Chimpanzees as an animal model for human norovirus infection and vaccine development

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Contributed by Robert H. Purcell, September 29, 2010 (sent for review May 26, 2010)

Noroviruses are global agents of acute gastroenteritis, but the development of control strategies has been hampered by the absence of a robust animal model. Studies in chimpanzees have played a key role in the characterization of several fastidious hepatitis viruses, and we investigated the feasibility of such studies for the noroviruses. Seronegative chimpanzees inoculated i.v. with the human norovirus strain Norwalk virus (NV) did not show clinical signs of gastroenteritis, but the onset and duration of virus shedding in stool and serum antibody responses were similar to that observed in humans. NV RNA was detected in intestinal and liver biopsies concurrent with the detection of viral shedding in stool, and NV antigen expression was observed in cells of the small intestinal lamina propria. Two infected chimpanzees rechallenged 4, 10, or 24 mo later with NV were resistant to reinfection, and the presence of NV-specific serum antibodies correlated with protection. We evaluated the immunogenicity and efficacy of virus-like particles (VLPs) derived from NV (genogroup I, GI) and MD145 (genogroup II, GII) noroviruses as vaccines. Chimpanzees vaccinated intramuscularly with GI VLPs were protected from NV infection when challenged 2 and 18 mo after vaccination, whereas chimpanzees that received GII VLPs vaccine or a placebo were not. This study establishes the chimpanzee as a viable animal model for the study of norovirus replication and immunity, and shows that NV VLP vaccines could induce protective homologous immunity even after extended periods of time.

Noroviruses are the most frequent cause of epidemic gastroenteritis (1) and responsible for over half of all gastroenteritis cases, in addition to causing as many as 200,000 deaths per year in developing countries (2). Research in the development of prevention strategies has been impaired because noroviruses causing human disease lack permissive cell-culture systems and robust animal models, leading to a continued dependence on human challenge studies to assess viral infection (3). Early human volunteer studies demonstrated that challenge with Norwalk virus (NV) conferred short-term but not long-term (>2 y) immunity to reinfection with the same virus (4–6). Moreover, cross-challenge studies with the serotypically distinct NV and Hawaii viruses [prototypes of the now-recognized genogroup I (GI) and genogroup II (GII) noroviruses, respectively], demonstrated the absence of heterotypic immunity (7). The mechanisms of host resistance to norovirus infection and disease are poorly understood, and such challenges demand new approaches for the evaluation of control strategies.

Human noroviruses are included in the genus *Norovirus*, which belongs to the family *Caliciviridae*, along with four other genera, *Nebovirus*, *Sapovirus, Vesivirus* and *Lagovirus.* Noroviruses form nonenveloped 30- to 35-nm virions with icosahedral symmetry that contain a 7.7-kb-long positive-sense single-stranded RNA genome (8). The RNA genome is organized into three ORFs (ORFs 1, 2, and 3). ORF1 encodes a large nonstructural polyprotein, and ORF2 and ORF3 encode the major (VP1) and minor (VP2) capsid proteins, respectively (8). The NV protruding (P) domain of the VP1 capsid protein was cocrystallized with certain saccharides of the histo-blood group antigens (HBGAs), following a proposed association of HBGA binding with viral entry into epithelial cells of the gastrointestinal tract (9). The amino acids involved in this interaction were identified, and two sites (interaction sites 1 and 2) that participate in trisaccharide A and B binding were mapped (10, 11).

Human volunteer studies to test the efficacy of potential norovirus vaccines are difficult to execute and depend on the availability of safety-tested norovirus inocula, which are characteristically 2% stool filtrates derived from previously infected volunteers. Animal models have been actively sought for the study of norovirus pathogenesis and immunity, but each animal model has limitations in the study of human noroviruses, such as short-term shedding and variable immune responses (12–16). Challenge of nonhuman primates, such as rhesus macaques and newborn pigtail macaques, with human norovirus strains results in only sporadic asymptomatic infections (13, 15). Chimpanzees were first described as permissive for asymptomatic NV infection by Wyatt et al. in 1978 (16). We chose to reevaluate chimpanzees as a viable model for human NV infection because they had been thoroughly studied as a model for other fastidious enteric viruses, such as hepatitis A and E virus (HAV, HEV) (17, 18), leading to crucial findings that aided the development of vaccines (19–21). The purpose of this study was to evaluate the chimpanzee as a model for the analysis of norovirus infection, pathogenesis, evolution within a host, and vaccine development with new molecular tools.

Results

Experimental Infection of Chimpanzees with NV. The chimpanzee animal model has played a key role in the study of several viral pathogens (22–30). During early HAV challenge experiments in nonhuman primates, it was determined that $10^{4.5}$-fold more virus was required to infect animals by the oral route compared with the i.v. route (31). Administration of HAV and other hepatitis viruses by the i.v. route became the standard method for virus challenge in chimpanzees, and these studies were instrumental in the development of a successful HAV vaccine (19–21). In addition, the i.v. route proved successful in the establishment of porcine enteric calicivirus infection in the gnotobiotic piglet model for sapoviruses (32). Prompted by these studies, we examined NV infectivity by inoculating chimpanzees i.v. with ascending concentrations of the NV inocula, starting with the highest dilution detected by qRT-PCR, to establish the lowest infectious dose via reverse titration. The i.v. route of adminis-

Author contributions: K.B., A.Z.K., S.V.S., R.H.P., and K.Y.G. designed research; K.B., G.I.P., T.M., E.A., C.K.S., D.B., R.E., C.Y., and S.V.S. performed research; K.B. and S.V.S. contributed new reagents/analytic tools; K.B., G.I.P., A.Z.K., S.V.S., R.H.P., and K.Y.G. analyzed data; and K.B., A.Z.K., R.H.P., and K.Y.G. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1014577107/-/DCSupplemental.

MICROBIOLOGY

Table 1. Summary of NV virus challenge studies in naïve chimpanzees

Chimpanzee ID	Inoculum: genome copies (CID*)	Day of onset of shedding, dpi	Duration of shedding, days	Peak shedding titer, genome copies/g stool	Peak seroconversion, EIA titer		Peak virus titer in endoscope biopsies (genome copies/100 ng of RNA†)	Peak virus titer in liver biopsies (genome copies/100 ng of RNA†)
					Week postinfection	Peak		
1622	4×10^6 (0.1)	3	23	3×10^7	6	1:1,600	NC	NC
1628	4×10^7 (1)	4	17	3×10^7	2	1:800	NC	NC
A3A023	4×10^7 (1)	5	22	6×10^6	3	1:3,200	22 (j/21 dpi)	NC
A3A008	4×10^8 (10)	3	42	6×10^7	3	1:25,600	1×10^6 (j/4 dpi)	NC
A2A014	4×10^7 (1)	3	31	8×10^8	4	1:3,200	3.5×10^4 (d/11 dpi)	6×10^2 (l/7 dpi)
A3A007	4×10^7 (1)	2	22	3×10^9	4	1:6,400	7×10^2 (d/ 9 dpi)	1.3×10^2 (l/7 dpi)

NC, no sample was collected for that chimpanzee.

*CID, chimpanzee infectious dose. The original inoculum contained 10 CID.

†In cases where more than one biopsy is NV positive, the highest genome copies/biopsy titer is reported, and the tissue of origin (j, jejunum; d, duodenum; l, liver)/date of collection (dpi) are reported in parentheses.

tration proved successful, and one chimpanzee infectious dose (CID) was established as 4×10^7 genome copies. Six chimpanzees inoculated i.v. with 0.1–10 CID of NV developed subclinical infections as determined by virus shedding in stools and a serum antibody response to the virus (Table 1). The chimpanzees shed virus in stools beginning 2–5 d postinoculation (dpi) and lasting 17 d to 6 wk. A typical pattern of virus shedding in stools is represented in the analysis of chimpanzee A2A014 (Fig. 1). The peak of genome copies in stool was equal to or higher than the viral genome copies present in the original inoculum in four of six chimpanzees. Although the chimpanzees were inoculated i.v., there was no evidence of viremia by real-time qRT-PCR in any of the chimpanzees studied, even in serum samples collected at 0, 3, 5, 7, 11, 14, and 21 dpi (CH A2A014). Moreover, NV genomic RNA was not detected in PBMCs collected from infected chimpanzees. No elevation of hepatic enzymes was detected throughout the study.

Serologic Response to NV Infection. Two weeks after infection all of the infected naïve chimpanzees (serum antibody <1:100 prechallenge) developed NV-specific antibodies that lasted for the duration of the study (4 wk after shedding was no longer detected in stools; Table 1). Antibody titers ranged from 1:800 to 1:25,600, the latter observed in the chimpanzee that received 10 CIDs (the highest CID administered; Table 1). The serum antibody response to NV infection consisted mostly of IgM and IgG, as illustrated by analysis of chimpanzee A2A014, compared with the viral genome copies present in stools (Fig. 1*A*). IgA isotype was present at very low levels or was undetectable, both in serum and stool samples.

Presence of NV in Biopsy Tissues. To study the histopathology of the gut of inoculated chimpanzees and identify infected cells, endoscopic biopsies from the jejunum and duodenum were obtained. No histological changes were observed between biopsies obtained before and after NV infection. However, viral genome was detected in the jejunum and duodenum biopsies of 4/4 chimpanzees studied (Table 1). This was detected in endoscopic biopsies from chimpanzee A2A014 for up to 21 dpi at concentrations lower than 3.5×10^4 genome copies/100 ng of RNA (Fig. 1*B*). Surprisingly, NV RNA was also detected in liver of two chimpanzees, although at lower concentrations than in gut (Table 1). The RNA was detected in liver for extended periods of time, up to 21 dpi (Fig. 1*B*).

Norwalk virus antigen expression in tissue was examined by immunofluorescence. At day 4, NV antigen was observed in the cytoplasm of cells of the lamina propria, both in jejunum and duodenum of chimpanzee A3A008, whereas no positive cells were observed in either biopsy before inoculation (Fig. 2). Norovirus (GII.4) antigen was previously observed in enterocytes in fresh fixed tissue of gnotobiotic piglets infected orally (12, 33);

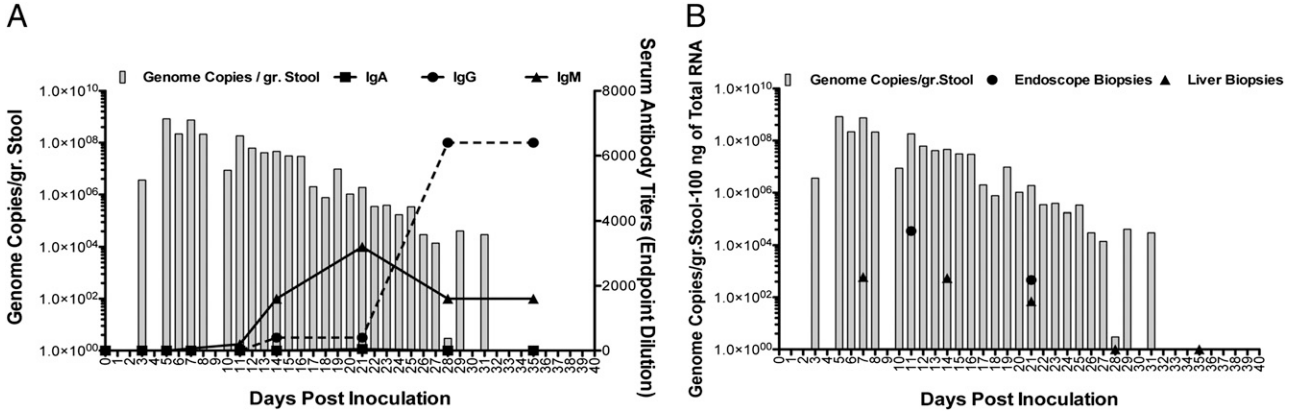

Fig. 1. Norwalk virus infection in chimpanzee A2A014. (*A*) Serum antibody isotype titers in chimpanzee A2A014. Titer of IgG, IgM, and IgA anti-NV (*Right y* axis) developed after NV virus infection, compared with norovirus shedding in stools (*Left y* axis). (*B*) Norovirus genome copies in biopsy samples from chimpanzee A2A014. The genome copies per 100 ng of total RNA in endoscope (duodenum, 11 dpi; jejunum, 21 dpi) and liver (7, 14, and 21 dpi) biopsies are shown in relationship to the titer of norovirus shedding in stools.

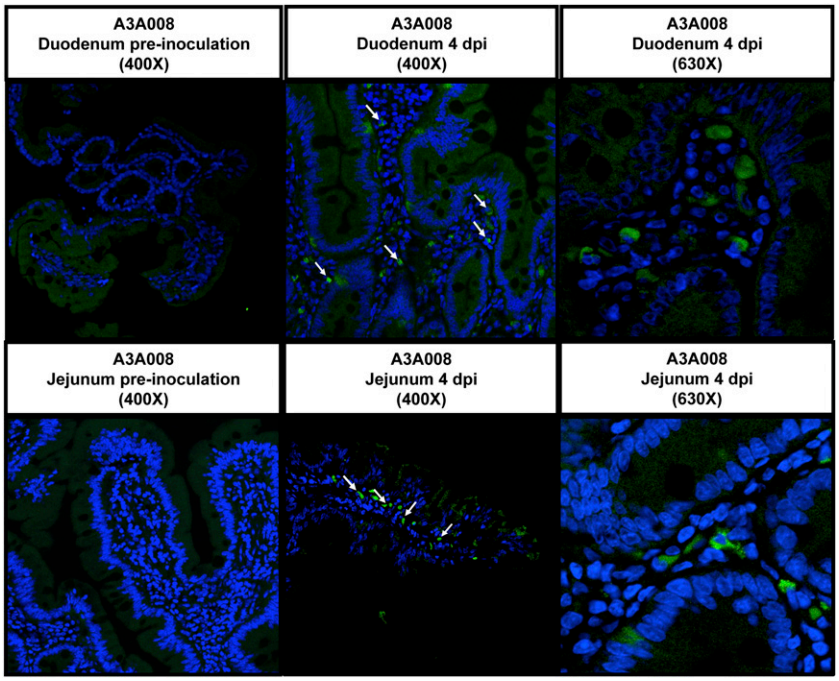

Fig. 2. Immunohistochemistry of biopsy samples of duodenum and jejunum in chimpanzee A3A008. Pre-inoculation and 4-dpi endoscopic biopsies were incubated with a monoclonal anti-NV antibody labeled with FITC (green). Cell nuclei are shown with DAPI staining (blue). White arrows indicate representative NV-positive cells at 400×.

however, NV (GI.1) was observed in cells of the lamina propria in a 24-hpi human biopsy from a NV-infected patient (34).

Characterization of NV-Infected Cells in Duodenum. To further characterize the cells in the lamina propria that showed evidence of NV infection, several cellular markers, including those specific for macrophages, B lymphocytes, CD4 lymphocytes, and dendritic cells, were examined (Fig. S1). The NV capsid-specific signal did not colocalize with cells that expressed CD68 (macrophages) and CD4 (Fig. S1 *A* and *B*). However, many (but not all) cells in the lamina propria of the duodenum that expressed DC-SIGN (dendritic cells) showed evidence of colocalization with NV capsid expression (Fig. S1 *C–E*). The presence of NV colocalization was detected also in B lymphocytes (Fig. S1 *F–H*).

Evolution of NV Within a Host. Infected chimpanzees shed the virus in feces for prolonged periods of time, allowing us to monitor changes in the NV genomic sequence as the infection progressed. We obtained the consensus sequence of the genome of NV in the original inoculum and in stool after 12, 20, and 34 dpi in four chimpanzees to assess whether changes in the predominant consensus sequence might occur over time (Fig. S2). The majority of changes occurred in the capsid-encoding region (12/22), and most of them corresponded to nonsynonymous substitutions (9/12). In contrast, 10 of 22 changes were found in the genomic region coding for nonstructural proteins, but only four of them corresponded to amino acid substitutions. The capsid sequence changes varied in position among the chimpanzees, with the exception of nucleotide position 6499. An A-to-G nucleotide change resulted in a histidine-to-arginine amino acid substitution at position 381 in the protruding region of the viral capsid protein in two different chimpanzees at 20 and 34 dpi. We modeled the location of the amino acid position (aa 381) onto the solved structure of the NV capsid (Fig. S3). The residue is predicted to lie immediately adjacent to the HBGA binding sites exposed on the surface of the viral capsid. The remaining changes of the sequences coding for the viral capsid observed in the infected chimpanzees were mapped also onto the NV capsid structure (Fig. S3). Most of the amino acid substitutions correspond to surface-exposed residues.

Protection of Chimpanzees Against Reinfection. Humans experience many norovirus infections during their lifetime, and correlates of protection against reinfection have been difficult to establish in volunteer studies (8). Humoral immunity elicited from vaccination with norovirus virus-like particles (VLPs) resulted in either lower viral loads or protection against diarrhea following challenge in mice and gnotobiotic piglets, respectively (35, 36). However, protection against reinfection after viral infection or immunization with VLPs has not been shown in humans. One group of chimpanzees was challenged with NV to determine the level of protection against challenge after an initial infection event (Table 2). The group included a control chimpanzee that showed no serological evidence of previous infection with NV [<100 antibody titer by enzyme immunoassay (EIA)]. Chimpanzees that had been previously infected with NV were resistant to reinfection (defined by the absence of NV shedding in stool and of a measurable serologic response to NV) after challenge with the same virus. One chimpanzee was rechallenged sequentially at 4 and 24 mo after the initial challenge, and another chimpanzee was challenged 10 mo after an initial challenge at 0 mo. The antibody titer at the time of challenge varied from 1:800 to 1:3,200, with the lowest titer found in the chimpanzee that had been infected a second time 20 mo previously.

Vaccination of Chimpanzees and Subsequent Challenge with NV. Once it was established that chimpanzees could be protected against reinfection, three additional studies were performed to test whether homologous and heterologous immunization with VLPs would also protect against challenge with NV. Each study protocol included two vaccinated chimpanzees and one placebo or control chimpanzee. Chimpanzees were vaccinated with two doses of either GI or GII VLPs or with placebo, and challenged with NV (1 CID) 1 mo (GI and GII) and 18 mo (GI) after the second dose (Table 2). Chimpanzees receiving either GI or GII VLPs developed a strong serum antibody response to the immunizing VLP after vaccination (≥1:50,000). Chimpanzees vaccinated with the homologous GI VLPs were protected against infection (NV shedding in stools) when challenged with NV either 1 or 18 mo after the second vaccine dose. In contrast, chimpanzees vaccinated with GII VLPs or placebo were susceptible to NV infection fol-

Table 2. Summary of NV virus challenge and rechallenge studies in naïve and vaccinated chimpanzees

Study	Chimpanzee ID	Initial inoculum	Challenge with NV*, mo p.i./p.v.	EIA antibody titer at time of challenge against VLP†	Outcome of challenge
NV (GI) rechallenge	A3A023	NV	4	1:3,200 (NV)	Protected
after initial NV	A3A008	NV	10	1:1,600 (NV)	Protected
Infection	A3A023	NV	24	1:800 (NV)	Protected
	A2A014	Seroneg. control	—	<100 (NV)	Infected
NV (GI) challenge after	AOA007	NV VLP	2	1:100,000 (NV)	Protected
NV VLP vaccination	AOA006	NV VLP	2	1:50,000 (NV)	Protected
	A3A007	Placebo control	2	<100 (NV)	Infected
NV (GI) rechallenge	AOA007	NV VLP	18	1:12,800 (NV)	Protected
after NV VLP	AOA006	NV VLP	18	1:3,200 (NV)	Protected
vaccination	A6A007	Seronegative control	—	<100 (NV)	Infected
NV challenge after MD	A6A014	MD VLP	2	1:100,000 (MD)	Infected
(GII) vaccination	A5A010	MD VLP	2	1:100,000 (MD)	Infected
	4X0533	Placebo control	2	<100 (MD)	Infected

*All chimpanzees were inoculated with 1CID.
†EIA antibody titer determined against VLP, which is indicated within the parenthesis next to the titer value. NV, Norwalk virus; MD, MD145 norovirus.

lowing challenge. The infection of GII VLP-vaccinated chimpanzees did not differ from that of naïvely infected chimpanzees in regard to duration and viral load of norovirus shedding in stools. Although the GII VLP-vaccinated chimpanzees developed high titers of serum antibodies specific for MD145 (GII) after vaccination, the antibodies did not cross-react with NV and did not protect. However, a serological response to NV was detected 2 wk after infection with NV (Fig. S4).

HBGA Binding Blocking Assay Using Sera from Vaccinated Chimpanzees. The HBGA binding blocking assay has been used as a surrogate of antibody-mediated neutralization for noroviruses (35, 37, 38). To test the correlation of this assay with the outcome of challenge studies in chimpanzees after GI or GII VLP vaccination, sera obtained at prevaccination and 60 d postvaccination (dpv) were assessed for their ability to block the binding of NV VLPs to H3 carbohydrate. The sera from chimpanzees vaccinated with GII VLPs or placebo, which were susceptible to infection after challenge with NV, did not inhibit NV VLP binding to H3 at dilutions ranging from 1×10^{-2} to 1.25×10^{-3} (Fig. 3). However, both chimpanzees vaccinated with NV VLPs, that were protected against infection after challenge with NV, developed antibodies that blocked the binding of NV VLP to H3 carbohydrate at dilutions ranging from 1×10^{-2} to 1.25×10^{-3} (Fig. 3). These findings suggest that the HBGA blocking assay might be suitable as a surrogate for norovirus neutralization.

Discussion

Increasing recognition of the role of noroviruses as important diarrheal pathogens, the lack of preventive measures, and the need to establish whether a monovalent norovirus vaccine would provide protection against two different genogroups prompted us to examine whether the chimpanzee could serve as a model in which to evaluate vaccine candidates. We showed that chimpanzees could be reproducibly infected with NV, and that the animals shed NV for 2 wk or longer. Naïve animals developed a serum antibody response following experimental infection with NV or immunization with norovirus VLPs, and the presence of serum antibodies correlated with resistance to infection. Moreover, three chimpanzees showed evidence for longer-term immunity when they continued to resist infection when rechallenged 18–24 mo later. However, immunization with a heterologous norovirus vaccine candidate (GII.4) did not confer protection against subsequent NV challenge, suggesting that the antibodies generated were not cross-protective between what is thought to be two distinct norovirus serotypes. This study shows that the development of an antibody response against norovirus through vaccination with VLPs confers resistance to reinfection with the homologous virus. It remains to be determined whether the NV GI VLPs confer protection against other genetic variants within the same genogroup.

It has been difficult to establish the role of neutralizing antibodies in the absence of a cell culture system. We showed that seroconversion to norovirus in chimpanzees may correlate with resistance to reinfection by the same virus. In addition, the HBGA blocking profile of sera from chimpanzees protected (vaccinated with GI) or susceptible (vaccinated with GII) to NV challenge correlated with the outcome of the vaccine efficacy studies. These findings are in agreement with recent reports that sera from NV-infected human volunteers had also the ability to block binding of NV to H type 3 carbohydrates (38). These data suggest that the HGBA binding sites either form or are near protective epitopes on the surface of the virion, and demonstrate that this assay may be applicable as a surrogate of antibody-mediated neutralization for noroviruses (35, 37, 38).

All challenged chimpanzees in this study with no history of previous exposure to NV were infected, although no diarrhea or

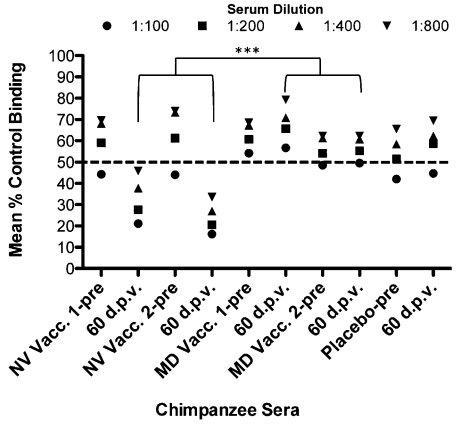

Fig. 3. Detection of HBGA blocking antibodies in sera from vaccinated chimpanzees. Sera from NV (GI) and MD145 (GII) vaccinated chimpanzees before and 60 dpv were tested for the ability to block the interaction between recombinant NV VLPs and H3 carbohydrates. A chimpanzee that was administered placebo was included as a control. The difference in BT50 (blocking titer 50%) between 60-dpv NV-vaccinated and MD145-vaccinated chimpanzees was significant (***$P < 0.001$).

histopathological changes of the gut tissue were observed. Moreover, all chimpanzees developed a specific serum antibody response to NV. Although chimpanzees did not show symptoms of disease, infected chimpanzees showed shedding patterns in feces of similar duration and titer as those observed in humans, as well as comparable antibody titers in serum samples (39). We did not detect evidence of NV (GI) viremia by the analysis of sera or PBMCs, even in samples collected soon after i.v. inoculation. These findings differ from those reported in certain animal models in which GII norovirus viremia was found in a subset of inoculated animals (12, 33). It was reported also that GII noroviruses were detected at very low levels in sera from ≈10% of GII norovirus-infected patients (40). Further study of norovirus pathogenesis is needed to establish whether viremia is a common feature of norovirus spread within the host.

The viral genome and VP1 capsid antigen were both detected in biopsy tissue using real-time RT-PCR and immunohistochemistry, respectively. NV-infected cells were located exclusively in the lamina propria. Both sections of the chimpanzee small intestine that were examined, the jejunum and duodenum, were infected and showed similar levels of virus genome, although the jejunum was typically infected for longer periods of time in most chimpanzees. In addition, this study provides evidence of virus in liver tissue, even in the absence of detectable viremia. The implications of this finding in the biology of the infection remain to be elucidated.

Further characterization of infected cells in jejunum and duodenum indicated that norovirus was present in cells from the dendritic cell lineage. DC-specific C-type lectin DC-SIGN has been identified as a pathogen receptor with broad specificity for viruses such as HIV, Ebola, and CMV, among others (41). However, it was recently reported that NV does not replicate in dendritic cells derived from human PBMCs (34), suggesting that NV might replicate in a subset of intestinal DC-SIGN–positive cells. Further analysis of the susceptibility to NV virus infection of DC-SIGN–positive cells is in progress.

Changes in the norovirus genome over time have been previously described in samples collected from immunocompromised patients infected with the virus, but not during the entire course of an infection (42). Most of the nonsynonymous amino acid changes observed 12–34 dpi were located in surface-exposed regions of the outermost viral capsid region, P2. This supports the theory that the virus continually evolves in an attempt to elude the host immune response. Furthermore, the observation of the same amino acid substitution in two independently infected chimpanzees suggests that there are preferred mutations (specific amino acid and location changes) that might help the virus evade the host immune response while providing the least possible impact in either viral fitness or other properties. In addition, this amino acid is located adjacent to the HBGA binding site for NV virus, although this particular site had not been implicated in HBGA binding (43).

Our findings in this study show that NV can infect chimpanzees, resulting in a similar duration of shedding and level of replication as observed in humans, albeit in the absence of clinical disease. We demonstrated a correlation between the presence of antibodies in sera and protection from reinfection, although the i.v. challenge route used in this study differs from that of a natural oral infection, and we were able to show protection after homologous but not heterologous vaccination. Characterization of the cells infected with virus might advance the development of a sustainable cell culture system for this virus. Future goals include infection of chimpanzees with additional norovirus strains, further evaluation of vaccine candidates or therapeutic treatments, and characterization of the host response to infection (and vaccination) at the molecular level. Equally important, challenge of chimpanzees with virus in late-shedding stool samples will determine if the virus is indeed adapting to replication in chimpanzees. Overall, the chimpanzee animal model should be an effective surrogate for human challenge studies and facilitate advances in the development of norovirus prevention and control strategies.

Materials and Methods

Norovirus Infection in Chimpanzees. The chimpanzees were individually housed at Bioqual Laboratories, an American Association for Accreditation of Laboratory Animal Care International-accredited institution under contract to the National Institute of Allergy and Infectious Diseases. In addition, the experimentation protocol was reviewed and approved by the National Institutes of Health Animal Care and Use Committee. Juvenile chimpanzees (2–8 y of age) typed as secretor positive, blood group A, and with no preexisting antibodies to NV were administered i.v. 1 mL of NV stool filtrate of human origin, designated 8FIIa (4×10^8 genome copies/mL) (44). Stool samples from chimpanzees were collected daily from 1 d preinoculation until 1 wk after the virus genome was no longer detected in stools as measured by qRT-PCR (45). Chimpanzees were observed for the appearance of clinical signs of gastroenteritis, including vomiting, diarrhea, and malaise, and were monitored weekly for elevation of hepatic enzymes. Whole-blood samples were collected preinoculation and every other week until the termination of the study (4 wk after norovirus shedding in stools was no longer detected). PBMCs were isolated on Ficoll-Histopaque density gradients (Sigma Chemical Co.), washed three times in HBSS. More than 1×10^7 cells were isolated and tested in total. Serum and liver biopsies fixed on formaldehyde or snap frozen with or without optimal cutting temperature compound (OCT) were collected preinoculation and weekly when indicated. Endoscopic biopsies of jejunum and duodenum were collected in TRIzol (Invitrogen), as well as processed in the same manner as the liver biopsies at the following times: preinoculation, 1–2 wk postinoculation (pi), 3 wk pi, after virus was no longer detected in stools, and later during the convalescent period. The rechallenge studies were conducted similarly to the initial challenge studies, with the addition for each group of a control chimpanzee that had no preexisting antibodies to NV.

Detection of Antibodies to Noroviruses in Sera and HBGA Blocking Binding Assay. Anti-norovirus (NV and MD145) antibodies were detected in sera by testing serial twofold dilutions in an EIA with norovirus VLPs as antigen, and performed similarly to that previously described by Jiang et al. (46). The HBGA blocking binding assay was performed as described in Table S1 and *SI Materials and Methods*.

Detection and Sequencing of NV RNA. Norovirus RNA extraction and sequencing is detailed in *SI Materials and Methods*.

Immunohistochemistry on Paraffin-Embedded Tissue. Additional pieces of intestinal and liver biopsies were stored in a 10% formaldehyde solution for the duration of the study. Tissue samples were embedded in paraffin, and sections were placed on positively charged glass slides (Fisher Scientific). Slides were deparaffinized and then washed twice in Dulbecco's PBS (Invitrogen), air-dried, and treated with proteinase K (DAKO) for 30 min to expose the antigens. Following three more 5-min washes in Dulbecco's PBS, a NV capsid-specific FITC or Alexa 594-labeled monoclonal antibody (NV-10, 1 mg/mL) was added at a 1:250 dilution in Dulbecco's PBS with 5% FBS, and incubated overnight at 4 °C. Slides were washed three more times, mounted using ProLong Gold anti-fade reagent with DAPI (Invitrogen), and observed under a confocal microscope 24 h later.

Several cell markers were used also in combination with NV-10 to further characterize the norovirus-infected cells. Briefly, duodenum slides were incubated overnight with a combination of FITC-labeled monoclonal antibodies to CD68 (Invitrogen), CD20 (Lifespan Biosciences), CD4 (Lifespan Biosciences), and DC-SIGN (Dendritics) in the dilutions recommended by the manufacturers, and Alexa 594-labeled NV-10. Slides were then washed and mounted as described previously.

Residue Mapping on Protein Structure. The amino acid differences between the inoculum and norovirus shed in stool by chimpanzees at various days postinoculation were inserted into the pdb files for the corresponding NV capsid protein structure (1IHM) (47). The resulting capsid protein structure was visualized using PyMOL (DeLano Scientific, LLC).

Preparation of VLP-Based Vaccine and Immunization. NV (GI) and MD145 (GII) VLP-based vaccines were prepared as follows: NV and MD145 VLPs containing VP1 and VP2 capsid proteins were expressed in a baculovirus system as de-

scribed previously (48). The VLPs were purified using a combination of sucrose and CsCl gradients and dialyzed in PBS overnight. The vaccine (50 μg per dose) was prepared by mixing 300 μg of VLP solution in 5.308 mL of Tris buffer [0.05M Tris (pH 7.8), 0.14 M NaCl] for the vaccine, or 5.308 mL of Tris alone for the placebo, with 0.692 mL of Alhydrogel (Brenntag Biosector) added to both vaccine and placebo for a total of 6 mL. The mixture was incubated at 4 °C overnight, and centrifuged at 1,500 rpm for 5 min (49). The pellet was resuspended in PBS for a final volume of 6 mL and stored at 4 °C in 1-mL aliquots. Two groups of three chimpanzees (two vaccinated and one placebo) were administered i.m. 1 mL of GI (NV) or GII (MD145) vaccine, respectively, or placebo at 0 and 30 d. The chimpanzees were challenged by the i.v. route at 60 dpv using the same NV inoculum described above. Stool, serum, and biopsy samples were collected at the same time intervals as chimpanzee challenge studies.

ACKNOWLEDGMENTS. We thank Dr. Anthony Cook and Jake Yalley-Ogunro for their expert assistance with the animal protocols. We are also very grateful to Alicia Brockington for support in conducting these studies. This research was funded by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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