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Immune Response to Experimental Norwalk Virus Infection

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Master of Public Health

Epidemiology

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B.S.A., University of Georgia, 1997

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Abstract

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In the US, it is estimated that 21 million people are infected with norovirus every year. For the vast majority of these individuals, the infection will cause a mild gastroenteritis lasting 1-2 days. However, more severe outcomes are possible. Despite this very high incidence, the immunological response to norovirus infection, and, more importantly, the markers of a protective response, are poorly characterized. In this study, the immune response of 16 infected and 35 challenged-but-uninfected volunteers was monitored after an experimental challenge with Norwalk virus (GI.1). The dynamics of the humoral response were assessed by measuring the α-Norwalk serum IgG concentration. Similarly, α-Norwalk salivary IgA was used as an indicator of the mucosal response. The humoral, but not the mucosal, response was found to be highly predictive of infection status, with all but one infected volunteer seroconverting. Conversely, whether a given infection was symptomatic was correlated with the general, but not specific mucosal response; volunteers with illness had a higher final total salivary IgA titer than did those without symptoms. The presence of symptoms was not correlated with an α-Norwalk serum IgG response. Pre-existing antibodies, either serum IgG or salivary IgA, were not correlated with infection. Finally, longer periods of viral shedding were correlated with stronger mucosal responses, as measured by total salivary IgA.

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Introduction to Norovirus

Norovirus Epidemiology

According to the most recent estimate from CDC, there are approximately 9.4 million cases of foodborne disease every year in the United States (2). While bacterial causes of foodborne illness receive the most attention, both from scientists and the public, viruses are estimated to account for 59% of all foodborne infections. By far the most common cause of viral gastroenteritis is norovirus, accounting for nearly all of the viral gastroenteritis and 58% of all foodborne illness. However, due to the relatively mild nature of the disease, only 0.03% of hospitalizations and less than 0.1% of deaths attributable to foodborne disease are due to norovirus. This estimate predicts over 5 million domestically-acquired cases of foodborne norovirus every year in the US, however, even this high number is an underestimate of the true burden of norovirus disease. Scallan et al. assumed that only 26% of norovirus infections are domestically-acquired foodborne infections. Thus, the true annual incidence of norovirus infection in the US could be as high as 21 million infections per year. A recent study in Georgia estimated the incidence of norovirus infection in the community to be 6,500 per 100,000 person-years (4), which is consistent with the national annual incidence predicted from Scallan et al.

The prevalence of norovirus globally is not well understood, particularly in developing nations where other infections with greater morbidity and mortality are prevalent. WHO estimated that 2.2 million deaths were attributable to diarrheal disease in 2004, representing 3.7% of total deaths (5). Given that diarrheal disease is generally non-fatal, particularly if it is treated promptly, this

high mortality is indicative of a very high disease burden, of which a substantial fraction is likely due to norovirus. Smaller, regional studies have attempted to answer this question using seroprevalence studies (6-8). All of the studies found very high rates of seropositivity, ranging from 71% in Brazilian children (8) to >95% in southern Africa (7). Interestingly, in all of these studies, the rate of seropositivity increases dramatically after the first year of life, indicating that norovirus exposure occurs during the first five years of life.

Due to the underreporting of norovirus disease and the relatively mild nature of the infection, it is difficult to estimate the economic burden of the disease. However, other estimates can be used to visualize the scale of the problem. Sandler et al. estimated that the total cost of infectious gastroenteritis in the US in 1998 was $344,600,000 (9), most of which is expected to be due to norovirus. This estimate includes direct medical care costs as well as indirect costs such as lost work hours. For norovirus-specific estimates, a few studies have focused on nosocomial outbreaks, where diagnosis rates and costs are likely to be high. Lopman et al. estimated that nosocomial gastroenteritis outbreaks cost the English National Health Service £115 million (US$184 million) in a single year, with the losses attributable to both ward closure and lost productivity of staffers (10). Danial et al. employed a similar analysis to determine the cost of norovirus outbreaks in a single community, Edinburgh, Scotland, which has a total of 2300 hospital beds (11). Across two norovirus seasons, nosocomial outbreaks in this community cost the National Health Service £1.2 million (US$1.9 million). Not surprisingly, the outbreaks had the highest impact in geriatric long-term care, general medicine, and rehabilitative medicine wards.

Encouragingly, Lee et al. used stochastic economic models to show that the costs of these outbreaks can be limited by early detection and implementation of control measures (12).

Norovirus incidence has a distinct seasonality, with cases peaking in the winter months (13-15). As with most seasonal infections, it is not clear what factors drive the seasonality of norovirus infections. Using laboratory data from England and Wales and regression modeling, Lopman et al. found that temperature and relative humidity were correlated with norovirus incidence, with colder, drier conditions being more favorable to infection (16). This model also included a community immunity predictor based on the size of the previous year's norovirus season and the variation in predominant strains from year to year. The significance of this predictor, along with the relatively short-lived immunity conferred by natural infection (17), suggests that cyclical oscillations in immunity on the community level could be driving the seasonality of the norovirus infection. Conversely, a similar study in Toronto, Canada found that norovirus incidence was correlated with not only temperature and precipitation, but also the average flow through the region's watershed and the water temperature in Lake Ontario (18). This study implicated the longer persistence of virus in a colder, more stagnant watershed as the driving factor in seasonality.

Viral Structure, Biology and Phylogeny

Noroviruses are small, non-enveloped viruses that are the causative agents of acute gastroenteritis (AGE). As with all members of the *Caliciviridae*, noroviruses have a positive-sense, single-stranded, non-segmented RNA genome

enclosed within an icosahedral capsid approximately 38 nm in diameter (19-21). The prototypical virus, and the strain for which the genus is named, is the Norwalk virus, which was isolated from an elementary school outbreak in Norwalk, OH in 1968. Prior to molecular identification techniques, these viruses had several designations: small, round, structured viruses (SRSVs), Norwalk agent, Norwalk-like virus, and Snow Mountain virus.

The genome of norovirus is ~7.5 kb and contains three open reading frames (ORFs)(20). ORF1 encodes the non-structural genes: a helicase, a protease and a RNA polymerase (22, 23). These products are initially translated directly from the viral genomic RNA as a polyprotein, and the mature proteins are produced by proteolytic cleavage. ORF2 encodes the major viral capsid protein VP1 (24) and ORF3 encodes a minor structural protein designated VP2 (25). ORF2 and ORF3 are translated from a bicistronic, subgenomic RNA (26), though there is evidence that these ORFs can also be translated directly from the viral genomic RNA (27).

The Norwalk virus capsid was first crystallized in 1999 (28). The icosahedral capsid consists of 180 monomers of the VP1 capsid protein, which assemble into 90 dimers via their N-terminal S domain (Fig. 1A). These dimers then associate to form a capsid (24). It has been shown that the S domain

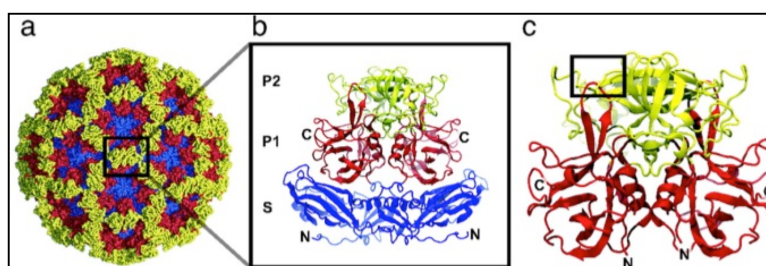

Figure 1. A) Crystal structure of the Norwalk virus capsid. B.) Ribbon structure of the major capsid protein VP1. C.) Region of VP1 involved in HBGA binding and antigenic variation of strains. Reproduced from (1).

is both necessary and sufficient for capsid formation (29), however, the antigenic diversity within the norovirus genus is mostly within the P1 and P2 domains of VP1 (30). These domains are joined to the S domain by a flexible hinge region, which allows the P1 and P2 domains to extend away from the capsid (Fig. 1B).

While both P1 and P2 regions exhibit strain-dependent variation, the P2 domain is much more variable than P1 (30), which is consistent with its location on the most exposed region of VP1 (Fig. 1B). Indeed, the P2 region contains epitopes that are targeted during infection, and changes in this region are correlated with immune escape (30-32).

Much less is known about the role of VP2, the small basic protein encoded by ORF3. Present in only a few copies per capsid, VP2 is not necessary for capsid assembly, though it does increase the stability of VP1 (25). Virus-like particles (VLPs) containing VP1 and VP2 can be produced by expressing ORF2 and ORF3 in mammalian cells, indicating that the inclusion of VP2 in capsids is independent of viral genome packaging. However, it has been suggested that VP2 may have a role in packaging of the viral RNA (33). Since there does not appear to be a point of entry for the viral genome in the assembled VP1 capsid (28), genome packaging is likely to be concurrent with capsid assembly. Due to its basic chemistry and ability to assemble into VP1 capsids, VP2 could interact with the genomic RNA during assembly, and act as a bridge between the VP1 capsid and the genome.

The molecular details of norovirus replication, particularly the role of the non-structural proteins, are limited due to the inability to culture the virus in the laboratory (reviewed in (34)). Direct transfection of cells with the viral genome

has resulted in genome replication and the production of RNA-bearing viral particles (35-37). The success of this approach suggests that the failure of in vitro culture systems is due to defects in the early stage of infection: viral binding, cell entry, and/or uncoating of the genome. Studies of virus binding, using either virus isolated from stools or recombinant VLPs, support this hypothesis (38). Using a variety of human and animal cell lines, White et al. demonstrated specific, but very low level, binding of Norwalk VLPs to cultured cells. The only cell line to show substantial binding was differentiated Caco-2 cells, which is consistent with binding studies in duodenal organ explants (39, 40). In that study, the virus was shown to bind to non-epithelial cells, specifically the lamina propria and Brunner's glands. Recently, two 3-dimensional organoid culture systems – one with Caco-2 cells (41) and one with INT-407 human embryonic intestinal epithelial cells (42) - have been reported to support human norovirus infection. While it is clear that a small proportion of the inoculated virus does enter the cells, and replication and transcription of viral RNA is occurring, the systems do not appear to produce infectious virions. Unfortunately, it does not appear that the cultivable, and closely related, murine norovirus will be useful as a model to gain insight into human norovirus replication. Lay et al. found that, unlike murine norovirus, human norovirus does not replicate in macrophages or dendritic cells (43).

Due to the high diversity within the VP1 capsid protein, the sequence of ORF2 is used as the basis for norovirus phylogeny. Based on sequence analysis of 164 norovirus isolates, Zheng et al. proposed a classification scheme that divides viruses into genogroups, which are further divided into genotypes (see Fig. 2) (3).

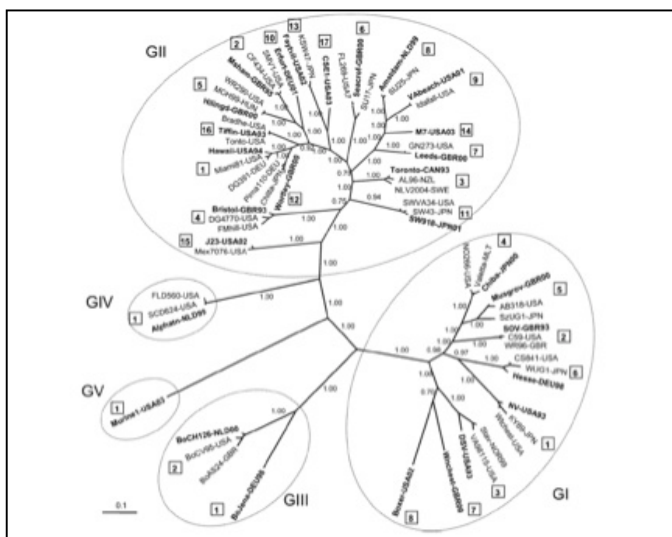

Figure 2. Norovirus classification tree proposed by Zheng et al. (3)

The majority of human norovirus isolates fall into genogroups I and II, with a small number also falling within genogroup IV. Genogroup III is comprised of non-human isolates, primarily of bovine origin, and genogroup V is comprised of murine isolates. Within genogroup I, there are 8 genotypes (44). The prototypical Norwalk virus defines genogroup I, genotype 1, which is designated GI.1 according to the standards of the field. Genogroup II is the most frequently isolated group in epidemic settings (45-47) and is comprised of 19 genotypes (44). Among those, the most frequently isolated type is GII.4 (48). Recent evidence suggests that new virus variants have evolved by recombination events at the ORF1/ORF2 overlap, confounding typical classification strategies (49-51).

Although the entirety of ORF2 is considered for strain designation, strain typing is generally completed using sequences from smaller regions within either ORF2 or ORF1 (52-56). The most broadly applicable typing methods utilize reverse-transcriptase polymerase chain reaction (RT-PCR) followed by sequencing and alignment to known isolates (53, 55, 56). These methods differ mainly in the genomic region targeted for amplification. These regions have been designated A, B, C, D, and E (53). Regions A and B fall in the polymerase region of ORF1, while regions C, D, and E are within ORF2. Because diagnostic methods

have moved towards quantitative real-time PCR (qRT-PCR), where the amplicons are not appropriate for sequencing, a complete diagnosis with genotyping requires two separate amplifications: qRT-PCR for quantitation and RT-PCR for genotyping. Newer methods have utilized TaqMan RT-qPCR, which produces amplicons that can be sequenced directly (54, 57).

Norovirus Infection and Susceptibility

Typically, norovirus infection causes a relatively mild episode of acute gastroenteritis (AGE), colloquially known as "winter vomiting disease" or the "stomach flu". After a short incubation period (24-48 hours), the disease is characterized by a very abrupt onset of diarrhea and/or vomiting. Nausea and stomach cramping often accompany these symptoms. Fever, headache and coughing are rare. The symptoms typically resolve without intervention after 24 hours (58). In rare cases, the severity of the diarrhea or vomiting requires hospitalization due to dehydration, and it is this dehydration that is responsible for the few fatalities associated with norovirus. Other than oral rehydration therapy for dehydration and anti-emetics for nausea, there is no specific treatment for norovirus.

Norovirus infection can result in other, less common clinical presentations. Individuals with severely compromised immune systems can become chronically infected with symptoms and viral shedding persisting for months (59-62). There is evidence implicating these long-term shedders as a source of hospital outbreaks of AGE (61). Additionally, human challenge studies suggest that roughly 30% of infections are asymptomatic (63, 64) and cross-

sectional studies estimate the prevalence of asymptomatic infection in the community to be around 12% (65, 66). Recently, necrotizing enterocolitis in infants, a very severe disease requiring emergency surgical intervention, has been associated with the presence of norovirus RNA in the stool (67, 68), but it is unclear whether norovirus infection is involved in the pathogenesis of the disease.

Diagnosis of norovirus infection, when it occurs, is generally a diagnosis by exclusion of bacterial causes. Since the virus cannot be cultured in vitro, diagnostics depend on detection of the virus in the stool or virus-specific antibodies in the sera or saliva. Initially, norovirus particles were detected in stool samples by direct visualization by electron microscopy (69). This method of diagnosis was expensive, time-consuming, and neither sensitive nor specific. The sensitivity and specificity of detection was greatly improved by the development of RT-PCR, which detects the viral RNA (70-72). Even more sensitive is quantitative real-time PCR (qRT-PCR), which can determine the number of viral genomes in a sample (54, 73-75). Viral load in the stool can also be estimated using an antigen capture ELISA, (76), which is the basis for an immunochromatographic dipstick test for infection (77). The presence of high levels of anti-norovirus antibodies, particularly IgM but also IgG, in sera can also be used to diagnose infections using ELISA-based techniques (78, 79). However, developing broadly reactive ELISAs is difficult due to the strain-specific nature of the immune response, which limits their utility in general diagnosis of norovirus disease. RT-PCR assays can be either strain-specific or broadly reactive based on the primers used.

Susceptibility to norovirus infection is strongly influenced by host factors. As previously discussed, chronic infections are associated with immunocompromised individuals, such as transplant recipients (60) and leukemia patients (59). In addition to the role of immune status, susceptibility to norovirus is correlated with blood type and histo-blood group antigens (HBGAs). Initial studies showed that individuals with type O blood had the highest odds of infection in an outbreak (64, 80). Further studies found that infection with Norwalk virus was completely dependent on secretor status, with the virus capable of infecting only secretor positive (Se+) individuals (64, 81).

Secretor status is determined by the *FUT2* gene (82). When this gene is functional (i.e. Se+), Lewis antigens are present in saliva and other body fluids and are free to bind to the surface of epithelial cells. When both *FUT2* alleles are non-functional, Lewis antigens are still produced (by the action of the other *FUT* genes) but they are no longer secreted into body fluids (i.e. Se-). Individuals who are heterozygous for functional *FUT2* are called partial secretors.

Although it cannot be tested directly due to the lack of a norovirus culture system, Lewis antigens are thought to act as the cellular receptor for norovirus. VLPs (81, 83-85), recombinant VP1 (86, 87), and P particles (88) have been shown to bind to Lewis antigens in Se+ saliva as well as synthetic glycans. The regions of VP1 involved in HBGA binding have been identified (32), and the interaction between the molecules has been mapped by structural studies (89). Additionally, changes in VP1 can be linked to changes in HBGA binding (86, 87, 90), providing a potential explanation for the strain-dependent differences in host susceptibility.

Recently, outbreak studies have found that not all noroviruses are restricted to Se+ individuals. Investigations of a GI.3 outbreak in Sweden (91) and a mixed GII.g/GII.12 outbreak in Ohio (92) identified symptomatic, Se- individuals with norovirus RNA in their stool, indicative of active infection. A mixed GII.4 outbreak in the Israeli military was reported to have a secretor-independent attack rate (93), however, there are potential methodological problems with the study (94, 95). Most convincingly, a human challenge study with Snow Mountain virus (GII.2) found that infection status was not correlated with ABO blood type, Lewis type, or secretor status (63).

Norovirus Transmission and Environmental Stability

Transmission of norovirus is primarily via the fecal-oral route (58), with fecal contamination of food and water being the main source of infection. Due to the inactivation of the virus by heat, foods implicated in outbreaks are generally served raw (e.g.- salads, fruits, etc.) or subject to handling prior to consumption (e.g.- sandwiches, sliced breads, etc.). Fomites have also been implicated in transmission during outbreaks (96-101). Evidence from outbreaks suggests that aerosolized virus, particularly in vomitus, could be contagious, making inhalation a possible route of infection (102).

The infectious dose is thought to be between 10 and 100 viral particles (58), though some studies estimate that it could be as low as one (103). Infected individuals shed large concentrations of virus in their stool, with reported numbers as high as 10^{11} genome equivalents per gram of stool (74, 75, 104). This shedding occurs throughout the symptomatic period and has been documented

to continue for up to 1-5 weeks after the resolution of symptoms (61, 105, 106). In immunocompromised patients with chronic infections, viral shedding can continue for months after the onset of symptoms (13).

In addition to high shedding rates, norovirus is extremely stable in the environment. The best evidence for this environmental stability comes from outbreak investigations implicating fomites in transmission. Transmission of the same strain on consecutive cruises has been documented multiple times, strongly implicating the boat itself as the source (96, 99). Outbreaks have also been linked to contaminated kitchen surfaces (100) and surface contamination in long term care facilities (97). In some cases, the contamination persisted despite documented, appropriate sanitization efforts (99, 100, 107).

This persistence has also been documented in the lab. Seitz et al. found that Norwalk virus in groundwater remained infectious for at least 61 days and the RNA was detectable for over 3 years (108). Consistent with this, Skraber et al. found that norovirus GI and GII can survive for at least 49 days in wastewater at 4°C, but increasing the temperature to 20°C shortened the survival to 25 days (109). The viruses have also been detected in biofilms associated with wastewater, which could serve as a reservoir of the virus (109). Perhaps most importantly, noroviruses can persist on refrigerated ready-to-eat foods for at least 10 days (110).

In addition to general environmental stability, norovirus is highly resistant to many types of disinfectants. Commonly used disinfectants such as quaternary ammonia compounds (111, 112), alcohols (113) and alcohol-based sanitizers (114) are ineffective against norovirus. The only commonly used disinfectant that is

effective against norovirus is hypochlorite (i.e. bleach), with 160 ppm reducing the number of genome copies at least 4-fold (113-115). A variety of less common disinfectants and disinfection protocols have been tested. Peroxide- and aldehyde-based disinfectants have been shown to be capable of reducing norovirus genome titers by at least 3 logs, but it requires substantial contact time (60 min) (116). Gas sanitization of rooms is particularly appealing due to the role of fomites in transmission. Unfortunately, gassing a room with ozone (117) or hydrogen peroxide vapors (118) has little effect on norovirus, reducing the detectable genomic copies by only 1 log.

The inability to culture norovirus in the lab greatly complicates the study of viral persistence in the environment and the development of inactivation strategies. In an attempt to overcome this limitation, many researchers have turned to surrogate viruses (for a review, see (119, 120)). Commonly used surrogates for human norovirus include murine norovirus, feline calicivirus, poliovirus, monkey calicivirus (i.e. Tulane virus) and bacteriophage MS2. However, the results of these surrogate studies are very difficult to interpret (119). It is not uncommon for results from one surrogate to conflict with those from another surrogate. Most alarmingly, all of the surrogates have been found to be in disagreement in at least one aspect with results obtained for human norovirus. For example, for several of the studies cited above, norovirus was found to be more resistant to disinfection than the surrogate virus (FCV or MNV) in all conditions tested (113, 116, 117) To avoid this confusion, studies exclusively utilizing viral surrogates will not be considered here.

Immune Response to Norovirus

Although much is known about the immune response to norovirus infection, it is still unclear how the immune response contributes to the disease process, how this response exerts selective pressure on the evolution of the virus, and how the response can be exploited to change the epidemiology of the disease. The current understanding of norovirus immunity is derived from outbreak investigations and human challenge studies. Both of these approaches have strengths and weaknesses. Outbreak investigations provide a realistic view of natural infections in a variety of populations; however, this diversity is also the greatest limitation of outbreak data. Conversely, human challenge studies provide well-controlled data that is relatively easy to interpret, but it is unclear how this data can be extrapolated to the diverse populations and settings outside the laboratory. In this review, outbreak data will be considered first, followed by data from the human challenge studies.

It is clear from outbreak studies that infection with norovirus, whether symptomatic or asymptomatic, results in a strong rise in serum IgG between the acute and convalescent phases (i.e. rise in titer ≥ 4-fold, seroconversion) (121-125). Less predictable is seroconversion for IgM (121) and IgA (123, 124). Several studies found that the initial titers of anti-norovirus antibody varied widely across individuals, likely due to previous exposures (123, 125). However, initial antibody titers were not correlated with infection status. The type and quality of this immune response change with age. Children predominantly mount an IgG response, with the likelihood of IgA seroconversion increasing with age (124).

The quality of the response, as measured by the avidity of the antibodies produced, also increases with age.

The extent of cross-reactivity of the antibody response is still unclear. Two studies have addressed this question using outbreak data. In the first study, samples from two outbreaks were tested against a panel of VLPs that spanned both genogroups and included the outbreak strain (122). As expected, reactivity was highest against the homologous outbreak VLP. Cross-reactivity was variable, but generally higher across genotypes than across genogroups. A similar study, focused exclusively on outbreaks of GII viruses, found similar variability in cross-reactivity to heterologous VLPs (126). However, there was no cross-reactivity in H type 3 blocking antibody, which is consistent with low levels of protection due to cross-reactivity.

As expected for a virus, norovirus infection stimulates a primarily Th1 response. In a cohort of Mexican children, infection led to increases in the fecal cytokines IL-5 and IL-8 (127). Similarly, in adults with traveler's diarrhea, fecal IL-2 and IFN-γ were elevated (128). These cytokine profiles are consistent with a Th1 response, which drives a cellular, as opposed to humoral, immune response.

Human challenge studies have facilitated a more detailed understanding of the immune response to norovirus infection. Consistent with the data from outbreaks, individuals challenged and infected with norovirus seroconvert (IgG) approximately 6 days after exposure (129-131) and most of those also had serum IgM and IgA seroconversions (129). Changes in salivary IgA and IgG titers are correlated with IgG seroconversion, but the rise in titer is not as strong and not all individuals exhibit the 4-fold rise in titer that defines conversion (63, 130).

Intriguingly, in a Norwalk challenge study, salivary IgA response was predictive of infection status (131). Volunteers who were not infected had a rise in salivary IgA during the first five days after challenge that was not present in those who became infected. These uninfected volunteers did not, however, have the sharp rise in antibody titer that corresponds with seroconversion.

Challenge studies have been an excellent source of information on the quality of the immune response to norovirus infection. Of particular interest is the level of HBGA blocking antibody (also called HBGA blockade), which is thought to be functionally equivalent to neutralizing antibodies to viruses that can be grown in the lab. Infection with Norwalk virus stimulates rises in the serum concentration of antibodies that can block virus binding to H type 1 and H type 3 polysaccharides, as well as Le^b antigens (83, 132, 133). As with total antibody responses, the strength of the blockade response varies by individual, as does the pre-challenge blockade titer (BT_{50}). Although the prechallenge total antibody titers are not correlated with infection status, the prechallenge BT_{50} is strongly correlated with asymptomatic infection (133).

As in the outbreak studies, the cross-reactivity of the immune responses generated in challenge studies is variable. Across genogroups, the level of cross-reactivity is moderate and varies considerably between individuals. Infection with Snow Mountain virus (GII) resulted in IgG seroconversion to Norwalk virus (GI) in 40% of volunteers in one study (129) and 0% of volunteers in another study (63). Similarly, infection with Hawaii virus (GII) induced an IgG seroconversion to Norwalk virus in only 13% of volunteers (129). No volunteers had a salivary IgA response that recognized Norwalk virus (63). Conversely,

within genogroups, cross-reactivity of both total and blockade antibodies can be relatively high (132, 134).

This variability in cross-reactivity was also seen in the cytokine response of individual volunteers. Peripheral blood mononuclear cells from infected individuals can be stimulated to produce Th1-type cytokines (i.e.-IFNγ, IL-2, and IL-5) by exposure to the challenge virus (63, 132). In some individuals, this response could also be elicited by stimulation with heterologous viruses, even across genogroups.

Potential Vaccines and Antivirals for Norovirus

With the high incidence and large economic impact of norovirus disease, there is much interest in developing specific interventions. Antivirals are historically difficult to develop and, due to the extremely short incubation period and disease duration, they are unlikely to have a significant impact on norovirus transmission. Thus, most of the research and development effort has been directed towards a vaccine. This approach has been very successful for rotavirus, another high impact enteric virus (135).

The most direct approach to a norovirus vaccine is to use VLPs, which contain the major antigen VP1 but cannot replicate and cause disease. Indeed, this is the approach used for the vaccine currently in clinical trials (136). Vaccination with a monovalent (Norwalk only) VLP results in a dose-dependent increase in serum IgG, as well as increases in mucosal IgA (137). Upon challenge with the homologous virus, vaccination reduced the infection rate (82% vs. 61%)

and the rate of symptomatic infection (69% vs. 37%) (136). Neither the duration of protection nor the extent of cross-protection are known.

Other approaches have been taken to develop a norovirus vaccine. The VP1 capsid protein can be expressed in transgenic potatoes, producing an edible vaccine (138). The capsid protein can also be expressed in a vesicular stomatitis virus vector system and these recombinant VLPs used as a vaccine (139). Another VLP-based approach inserted an antigenic influenza peptide into the P loop of VP1 and used this chimeric protein to produce P-particles (140). Using these chimeric particles as vaccine antigens protected mice against influenza infection and stimulated the production of HBGA blockade antibodies, supporting the possibility of a dual vaccine.

Although a vaccine is the most likely solution to the burden of norovirus disease, there specific antivirals are also under investigation. A compound that is able to block the binding of the virus to HBGAs should be able to prevent infection. With this goal in mind, Feng et al. developed a high-throughput ELISA for HBGA binding inhibitors and screened a library of synthetic compounds (141). A few potential candidates were identified, but their ability to block infection has not been tested.

Another approach to antiviral development is to block viral replication within the cell. Bok et al. used phosphorodiamidiate morpholino oligomers (PMOs) to prevent translation of the viral genome (142). This approach has been shown to prevent replication of murine norovirus in culture. PMOs specific to human norovirus were shown to block translation of both GI and GII reporter constructs, indicating that this approach could be successful. It is not known

whether these oligomers can inhibit human norovirus replication nor what impact this could have on infection.

Outstanding Research Questions

Although there are many research questions to be addressed in the norovirus field, there are two research needs that will significantly advance the field. 1) A method to accurately assess the infectivity of norovirus is needed. This would allow true comparisons of disinfection and neutralizing procedures. A reliable cell infection method would also allow further studies of the viral life cycle and replication. The results of these studies could then be used to develop norovirus-specific therapies. 2) A reliable immune correlate of protection must be identified to further vaccine development. Although human challenge studies are available to test vaccine efficacy, challenge studies are not practical for the large-scale studies necessary to bring a vaccine to market. If the characteristics of a protective immune response can be identified, monitoring of this correlate could predict vaccine efficacy. Understanding the markers of a protective immune response would also allow the identification of susceptible and immune individuals in an outbreak situation. Achieving both of these research goals would give public health officials the tools to reduce the burden of norovirus disease.

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Assay Optimization

Previous studies have published successful enzyme-linked immunosorbent assays (ELISAs) for detection and quantitation of anti-norovirus antibodies (1-5). A critical component of these assays is the antigen used to bind the antibodies. For this study, a new protocol was developed for production and purification of Norwalk virus-like particles (VLPs). The new antigen production protocol required optimization of both the serum IgG and salivary IgA ELISA protocols.

Serum IgG ELISA

The initial concern with the serum IgG protocol was the substitution of baculovirus-produced Norwalk VLPs for the previously optimized VLPs produced in Venezuelan Equine Encephalitis (VEE) virus replicons (1). Using the current laboratory protocol for serum antibody detection ELISAs, the reactivity of the baculovirus-produced VLPs was compared to the VEE-produced VLPs (Fig. 3). A human serum sample from a previous Norwalk virus challenge study that was known to have a high α-Norwalk IgG titer was used for assay optimization. With this serum sample, the baculovirus-produced VLPs had roughly half the reactivity of the VEE-produced VLPs. To address this lower reactivity, different concentrations of VLPs were used to coat the plate (Fig. 3). Doubling the VLP coating concentration dramatically increased the level of antibody detection and this higher concentration was chosen for future use.

The original lab protocol had high inter- and intra-assay variation, which made validation of the results and detection of differences difficult. In accordance with the recommendations in (6), all incubation steps, except the

overnight blocking step, were changed to room temperature incubations with rotation. By adding motion to the incubation steps, the dependence on Brownian motion is reduced and slight differences in handling do not result in signal variation. In addition, both the sample and the secondary antibody were diluted in the blocking solution, as opposed to PBS as directed by the original lab protocol.

ELISA sensitivity and variation can be sensitive to the components of the blocking solution (6). For some antigen-antibody combinations, BLOTTO (i.e. non-fat milk) is best, while for others, bovine serum albumin (BSA) is best. Other blocking options are available, but BLOTTO and BSA are the most common. To further optimize the serum IgG ELISA, BLOTTO and BSA were compared as blocking agents (Fig. 4). Although BSA resulted in higher antibody measurements, the variation was similar to that with BLOTTO. Due to the significantly increased cost of BSA and lack of a significant assay improvement, BLOTTO was used in the final optimized protocol.

Heat inactivation of the serum sample was also tested, but it increased the variation in the assay and was not pursued.

To further standardize the protocol, all samples were tested at a single dilution in quadruplicate. If the signal was below the lowest standard (31.25 ng/ml), the assay was repeated with the sample diluted at 1:200. If the signal was still below that of the lowest standard, a value of one-half of the lowest standard was used for analysis.

After the assay optimization described above, the α-Norwalk serum IgG ELISA is highly reproducible, both intra- and inter-assay. The intra-assay

coefficient of variation is routinely around 5%. The inter-assay CV is a bit higher, around 10%, but still in the acceptable range for ELISAs. Limit of detection analysis was not performed for this assay, however, theoretical limits can be calculated from the IgG standard concentrations and the dilutions tested. From these calculations, the dynamic range of the assay for this study is 6.25 μg/ml to 800 μg/ml.

Salivary IgA ELISA

Two previous studies reported ELISAs for α-Norwalk salivary IgA (3, 4). Using these studies, as well as the results of the serum IgG optimization, an initial protocol for salivary IgA was designed. This protocol included heat inactivation of the saliva (56°C, 60 min), BLOTTO blocking solution and antibody diluent, and incubation with rotation.

As with the serum IgG ELISA, the salivary IgA assay needed to be validated with the baculovirus-produced Norwalk VLPs. In addition, a new protocol for VLP purification had been developed, and the VLPs produced by this new method were also tested (Fig. 5). To make this comparison, several saliva samples were used. Two archived specimens, shown to have high salivary IgA titers in a previous study (4), were used as positive controls. In addition, saliva samples from a volunteer with a strong serum IgG conversion were also tested. As seen with the serum IgG ELISA, the baculovirus-produced VLPs were less reactive than the VEE-produced VLPs, however, the new purification procedure dramatically increased the reactivity of the baculovirus-produced VLPs.

Prior studies used dilutions ranging from 1:4 to 1:16 for detecting salivary IgA (3, 4). To determine the optimal dilution for this assay, a two-fold dilution series, from 1:4 to 1:32, of the known positive saliva was tested. The reactivity was very low (data not shown), necessitating the use of the 1:4 dilution for the optimized assay. The same dilution testing was performed for the total IgA assay, using a dilution series from 1:200 to 1:1600. The dilution that resulted in a signal roughly at the midpoint of the standard curve (1:400) was selected.

For all salivary IgA assays, the saliva was heat-inactivated and clarified immediately prior to dilution. It is possible that this inactivation step affected the assay, either by reducing the overall reactivity or increasing the variation. However, the assay was not tested without this step.

Unlike the α-Norwalk serum IgG ELISA, the α-Norwalk salivary IgA ELISA is more variable. The inter- and intra-assay CVs are between 10 and 20%. One possible explanation for this is the heat-inactivation of the saliva for each assay. Additionally, protease activity was detected in the low saliva dilutions, which could interfere with the assay. The total salivary IgA ELISA is more consistent, with CVs around 10%. This is likely due to the much higher concentrations of total IgA, and, thus, higher saliva dilutions.

Limit of detection analysis was not performed for these assays, however, the theoretical limits can be calculated as for the α-Norwalk serum IgG. For the α-Norwalk salivary IgA ELISA, the theoretical range of detection is 125 ng/ml to 8,000 ng/ml. For the total IgA ELISA, the theoretical range of detection is 12.5 μg/ml to 800 μg/ml.

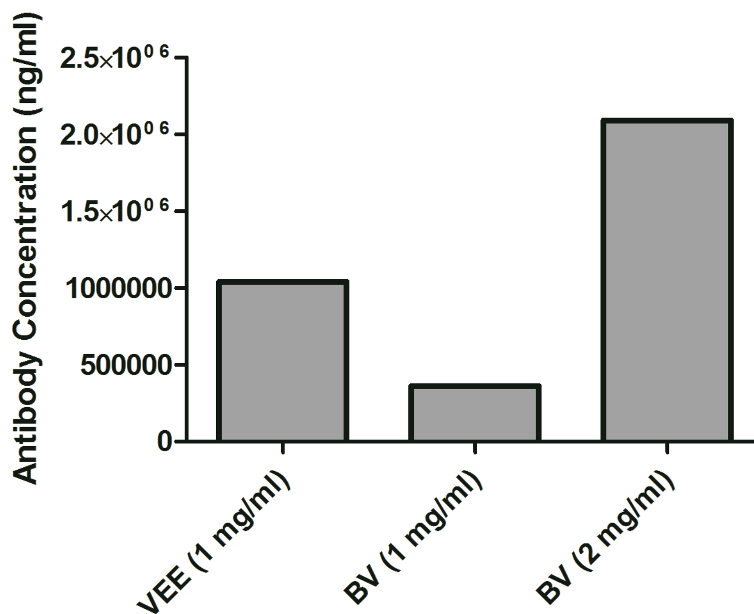

Figure 3. Comparison of baculovirus- and VEE-produced Norwalk VLPs. Plates were coated with 1 mg/ml VEE-produced VLPs (VEE), or 1 or 2 mg/ml baculovirus-produced VLPs (BV). A known high titer α-Norwalk serum sample was used to compare the reagents. Antibody concentrations were calculated by comparison to an IgG standard curve.

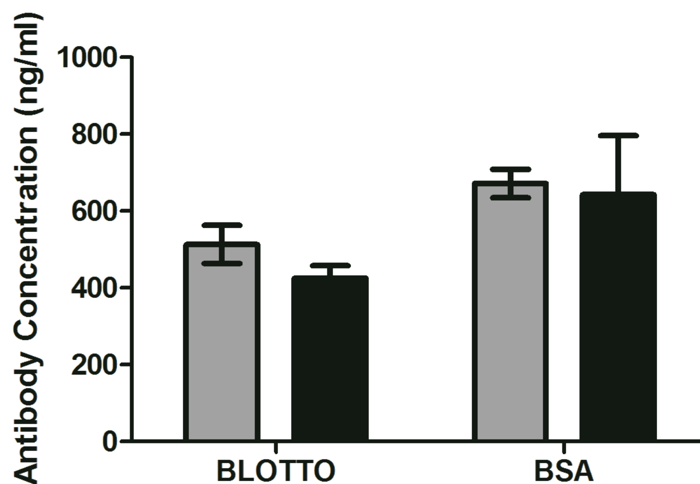

Figure 4. Evaluation of BLOTTO and BSA as blocking agents and heat inactivation of serum. Plates were coated in the indicated blocking solutions and the same blocking solution was used as diluent for the samples and the secondary antibody. The same serum sample was heat-inactivated (56°C, 30 min) and both serum samples were tested at a 1:400 dilution. Gray bars, no heat inactivation; black bars, with heat inactivation. BSA, 1% BSA; BLOTTO, 5% BLOTTO, both prepared in PBS-Tween.

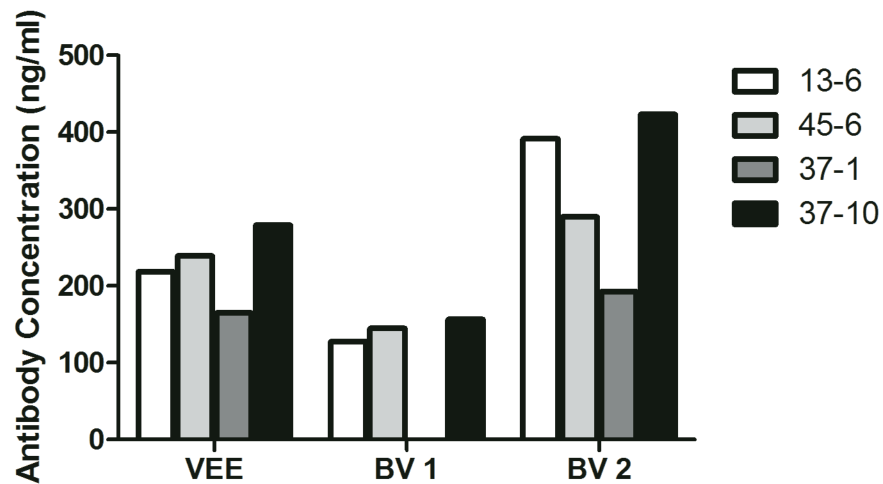

Figure 5. Comparison of different VLPs in the salivary IgA ELISA. VLPs produced in the VEE replicon (VEE) were compared to the baculovirus-produced VLPs used for the serum IgG ELISAs (BV 1) and the same VLPs purified with an optimized protocol (BV 2). 13-6 and 45-6 are archived saliva samples previously shown to have high α-Norwalk IgA titers. 37-1 and 37-10 are the pre-challenge and day 35 saliva samples from a volunteer with a very strong serum IgG conversion.

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Immune Response

to

Experimental Norwalk Virus Infection

Abstract

In the US, it is estimated that 21 million people are infected with norovirus every year. For the vast majority of these individuals, the infection will cause a mild gastroenteritis lasting 1-2 days. However, more severe outcomes are possible. Despite this very high incidence, the immunological response to norovirus infection, and, more importantly, the markers of a protective response, are poorly characterized. In this study, the immune response of 16 infected and 35 challenged-but-uninfected volunteers was monitored after an experimental challenge with Norwalk virus (GI.1). The dynamics of the humoral response were assessed by measuring the α-Norwalk serum IgG concentration. Similarly, α-Norwalk salivary IgA was used as an indicator of the mucosal response. The humoral, but not the mucosal, response was found to be highly predictive of infection status, with all but one infected volunteers seroconverting. Conversely, whether a given infection was symptomatic was correlated with the general, but not specific mucosal response; volunteers with illness had a higher final total salivary IgA titer than did those without symptoms. The presence of symptoms was not correlated with an α-Norwalk serum IgG response. Pre-existing antibodies, either serum IgG or salivary IgA, were not correlated with infection. Finally, longer periods of viral shedding were correlated with stronger mucosal responses, as measured by total salivary IgA.

Introduction

According to the most recent estimates, norovirus is responsible for the vast majority (58%) of all foodborne illness in the United States (1). Including non-foodborne sources, the estimated incidence of norovirus infection is 21 million infections per year in the US alone. Although the typical course of norovirus disease is short and mild, environmental stability (2) and low infectious dose (3) make the virus highly contagious and, thus, responsible for large, costly outbreaks (4, 5).

In general, the immune response to norovirus is typical of the response to any viral infection. Infection induces a characteristic ≥4-fold increase in serum IgG approximately 6 days after exposure (e.g. seroconversion) (6-13). This IgG seroconversion is often coupled with IgA and IgM seroconversions (6, 10, 11). Additionally, the few studies examining the cellular response to infection report that the response is predominantly Th1, as expected for a viral infection (14-17).

Some characteristics of the α-norovirus immune response remain unexpected and unexplained. Seroconversion, the major marker of an effective immune response, does not appear to be necessary to clear an infection; in rare cases, individuals may never seroconvert despite having a typical disease course (9, 15). The mucosal response is even less understood. In general, increases in salivary IgA and IgG are correlated with seroconversion, but the extent of the response varies greatly (9, 15). Neither the determinants nor the implications of the strength of the mucosal response are known.

Perhaps the most intriguing aspect of the mucosal response is that reported by Lindesmith et al. (8). In volunteers challenged with Norwalk virus (GI.1), the dynamics of the norovirus-specific salivary IgA response were different in infected and uninfected volunteers. When productive infections were established, the temporal dynamics of salivary IgA mirrored those of serum IgG, with an increase around day 6 post-exposure. Conversely, in uninfected volunteers, salivary IgA increased immediately after exposure and returned to pre-challenge levels around day 6. These response profiles are highly suggestive of a protective effect of an early salivary IgA response, however, further studies with more volunteers are needed.

In this study, the humoral and mucosal immune response to experimental infection with Norwalk virus was assessed by determination of antibody concentrations in archived serum and saliva samples. In addition to the temporal dynamics of the immune response, characteristics of the response were examined for correlation with disease outcomes, such as infection status, symptoms and viral shedding.

Materials and Methods

Human Challenge Study. The serum and saliva samples utilized in this study were collected from a human challenge study described in (18). Briefly, 51 volunteers were challenged with Norwalk virus inoculum 8fIIb isolated from stool filtrates from previous challenge studies. The inoculum dose was delivered in oysters that had been injected with virus such that three oysters contained 1 x 10^4 genome equivalent copies (GEC). The study was divided into three phases,

grouped by the high-pressure hydrostatic (HPP) treatment of the oysters, plus a pilot study. Phase I received inoculated oysters that had been subjected to 400 MPa for 5 min at 25°C. Phase II received inoculated oysters that had been subjected to 600 MPa for 5 min at 6°C. Phase III received inoculated oysters that had been subjected to 400 MPa for 5 min at 6°C. Controls were distributed across the three phases and received inoculated, but untreated oysters, serving as positive controls for exposure. Samples from the pilot study to assess the infectivity of the inoculum without HPP treatment were also included in this analysis.

Volunteers were admitted to the Emory University Hospital Clinical Interaction Site (a member of the Clinical Interactions Network of the Atlanta Clinical and Translational Science Institute) for the first 5 days of the challenge, with day 1 being the challenge day. Blood and saliva samples were collected pre-challenge and daily for the first 5 days, then at followup visits around days 8, 14, 21, 28, and 35. All stools were collected during the inpatient stay, and a stool sample was collected at each followup visit. Vomitus was collected when present. GCRC staff recorded vital signs 2-3 times daily during the inpatient stay and monitored the volunteers for symptoms of gastroenteritis.

Determination of Specific Antibody Levels. Direct enzyme-linked immunosorbent assays (ELISAs) were used to determine serological and salivary antibody titers to Norwalk virus. For serum IgG titers, medium binding polystyrene plates were coated with 2 µg/ml Norwalk virus-like particles (VLPs) produced in a baculovirus expression system. Unbound VLPs were removed by

washing the plate 5 times in PBS-T (PBS + 0.025% Tween 20), and the plates were blocked overnight at 4°C with 5% Blotto in PBS-T. After 5 washes, the plates were incubated for 1 hr at room temperature with quadruplicate serum samples diluted 1:200 or 1:400 in blocking solution, after which unbound antibody was removed by washing. The secondary antibody (alkaline phosphatase-labeled rabbit α-human IgG, Sigma-Aldrich Co., St. Louis, MO) was diluted 1:2500 in blocking solution and incubated for 30 min at room temperature. After a final 5 washes in PBS-T, 100 µl of *p*-nitrophenyl phosphate solution (Sigma-Aldrich Co., St. Louis, MO) was added to each well and the plate was incubated at room temperature in the dark for 10-30 min. The optical density at 405 nm was determined using an ELx800 plate reader (BioTek Instruments, Inc. Winooski, VT).

The antibody concentration in each sample was determined by comparison to a standard curve generated with known concentrations of IgG (31.25 – 2000 ng/ml) and fitted with a 4-point parametric curve fitting procedure. Samples that were below the level of detection were assigned a value of one-half of the lowest detectable concentration.

The protocol for Norwalk-specific salivary IgA ELISAs was the same as that described above with the following exceptions. Prior to dilution, the saliva samples were heat inactivated at 56°C for 60 min, followed by centrifugation at 16,000 x g for 10 min to pellet mucins and other debris. The clarified samples were then diluted 1:4 in blocking solution and used as described above. The secondary antibody was alkaline phosphatase-labeled anti-human IgA (Sigma-

Aldrich Co., St. Louis, MO), diluted 1:2500 in blocking solution. Finally, the standard curve was generated with known concentrations of IgA (31.25 – 2000 ng/ml).

To correct for changes in salivary flowrate, IgA levels are reported as the negative logarithm of the ratio of α-norovirus IgA to total IgA. To determine the total IgA concentration, the plates were coated with rabbit α-human IgA (Sigma-Aldrich Co., St. Louis, MO), and the clarified saliva samples were diluted 1:400 in blocking solution. Otherwise, the protocol was identical to that for norovirus-specific IgA.

Quantitation of Virus in Stool. Viral shedding in stool was measured by quantitative RT-PCR as previously reported (19). Briefly, total RNA was extracted from a 10% stool suspension (wt/vol in water) with an equal volume of Vertrel XF (DuPont, Wilmington, DE). After clarification by centrifugation, RNA was purified from the supernatant using the QiaAmp Viral RNA kit (Qiagen, Valencia, CA). Norwalk RNA was detected by quantitative real-time reverse transcription PCR (qRT-PCR) using the Qiagen One-step RT-PCR kit (Qiagen, Valencia, CA). Norwalk-specific primers (NVKS1 and NVKS2) and probe (NVKS3) were used as previously described (19). RNA copy number was determined by comparison to a RNA standard curve.

Data Analysis. Because the sample collection did not always occur on the same days relative to the baseline, the data was grouped into the following sample groups based on days post challenge: 1, pre-challenge (and challenge day); 2, day 2; 3, day 3; 4, day 4; 5, day 5; 6, days 6-9; 7, days 13-16; 8, days 21-26; 9, days 27-30; and 10, days 35-41. All data types were grouped according to this scheme. If

more than one sample was collected in a given timeframe (stool, for example), the average of the values was used.

Additional variables were created to facilitate the data analysis. "IgG conversion" is a dichotomous variable indicating a 4-fold or greater increase in α-norovirus serum IgG in the 10th sample relative to the serum α-norovirus IgG in the pre-challenge sample. "Fold increase in IgG" is a numerical variable calculated by dividing the serum α-norovirus IgG concentration at the 10th sample by the pre-challenge serum α-norovirus IgG concentration, e.g. the magnitude of the seroresponse. "HPP treatment" is a dichotomous variable that indicates high pressure processing of the inoculated oysters, i.e. an indicator of inclusion in the control group. "Duration of shedding" is calculated by subtracting the date of the first positive stool from the date of the last positive stool.

All analysis was performed using SAS version 9.3 (SAS Institute, Inc., Cary, NC). For all analyses, the α value for significance was 0.05.

Results

A previous human challenge study examined the effect of high pressure processing (HPP) treatment on the infectivity of norovirus-contaminated oysters (18). This study resulted in 16 infected volunteers and 35 uninfected volunteers, and these individuals provided the serum and saliva samples used in the current study. The relevant demographics of the study population are presented in Table 1. Sex, race, and age are similar for the infected and uninfected groups and are

not correlated with infection status in either the full study or the control group. Infection was more common in blood types A and O, consistent with previous studies with Norwalk virus (8). However, blood type was not correlated with infection status (Table 2), likely due to the small number of volunteers and the effect of HPP processing on overall infection frequencies. Because previous studies showed that Norwalk infection is dependent upon secretor status (8), study volunteers were screened for secretor status, and only Se+ individuals were enrolled.

Figure 1 presents the temporal dynamics of the humoral IgG response to Norwalk virus infection. As expected, infected individuals exhibited a characteristic seroconversion (≥4-fold increase in titer) around day 7 after challenge. Although all infected individuals seroconverted, the extent and timing of the conversion varied considerably. Among infected volunteers, the minimum fold increase over baseline was 4.5 and the maximum was 21.1. Of those volunteers who seroconverted, all but two had converted by day 14 post-challenge. Of the remaining two volunteers, one converted by day 21 post-challenge and the other converted by day 28 post-challenge. Not surprisingly, bivariate analysis revealed that seroconversion was strongly correlated with infection (Table 3). The distribution of IgG concentrations are shown in Table 2.

The temporal dynamics of the salivary IgA response are less clear. To correct for changes in salivary flow rate, the ratio of Norwalk-specific IgA to total IgA was calculated. For ease of interpretation, this ratio is presented as a negative logarithm (Fig. 2). Unlike the serum IgG titers, salivary IgA did not display the characteristic increase around day 7 post-challenge, however, there

was a detectable response by day 14 which continued until the end of the study. Although there was clearly a trend toward higher specific responses in infected individuals, the final IgA measures (i.e. specific titer, total titer, and ratio of specific to total) were not significantly different between infected and uninfected individuals (Tables 2 and 3 and Fig. 2).

Bivariate analysis did not find a correlation between infection status and any of the pre-challenge antibody measurements (Table 3). Pre-challenge IgG titers were considered for both the full study (N=51) and the control group (N=22), to account for the possible confounding effect of the HPP treatment. Additionally, the pre-challenge IgG titer was not correlated with age, initial Norwalk-specific salivary IgA, nor the fold increase in IgG in infected volunteers (data not shown). Infection status was, however, strongly correlated with final (day 35) IgG titer and the related measure of seroconversion.

Similar results were obtained when the occurrence of symptomatic infection was considered. Symptomatic infection was defined as the presence of any two or more of the following, regardless of severity: diarrhea, vomiting, nausea, abdominal cramping, fever, myalgia, headache, chills, or fatigue. The frequency of asymptomatic infection among infected volunteers was not correlated with any of the initial antibody measures nor with final IgG titer (Table 4). Interestingly, symptomatic infection was correlated with the final total IgA titer, but not with the final Norwalk-specific IgA titer or the ratio of Norwalk-specific IgA to total IgA.

Finally, the duration of viral shedding was examined in relation to the antibody results. Although the duration of viral shedding varied considerably

among volunteers (mean, 19.3 days; minimum, 4; maximum, 34), it was not correlated with the magnitude of the seroconversion, which also varied considerably (Table 5). As seen with symptomatic infection, the final total, but not specific, salivary IgA titer was correlated with the duration of shedding, with longer shedding periods associated with higher final total IgA titers.

Discussion

Despite many outbreak and challenge studies, the immune response to human norovirus infection remains poorly understood. By examining the immune response of volunteers challenged with norovirus, both the temporal dynamics of the immune response and the extent of the response can be assessed. In the current study, the temporal dynamics of both the humoral IgG response and the salivary IgA response to norovirus were monitored. These responses could then be examined with respect to infection status, illness, and viral shedding.

The humoral response to Norwalk virus infection was consistent with the general IgG response to infection and that previously reported for norovirus (20), with seroconversion occurring around 7 days after the exposure and remaining high for the duration of followup (Fig. 1). The mucosal response was less consistent. Although the average salivary IgA level in infected individuals was higher than that in uninfected individuals 14 days after exposure (Fig. 2), this trend was not evident in the samples for any given individual. Unlike serum IgG, where paired samples would show a strong increase in infected individuals, for most of the volunteers, salivary IgA did not show a strong increase, rarely more

than 2-fold, which is within the normal variation for the ELISA. Further complicating the issue, increases in salivary IgA were not maintained, and the values for any volunteer fluctuated considerably over time.

The lack of correlation between the mucosal IgA response and infection is in contrast to previous reports (8, 9). There are several possible explanations for this discrepancy. There were some variations in the salivary IgA assay, including the conditions of heat inactivation, the sample storage conditions, the production method for the virus-like particles used as antigen, and the saliva dilutions tested. In addition, protease activity was detected in some of the saliva samples, which could have interfered with the ELISA. There was also variation in the exposure conditions for each study. Here, a known titer of Norwalk virus was delivered in HPP-treated oysters. In the other studies, Norwalk virus was delivered as a liquid inoculum (8, 9).

In general, prior immunity reduces host susceptibility to infection, and this concept is exploited to develop vaccines that prevent infection. However, studies of norovirus immunity have yielded conflicting results on this issue (21). In outbreak and challenge studies of Norwalk virus (6, 10, 12, 22) and Snow Mountain virus (12, 15), infection was not correlated with prior immunity. Similarly, the results of a recent clinical trial of a norovirus vaccine indicated that the total α-norovirus IgG response elicited by the vaccine was not predictive of infection, but a subset of the α-norovirus IgG, specifically that able to block carbohydrate binding, was predictive and protective (23, 24). In this study, initial humoral and mucosal immunity were not correlated with either infection status or symptoms. This lack of correlation was not due to homogeneity of these

measures; both serum IgG and salivary IgA (total and Norwalk-specific) varied over a fairly wide range of values (data not shown).

It is reasonable to hypothesize that individuals with a higher pre-challenge antibody level, either humoral or mucosal, would have a stronger response to infection, reflecting either a memory response or a generally stronger immune system. However, this was not supported by the data. Pre-challenge antibody levels were not correlated with the magnitude of the humoral response. Also, pre-challenge IgG levels were not correlated with age, as might be expected if prior exposures were responsible for the higher pre-challenge values. The pre-challenge serum IgG level was not correlated with the pre-challenge IgA levels, suggesting that the humoral and mucosal responses are independent of each other. This independence is also reflected in the lack of association between the humoral response and illness and duration of shedding, both of which are associated with the mucosal response, as measured by total salivary IgA (Tables 4 and 5).

The fact that symptomatic infection and duration of shedding are only associated with total IgA and not other immunological measures is unexpected but not unreasonable. IgA is the predominant immunoglobulin of the mucosal response (25). Norovirus replication occurs in the gut epithelium, where a mucosal response is more effective than a humoral response. However, it is unusual that the norovirus-specific response is not correlated with duration or symptomatic infection. It could be that the total IgA response is correlated with another component of the mucosal immune response, and it is this second component that is important during norovirus infection. Alternatively, the lack

of correlation could be due to sensitivity issues in the VLP-based salivary IgA ELISA. It is not known to what extent the VLPs differ from intact virions, but it is conceivable that such differences could cause changes in antibody binding.

The main limitation of this study, like all human challenge studies, is sample size. Detecting trends in highly variable measures, such as viral shedding and salivary IgA is difficult, if not impossible, in studies of this size. One must also consider the primary goal of the initial challenge study was to evaluate the efficacy of high pressure processing of oysters on norovirus infectivity. Thus, it is possible that the HPP-treated inocula produced immunologically unique infections and that uninfected-but-exposed individuals mounted some response to the virus. Finally, only Norwalk virus was used as an inoculum for this study, so the results may not be applicable to other norovirus strains.

Tables

Table 1. Characteristics of Volunteers by Infection Status.

	Uninfected N=35	**Infected N=16**	**Total N=51**
Mean Age (years)[a]	26.2 (18, 48)	25.7 (19, 51)	26.5 (18, 51)
% Male	29.4	13.7	43.1
Race[b]			
Asian	4 (7.8)	1 (2.0)	5 (9.8)
African American	15 (29.4)	6 (11.8)	21 (41.2)
Caucasian	13 (25.5)	7 (13.7)	20 (39.2)
Hispanic[c]	2 (3.9)	0 (0.0)	2 (3.9)
Multi-racial	0 (0.0)	1 (2.0)	1 (2.0)
Other	1 (2.0)	1 (2.0)	2 (3.9)
ABO Blood Type[b]			
Missing	3 (5.9)	4 (7.8)	7 (13.7)
A	10 (19.6)	3 (5.9)	13 (25.5)
B	4 (7.8)	0 (0.0)	4 (7.8)
AB	1 (2.0)	0 (0.0)	1 (2.0)
O	17 (33.3)	9 (17.7)	26 (51.0)
Secretor +[b]	35 (68.6)	16 (31.4)	51 (100.0)

[a] Number in parentheses is the range.
[b] Number of volunteers in each category. Number in parentheses is percent of the total study group in each category.
[c] Non-Caucasian Hispanic

Table 2. Distribution of Antibody Concentrations By Infection Status Over Time.

Antibody Measure[a]	Sample Day									
	1	2	3	4	5	7	14	21	28	35
α-Norwalk IgG										
Infected	38.4 (27.8)	37.6 (28.0)	34.9 (30.9)	36.0 (26.1)	30.3 (21.6)	80.7 (57.1)	269.5 (169.7)	305.8 (180.1)	323.3 (185.2)	338.3 (215.5)
Uninfected	39.0 (34.8)	ND	ND	ND	ND	40.0 (33.0)	ND	ND	ND	42.5 (33.3)
α-Norwalk IgA[b]										
Infected	0.35 (0.25)	0.51 (0.50)	0.29 (0.22)	0.25 (0.18)	0.45 (0.29)	0.33 (0.28)	0.37 (0.35)	0.50 (0.36)	0.40 (0.25)	0.39 (0.31)
Uninfected	0.37 (0.28)	0.45 (0.42)	0.34 (0.29)	0.39 (0.38)	0.28 (0.15)	0.33 (0.20)	0.41 (0.31)	0.42 (0.32)	0.37 (0.20)	0.31 (0.18)
Total IgA[b]										
Infected	52.4 (35.7)	93.3 (74.4)	87.8 (82.0)	67.2 (61.9)	91.7 (57.4)	56.8 (36.9)	64.7 (27.2)	74.4 (46.7)	71.7 (37.0)	69.2 (41.7)
Uninfected	54.4 (48.9)	44.5 (32.1)	42.8 (43.4)	57.9 (26.5)	53.8 (40.2)	63.7 (35.6)	37.2 (17.0)	47.7 (19.7)	38.3 (26.9)	41.2 (29.9)

[a] In µg/ml. Value is the mean. Number in parentheses is the standard deviation.

[b] Control group only (N=22)

Table 3. Bivariate Analysis for Correlates of Infection

Variable 1	**Variable 2**	**Test Statistic**	
		R-squared	**P value**
Infection status	Pre-challenge IgG titer	0.000083	0.95
	Pre-challenge IgG (controls only)	0.017752	0.58
	Pre-challenge NV-specific IgA[a]	0.008591	0.71
	Pre-challenge total IgA[a]	0.000670	0.92
	Final IgG titer	0.561861	<0.0001
	Final NV-specific IgA[a]	0.024457	0.56
	Final total IgA[a]	0.132666	0.15
		Chi-square	**P value**
Infection status	ABO blood group[b]	4.99	0.29
	ABO blood group[a]	1.20	0.75
	Seroconversion	36.56	<0.0001

[a] Control group only (N=22)
[b] Full study (N=51)

Table 4. Bivariate Analysis for Correlates of Symptomatic Infection[a]

Variable 1	**Variable 2**	**R-squared**	**P value**
Symptoms	Pre-challenge IgG titer	0.044428	0.43
	Final IgG titer	0.224416	0.07
	Pre-challenge NV-specific IgA[b]	0.013083	0.75
	Pre-challenge total IgA[b]	0.198694	0.20
	Final specific IgA[b]	0.217716	0.21
	Final total IgA[b]	0.499697	0.02

[a] Among infected volunteers only (N=16)
[b] Control group only (N=11)

Table 5. Bivariate Analysis for Correlates of Duration of Viral Shedding[a]

Variable 1	Variable 2	Pearson's r	P value
Duration of shedding	Magnitude of seroconversion	0.05779	0.84
	Final NV-specific IgA[b]	0.23584	0.54
	Final total IgA[b]	0.64967	0.04

[a] Among infected volunteers only (N=16)
[b] Control group only (N=11)

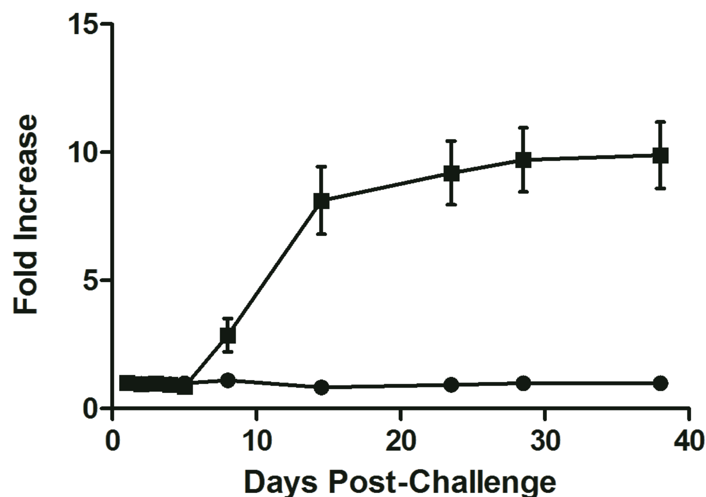

Figure 6. Temporal dynamics of serum IgG response to Norwalk virus infection. Serum IgG titers of Norwalk-specific IgG were determined by ELISA. The fold increase in titer was calculated by dividing each sample value by the pre-challenge value for that volunteer. The mean fold increase for infected and uninfected volunteers is plotted. Infection status was determined by the presence of Norwalk RNA in one or more stool samples. Circles, uninfected (N=35); squares, infected (N=16). Error bars represent the standard error.

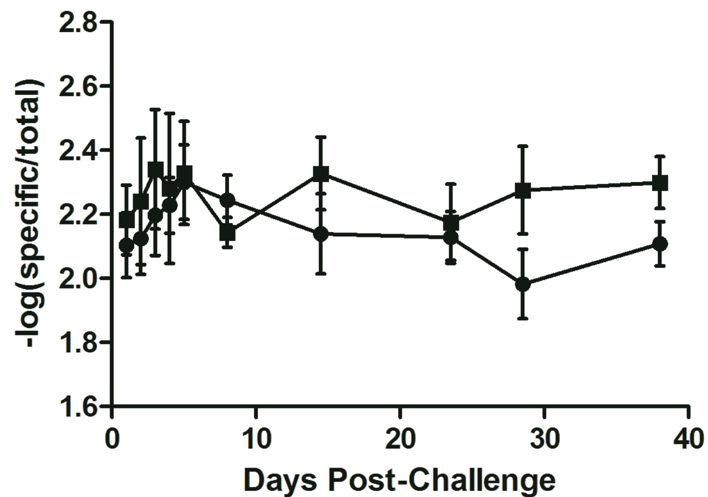

Figure 7. Infection with Norwalk virus did not induce a virus-specific salivary IgA response. Salivary Norwalk-specific and total IgA titers were determined by ELISA. To correct for changes in salivary flow, the negative logarithm of the ratio of Norwalk-specific IgA to total IgA is presented. The mean of this value for infected and uninfected volunteers in the control group is presented. Infection status was determined by the presence of Norwalk RNA in one or more stool samples. Circles, uninfected (N=11); squares, infected (N=11). Error bars represent the standard error.

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Public Health Implications

and

Future Directions

Public Health Implications

Understanding the immune response to norovirus is critical to the reducing the incidence and morbidity associated with this infection. Current estimates place the annual incidence of norovirus infection in the US at 21 million infections per year (1) or approximately 6,500 cases per 100,000 persons per year (2). Although the infection is generally mild, serious complications and even death can occur (3-6). Without an effective virus-specific treatment, intervention is dependent upon reducing the contagiousness of infected individuals (and by extension, their environment) and increasing the resistance of uninfected individuals. Knowledge of the immune response and its characteristics can inform both of these efforts through improved diagnostics, vaccines and, potentially, treatments which could reduce viral shedding. It will also allow validation of animal models that could be used to further development of these tools.

Currently, laboratory diagnosis of norovirus infection is by detection of the virus in stool, however, the diagnosis can also be made by serology. Although IgG seroconversion is very specific for an active infection, it is not used often. This is likely due to the greater technical difficulty of the assay and the relative difficulty of obtaining paired serum samples. There is some indication from this study and others (7, 8) that the salivary immune response may also be predictive of infection status. If this is the case, saliva samples could be used for diagnosis. The ease of collecting saliva samples, both on the part of the clinician and the patient, would be a great advantage. For epidemiological and clinical surveys, it

would allow greater sampling and compliance. For routine diagnostics and outbreak investigations, it would enable sample collection to be performed by untrained individuals, allowing the trained staff to better utilize their time.

The development of an effective norovirus vaccine will require a thorough understanding of the immune response to infection. In particular, the characteristics of a protective immune response must be defined so that they can be replicated by vaccination. The role of pre-existing antibodies in preventing infection is still unclear (9-12). However, some protection is conferred by infection (13-15), so there must be a component(s) of the immune response that is protective. Because norovirus is an infection of the mucosa, it is reasonable to expect that a protective response will include the mucosal immune system.

Long durations of high titer viral shedding are largely responsible for the highly contagious nature of norovirus, which, in concert with its environmental stability, makes halting outbreaks difficult. Any intervention that could decrease the duration or magnitude of viral shedding would be of great utility. In untreated infections, the virus is generally cleared within a month, as seen in this study and others (16-18). However, in immunocompromised individuals, the duration of viral shedding is much longer (19), indicating a role for the immune system in viral clearance. By understanding the characteristics of the immune response that are correlated with less viral shedding, it may be possible to design therapies that will modify the immune response and reduce viral shedding.

Although it is possible to develop diagnostics and therapeutics without an animal model of infection, it is more difficult. Human challenge studies are limited by cost, sample size, and ethical considerations. These limitations also

apply to animal models, but to a lesser extent. Thus, animal models are more amenable to the early, riskier stages of development. Animal models are also advantageous because prior exposure to the virus is known, where that cannot be conclusively determined for human challenge subjects. The biggest limitation of animal models is comparability to the human disease. To overcome this limitation, the animal model must be validated by comparison to the human disease course. To properly validate disease models like the chimpanzee model (20, 21), the factors determining the human immune response must be defined using studies such as this one.

Future directions

Moving forward, the priority for this work is to resolve the discrepancy between the findings in this study and those by Lindesmith et al. (7) regarding the salivary IgA response to infection. There are differences in the assay conditions - for example, storage conditions, tested dilutions, heat inactivation protocols – that can be explored using the archived specimens. Additionally, strong protease activity was detected in some of the saliva samples, and this activity was negatively correlated with detected α-norovirus antibody levels. Thus, it is possible that this enzymatic activity skewed the results of the assay. This hypothesis could be tested by further dilution of the saliva samples to reduce the protease concentration or the addition of protease inhibitors to the saliva samples. Protease activity was not detected in the saliva dilutions used for measuring total IgA, which were diluted 1:400 versus 1:4 for the specific antibody assay.

To better understand the genesis of the immune response, the serological IgA profile should also be measured. In this study, the predominant immunoglobulin class in each compartment (i.e. IgG in serum and IgA in saliva) was assessed. From these results, it appears that the humoral and mucosal responses are independent of each other, however, it is difficult to assess if the difference is due to the compartment (i.e. humoral vs. mucosal) or the immunoglobulin (i.e. IgG vs. IgA). Measuring the IgA in serum would allow a more direct and easily interpretable comparison between the humoral and mucosal responses. Salivary IgG could also be measured, however, this assay is more challenging due to the low concentration of IgG in the saliva.

To fully understand the immune response to norovirus infection, this type of study must be expanded to other strains of the virus, particularly GII.4 viruses, which are responsible for most infections (22). In addition to confirming the general characteristics of a norovirus-specific response, these studies will identify any strain-specific responses to infection. This data will be important for developing vaccines and assessing their efficacy.

Once the immune response to challenge with different strains of norovirus is better understood, it will be important to examine the characteristics of cross-reactive immune responses. For example, is the response to Norwalk virus different if the individual has previously been infected with a GII.4 virus? Since most people will be exposed to norovirus very early in life (23-25), the effect of previous exposures will need to be taken into account when developing immune-based therapies. Also, the components of a cross-reactive immune response that are protective need to be identified and characterized for future vaccine studies.

By exploiting effective cross-reactive responses, the number of strains in a vaccine can be reduced while still maximizing the utility of the vaccine.

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