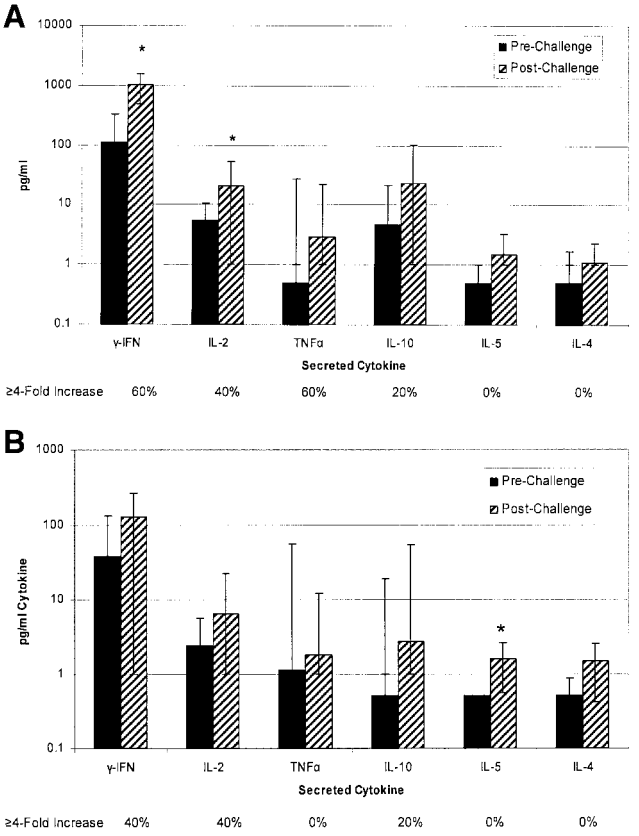

FIG. 4. SMV challenge elicits a cross-reactive, predominately Th1 cytokine response. PBMCs from two uninfected and three infected SMV-challenged volunteers were collected prechallenge and on days 8 and 21 and stimulated separately in vitro with HV VLP (A) or NV VLP (B), and the culture supernatants were analyzed for secreted Th1 cytokines (IFN-γ, TNF-α, and IL-2) and Th2 cytokines (IL-4, IL-5, and IL-10) using the CBA assay. The data shown represent day zero compared to the peak day of cytokine secretion for each volunteer (day 8 or 21). *, $P < 0.05$. Also shown are the percentages of volunteers with a ≥4-fold increase over the day zero titer. The error bars indicate standard deviations.

NV after SMV challenge (data not shown). Volunteers who had a significant increase in SMV-induced IFN-γ secretion post-SMV challenge also had a significant increase in response to HV stimulation ($P = 0.0131$), but not to NV stimulation ($P = 0.631$). Among the six infected volunteers, three (50%) had a ≥4-fold increase in IFN-γ secretion after HV stimulation, with a median increase of 61-fold (range, 0.17- to 183-fold) (Fig. 3). There was a median increase of 1.6-fold (range, 0.06- to 5.37-fold) among the four uninfected volunteers, and one volunteer had a ≥4-fold increase in IFN-γ secretion after HV stimulation (Fig. 3). None of the uninfected volunteers had a ≥4-fold increase in IFN-γ secretion after NV VLP stimulation. The median change was 0.58-fold (range, 0.11- to 1.3-fold) (Fig. 3). As identified with antibody responses, some SMV-induced Th1 cells cross-react with HV but not NV, indicating that strains of the same genogroup share common epitopes that may elicit cross-protection.

IFN-γ secretion is dependent upon CD4$^+$ cells. Both CD4$^+$ and CD8$^+$ T cells secrete IFN-γ (44). Since our method of antigen presentation relies on VLP uptake, processing, and presentation by antigen-presenting cells within the PBMCs, a bias toward detection of CD4$^+$ cells may be expected, although other means of presentation could also occur (44). To identify the cellular source of the Th1 cytokines produced in response to SMV challenge, PBMCs collected on day 8 or 21 postchallenge from five volunteers (three infected and two uninfected) were depleted of either CD4$^+$ or CD8$^+$ cells via antibody-coupled magnetic beads (15) and stimulated with SMV, and the culture supernatant was assayed for secreted IFN-γ (Fig. 5). Cell depletion (≥90%) was monitored by fluorescence-activated cell sorter analysis of depleted and nondepleted cultures (data not shown). Depletion of CD8$^+$ cells did not have a significant effect on IFN-γ secretion ($P = 0.6745$). However, it is noteworthy that in the two uninfected volunteers (SM07 and SM08 in Fig. 5), depletion of CD8$^+$ cells led to increased IFN-γ secretion after stimulation, suggesting that in these two uninfected volunteers, CD8$^+$ cells may be down-regulating the IFN-γ response in vitro (16), possibly providing a balanced immune response to a neutralized virus. In contrast, depletion of CD4$^+$ cells before SMV stimulation resulted in a significant decrease in IFN-γ secretion after stimulation in all of the volunteers tested ($P = 0.0214$). IFN-γ secretion decreased an average of 93% (range, 81.5 to 97%) when CD4$^+$ cells were absent from the PBMC culture during stimulation (Fig. 5), identifying CD4$^+$ cells as the primary source of IFN-γ secretion in PBMCs stimulated in vitro with norovirus VLPs.

DISCUSSION

Human challenge studies have indicated that a subset of individuals may be genetically resistant to noroviruses (30, 35). The characterization of the BGA H type 1 as a ligand that

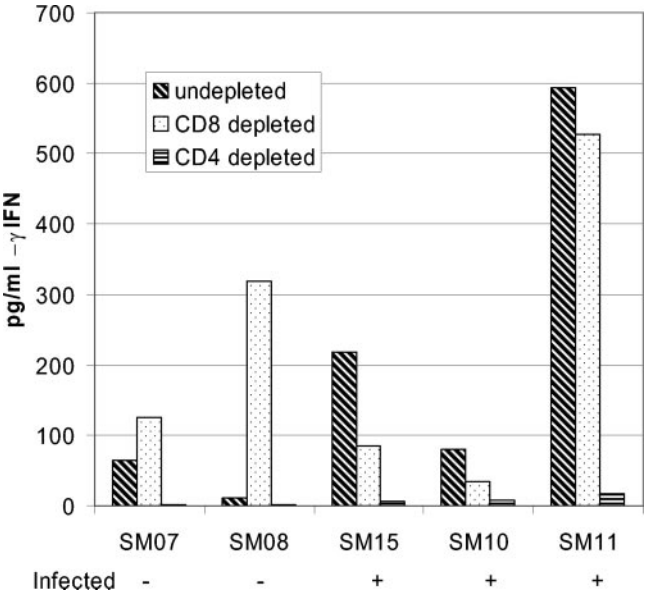

FIG. 5. CD4$^+$ T cells secrete IFN-γ after SMV challenge. PBMCs collected postchallenge from two uninfected (SM07 and -08) and three infected (SM10, -11, and -15) SMV-challenged volunteers were depleted of CD4$^+$ or CD8$^+$ cells using antibody-coupled magnetic beads. The depleted cells were stimulated in vitro with SMV VLPs, and the culture supernatants were analyzed by ELISA for secreted IFN-γ.

binds NV VLPs (34) led to the observation that individuals who do not express H type 1 (the secretor-negative phenotype) do not become infected with NV even when challenged at very high doses (32), establishing *FUT2* as a susceptibility gene for NV and suggesting that H type 1 may be a receptor for virus entry. Further work with other norovirus strains has demonstrated that the noroviruses can use a wide range of molecules in the histo-blood group antigen family as ligands (20, 22). In contrast to NV, SMV infection was not associated with the presence of a functional *FUT2* gene or with expression of any of the H type 1-related molecules, as one of three secretor-negative volunteers became infected with SMV. The effect of the BGA genes on SMV infection will need to be further studied in subsequent human challenge studies with larger groups with varied antigen expression. The small number of subjects characterized here is not sufficient to identify genes with weak penetrance, as was noted for the effect of B antigen expression and NV infection (32, 23). Moreover, our data indicate that saliva-VLP binding cannot predict norovirus susceptibility as strictly as suggested (22) and that virus binding and entry mechanisms may be considerably more complex.

In these studies, we used VLPs as the source of norovirus antigen. These VLPs are composed of the major capsid protein expressed from ORF2 of SMV, HV, or NV. While morphologically and antigenically very similar to native virions (29), they do not represent the entire norovirus strain genome, and thus, any immune response measured represents a partial response. Furthermore, the cross-reactivities of antibodies and T cells measured here are dependent upon several factors, including the degree of genetic relatedness between strains and the major histocompatibility complex (MHC) haplotype of each volunteer, as some MHC haplotypes may preferentially present peptides that are not conserved between strains from different genetic clusters (9, 36).

All of the SMV-infected volunteers and none of the uninfected volunteers produced a significant serum IgG response to SMV, indicating that SMV infection, not just exposure, is essential for activation of an anti-SMV serum IgG response, as reported for NV (32). Furthermore, the IgG elicited during an SMV infection cross-reacts with another strain within G2 but not with a G1 strain, supporting previous findings (28, 39). While these results provide a description of the antigenic relatedness of these viral strains to each other and an assessment of general norovirus exposure in the past, interpretation of their relevance to vaccine design and outbreak investigations is complicated. Studies have used the presence of a norovirus VLP-reactive IgG titer as an indication of strain exposure. However, the cross-strain reactivity of anti-norovirus IgG shown here makes it difficult to determine the infection strain history, as proposed earlier (12, 32). This point is particularly important in outbreak investigations, where, in our experience, more subjects are willing to donate serum samples than are willing to donate stool samples for diagnosis. The data presented here suggest that strain identification by serum IgG titer increase would be imprecise. A comprehensive panel of VLPs and refined diagnostic tests will be needed before IgG can be used to unravel prechallenge histories and current infections with norovirus.

Furthermore, data indicating that a subset of uninfected SMV- and NV-challenged volunteers mount a cellular immune response and a mucosal IgA response, respectively, after norovirus challenge support earlier work suggesting that some volunteers are uninfected due to protective immunity (32). None of these "protected" volunteers produced IgG reactive with the challenge strain. It is possible that a pathogen limited to the gut mucosal surface without a systemic distribution may not be significantly affected by serum IgG. Elegant studies of rotavirus infection in gnotobiotic piglets have shown that sIgA, not neutralizing IgG, correlated with protection from rotavirus reinfection (49–51). Concordant with these findings, NV-challenged susceptible volunteers who produced sIgA within the first 5 days after viral challenge were less likely to become infected than susceptible volunteers who did not produce sIgA, indicating that memory sIgA may be a component of protective immunity to NV infection (32). However, this early sIgA response was not detected in all of the susceptible uninfected volunteers. Thus, other mechanisms must participate in protection against norovirus infection as well. Although the numbers are small, among the SMV-challenged uninfected volunteers, an early (day 1 to 5) sIgA response was not detected, supporting earlier evidence that multiple mechanisms of protection from noroviruses exist (32).

This is the first reported characterization of the Th1 and Th2 cellular immune responses to any live norovirus challenge. PBMCs were stimulated in vitro with intact VLPs (43) from three norovirus strains. This approach allowed antigen-presenting cells to internalize native conformation protein, digest it, and present it in the context of syngeneic MHC class II, as would occur in vivo during challenge, resulting in norovirus-specific $CD4^+$-T-cell activation (44). Comparison of Th1 and Th2 cytokines produced after SMV stimulation of PBMCs indicated that the Th response to SMV was weighted toward a Th1 response, with significant increases in secretion of IFN-γ and IL-2 and serum IgG1 production, similar to results seen in a subset of NV VLP-challenged volunteers (46). These cytokines enhance macrophage activation and production of IgG subclasses that favor opsonization and support $CD8^+$ cytotoxic T cells (27). IFN-γ was up-regulated postchallenge in most of the volunteers, regardless of infection. Both infected and uninfected volunteers could be classified as responders or nonresponders based on a ≥4-fold increase in IFN-γ secretion postchallenge. The elapsed time between challenge and the first postchallenge PBMC sample (8 days) may have been too great to allow identification of a difference in the timing of activation of the Th1 cells between the infected and uninfected volunteers. Furthermore, the T cells examined here were collected from blood, while memory T cells to a mucosal pathogen would be located primarily at the site of initial antigen encounter, the gut-associated mucosa (25). Analyses of cytokines and IgG subclasses in daily serum samples support the hypothesis of a preferential but not exclusive Th1 cytokine response following SMV challenge. Increases in both IFN-γ and IL-2 were detected during the initial 48 h postchallenge, and the IgG response was IgG1, with little change in the IgG2 titer in serum samples from the infected volunteers. Furthermore, the only Th2 cytokine in which significant increases were detected after SMV challenge was IL-5, associated with enhanced sIgA production. sIgA has been shown to be associated with protection from NV infection among susceptible volunteers (32). Identification of IL-5 secretion by postchallenge

PBMCs in response to SMV and NV in vitro stimulation supports the hypothesis that the mucosal immune response plays an important role in norovirus infection. Additional human challenge studies will be needed to decipher the roles of sIgA and the Th2 immune response in protection from norovirus. Future studies should include measurement of gut cytokine profiles and evaluation of PBMCs collected soon after infection to accurately determine the importance of Th2 responses to infection.

Although these experiments do not address SMV protective immunity, the Th1 response identified in a subset of uninfected volunteers suggests that activation of a Th1 cellular immune response may be associated with protection from infection after challenge in some SMV-susceptible volunteers. Supporting a role for the Th1 response in mediating SMV infection outcome, the only infected volunteer without an increase in IFN-γ secretion after SMV challenge produced IL-10 (data not shown), suggesting that the Th1 response elicited by SMV in the other infected volunteers was being suppressed. This volunteer had high prechallenge titers of HV-reactive serum IgG and salivary IgA and Th1 cytokine secretion. These high titers decreased throughout the course of the study, suggesting that this volunteer had experienced a recent infection with HV or a closely related strain of norovirus before entering the SMV study. SMV-reactive IgG developed normally over time and in magnitude, but the volunteer did not develop a salivary IgA or Th1 immune response to SMV after infection. Although speculative, these data provide suggestive evidence that previous viral infection history may affect the immune response to a current norovirus challenge, as has been shown for dengue serotypes (38) and between lymphocytic choriomeningitis and vaccinia virus infections (31). Clearly, further studies are needed to identify the components of protective immunity and the effect of previous exposure history on infection outcome.

No change in T-cell cytokine secretion, serum IgG, or salivary IgA was detected in any of the uninfected volunteers who did not respond to SMV challenge with enhanced IFN-γ secretion (three volunteers). Lack of activation of an adaptive immune response, as seen in the other three uninfected volunteers who received at least the same dose level, suggests that these volunteers were protected by another mechanism, such as innate immunity or inherent genetic resistance to the virus (lack of receptors), or that the SMV ORF2 capsid may not be as antigenic for the HLA haplotype of these volunteers, hampering our ability to detect an immune response (9, 36).

Importantly, the cellular response elicited by SMV challenge was cross-strain reactive with HV but not NV, suggesting that the antigenic relatedness of the strains we examined mirrored the genetic relatedness. These data are in agreement with previous studies of strain-cross-reactive Th1-mediated responses in HIV (7), dengue virus (33), and influenza virus (26). Additional G1 and G2 VLPs are needed to assess the T-cell cross-reactivity between genoclusters and genogroups, given the high degree of genetic heterogeneity among norovirus strains. In addition to the capsid protein, ORF3 and nonstructural proteins should be examined as a source of cross-reactive T-cell antigen, as anti-influenza A virus (26) and anti-cytomegalovirus (45) CTLs recognized internal antigens and were subtype cross-reactive.

The data provided here indicate that antibody and cellular immune responses generated after SMV challenge are cross-reactive within genogroups. Production of new inocula of additional norovirus strains representing different genetic clusters and further human challenge studies are needed to define the antigenic relationships among strains and the components of protective immunity to each and to determine if specific heterotypic immune responses are cross-protective.

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