Adjuvanted Intranasal Norwalk Virus-Like Particle Vaccine Elicits Antibodies and Antibody-Secreting Cells That Express Homing Receptors for Mucosal and Peripheral Lymphoid Tissues

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(See the editorial commentary by Vinjé, on pages 1623–1625.)

Background. Noroviruses cause significant morbidity and mortality from acute gastroenteritis in all age groups worldwide.

Methods. We conducted 2 phase 1 double-blind, controlled studies of a virus-like particle (VLP) vaccine derived from norovirus GI.1 genotype adjuvanted with monophosphoryl lipid A (MPL) and the mucoadherent chitosan. Healthy subjects 18–49 years of age were randomized to 2 doses of intranasal Norwalk VLP vaccine or controls 21 days apart. Study 1 evaluated 5-, 15-, and 50-μg dosages of Norwalk antigen, and study 2 evaluated 50- and 100-μg dosages. Volunteers recorded symptoms for 7 days after dosing, and safety was followed up for 180 days. Blood samples were collected for serological profile, antibody secreting cells (ASCs), and analysis of ASC homing receptors.

Results. The most common symptoms were nasal stuffiness, discharge, and sneezing. No vaccine-related serious adverse events occurred. Norwalk VLP-specific immunoglobulin G and immunoglobulin A antibodies increased 4.8- and 9.1-fold, respectively, for the 100-μg dosage level. All subjects tested who received the 50- or 100-μg vaccine dose developed immunoglobulin A ASCs. These cells expressed molecules associated with homing to mucosal and peripheral lymphoid tissues.

Conclusions. The intranasal monovalent adjuvanted Norwalk VLP vaccine was well tolerated and highly immunogenic and is a candidate for additional study.

Trial Registration. ClinicalTrials.gov identifier: NCT00806962.

Noroviruses (NoV), a member of the family *Caliciviridae*, cause acute epidemic gastroenteritis in humans of all ages in both developed and developing countries [1]. The Norwalk agent was the first virus identified in this family, and sequencing of its RNA classified it as a new genus designated "norovirus." Noroviruses are

genetically variable and include 25 genotypes that infect humans. The prototype Norwalk virus is a member of genogroup I, genotype 1 (GI.1). Currently, genogroup II, genotype 4 (GII.4) viruses are responsible for most outbreaks. Although gastroenteritis is short-lived in healthy individuals, it has a significant socioeconomic toll on businesses, hospitals, schools, and other settings [2]. Noroviruses are highly infectious and spread rapidly by person-to-person contact with aerosol droplets, environmental surfaces, and ingestion of contaminated foods, especially in hospitals, nursing homes, university campuses, military barracks, and cruise ships [3–7]. Recent estimates indicate that >90% of nonbacterial gastroenteritis outbreaks in the United States are caused by noroviruses [8, 9]. Recently, the Centers for Disease Control and Prevention estimated that 23 million cases occur annually in the United States, and up to 200,000 deaths of children <5 years old occur each year in developing countries [10].

A major development in norovirus research came with the successful cloning, sequencing, and expression of noroviruses in insect cells using baculovirus recombinants with the major capsid protein (VP1) that spontaneously folds into virus-like particles (VLPs) [11, 12]. Lacking a viral genomic RNA, these noninfectious VLPs have a preserved antigen conformation and interact with cellular receptors, eliciting a strong host immune response [11, 12]. Norovirus VLPs are highly immunogenic when given to animals parenterally, orally, or intranasally without adjuvant [13, 14] and are stable following lyophilization and when exposed to acid (pH 2.5). In phase 1 studies, VLPs administered orally without adjuvant or in edible transgenic plants were safe but only modestly immunogenic as measured by serum antibody and specific antibody-secreting cells (ASCs) [5, 15, 16]. The purpose of these studies was to determine the safety and immunogenicity of adjuvanted Norwalk VLP vaccine administered intranasally for the first time to humans.

METHODS

Vaccine

Norwalk VLPs, derived from norovirus GI.1 genotype, were prepared under good manufacturing practice at Protein Sciences, Inc. The vaccine consists of (1) Norwalk VLPs produced by a recombinant baculovirus expression system; (2) monophosphoryl lipid A (MPL) adjuvant (GlaxoSmithKline Pharmaceuticals Inc), a Toll-like receptor 4 (TLR-4) agonist, derived from detoxified *Salmonella minnesota* lipopolysaccharide; (3) chitosan (ChiSys; Archimedes Development Ltd), a linear polysaccharide produced by alkaline hydrolysis (deacetylation) of chitin from shrimp shells, a mucoadhesive to nasal mucus and epithelial cells prolonging antigen adherence [17]; and (4) sucrose and mannitol excipients as bulking agents and preservatives to stabilize VLP structure during lyophilization. The

vaccine was formulated under good manufacturing practice as a dry powder by Archimedes Development, Ltd.

Vaccine was administered intranasally using Bespak UniDose DP delivery devices (Milton Keynes). A dose of vaccine consisted of 2 loaded Bespak devices discharging 10 mg of dry powder vaccine formulation into each nostril for a total dose of 20 mg.

Study Design

Two phase 1 clinical studies were performed. Study 1 was a step-wise, dosage escalation trial, with safety reviews before each dose escalation. Study 2 was a dose comparison study of the 2 highest dosages. Subjects were 18–49-year-old healthy H type 1 secretor adults, given that only individuals with blood H type 1 antigen are susceptible to Norwalk infection [18]. Study 2 also excluded subjects with blood types B or AB because those individuals were reported to be less susceptible to Norwalk infection [19]. Subjects were counseled to ensure comprehension of the study and the risks, benefits, and procedures involved. The respective Institutional Review Boards approved the studies, and all subjects provided informed signed consent.

Study 1 was a single-site (University of Maryland), randomized, double-blind study of 3 dosage levels of adjuvanted Norwalk VLP vaccine (Norwalk VLP vaccine, MPL, and chitosan) compared with adjuvant control (MPL and chitosan). Twenty-eight sequentially randomized adults received 2 intranasal doses of (1) 5 µg of Norwalk VLP vaccine ($n = 5$) or adjuvant control ($n = 2$), (2) 15 µg of Norwalk VLP vaccine ($n = 5$) or adjuvant control ($n = 2$), or (3) 50 µg of Norwalk VLP vaccine ($n = 10$) or adjuvant control ($n = 4$). The 2 doses were separated by 21 days. The vaccine and control preparations were administered with Bespak intranasal delivery devices.

Study 2 was a multicenter, randomized, double-blind study at 4 sites. Sixty-one healthy adults were enrolled and randomized 2:2:1:1, respectively, to receive either 2 doses of 50 µg of Norwalk VLP vaccine ($n = 20$), 100 µg of Norwalk VLP vaccine ($n = 20$), adjuvant control ($n = 10$), or true placebo ($n = 11$) consisting of a puff of air (no dry powder). All doses were administered intranasally with the Bespak device. The 2 doses were separated by 21 days.

Safety Evaluation

Safety was assessed after each dose by review of a 7-day symptom record for interim medical histories and clinical laboratory tests. The following 7 local symptoms were sought: runny nose and/or nasal discharge, nasal pain and/or discomfort, nasal stuffiness and/or congestion, nasal itching, sneezing, blood-tinged nasal mucus, nasal bleeding, and the following 8 systemic symptoms: headache, fever, malaise, nausea, vomiting, abdominal cramps and/or pain, diarrhea, and loss of appetite. In addition to these solicited adverse events, unsolicited adverse

Table 1. Frequency of Solicited Adverse Events Reported during the First 7 Days after Administration of Vaccine or Placebo in Study 2, by Group

| Solicited event | 50 µg of Norwalk vaccine (n = 20) | | | | 100 µg of Norwalk vaccine (n = 20) | | | | MPL plus chitosan control (n = 10) | | | | Placebo control (n = 11) | | | |
| | After V1 | | After V2 | | After V1 | | After V2 | | After V1 | | After V2 | | After V1 | | After V2 | |
	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI
Systemic symptoms																
Elevated oral temp	0	0.0–16.8	0	0.0–18.5	0	0.0–16.8	0	0.0–17.6	0	0.0–30.8	0	0.0–33.6	0	0.0–28.5	0	0.0–28.5
Fatigue/malaise	3 (15.0)	3.2–37.9	2 (11.1)	1.4–34.7	4 (20.0)	5.7–43.7	5 (26.3)	9.1–51.2	2 (20.0)	2.5–55.6	1 (11.1)	0.3–48.2	1 (9.1)	0.2–41.3	1 (9.1)	0.2–41.3
Headache	7 (35.0)	(15.4–59.2)	5 (27.8)	9.7–53.5	7 (35.0)	(15.4–59.2)	9 (47.4)	(24.4–71.1)	3 (30.0)	6.7–65.2	2 (22.2)	2.8–60.0	4 (36.4)	(10.9–69.2)	2 (18.2)	2.3–51.8
Nausea	2 (10.0)	1.2–31.7	0	0.0–18.5	3 (15.0)	3.2–37.9	1 (5.3)	0.1–26.0	0	0.0–30.8	0	0.0–33.6	2 (18.2)	2.3–51.8	0	0.0–28.5
Abdominal cramps	1 (5.0)	0.1–24.9	0	0.0–18.5	2 (10.0)	1.2–31.7	1 (5.3)	0.1–26.0	0	0.0–30.8	0	0.0–33.6	0	0.0–28.5	0	0.0–28.5
Loss of appetite	1 (5.0)	0.1–24.9	0	0.0–18.5	1 (5.0)	0.1–24.9	1 (5.3)	0.1–26.0	0	0.0–30.8	0	0.0–33.6	0	0.0–28.5	0	0.0–28.5
Vomiting	1 (5.0)	0.1–24.9	0	0.0–18.5	0	0.0–16.8	1 (5.3)	0.1–26.0	0	0.0–30.8	0	0.0–33.6	0	0.0–28.5	0	0.0–28.5
Diarrhea	2 (10.0)	1.2–31.7	1 (5.6)	0.1–27.3	1 (5.0)	0.1–24.9	2 (10.5)	1.3–33.1	0	0.0–30.8	1 (11.1)	0.3–48.2	0	0.0–28.5	0	0.0–28.5
Any systemic	11 (55.0)	31.5–76.9	7 (38.9)	17.3–64.3	10 (50.0)	27.2–72.8	13 (68.4)	43.4–87.4	3 (30.0)	6.7–65.2	3 (33.3)	7.5–70.1	5 (45.5)	16.7–76.6	3 (27.3)	6.0–61.0
Local symptoms																
Nasal pain	9 (45.0)	23.1–68.5	7 (38.9)	17.3–64.3	8 (40.0)	19.1–63.9	1 (5.3)	0.1–26.0	1 (10.0)	0.3–44.5	1 (11.1)	0.3–48.2	2 (18.2)	2.3–51.8	0	0.0–28.5
Nasal stuffiness	15 (75.0)	50.9–91.3	15 (83.3)	58.6–96.4	16 (80.0)	56.3–94.3	15 (78.9)	54.4–93.9	8 (80.0)	44.4–97.5	6 (66.7)	29.9–92.5	6 (54.5)	23.4–83.3	3 (27.3)	6.0–61.0
Nasal discharge	15 (75.0)	50.9–91.3	14 (77.8)	52.4–93.6	11 (55.0)	31.5–76.9	11 (57.9)	33.5–79.7	9 (90.0)	55.5–99.7	6 (66.7)	29.9–92.5	5 (45.5)	16.7–76.6	2 (18.2)	2.3–51.8
Nasal itching	9 (45.0)	23.1–68.5	5 (27.8)	9.7–53.5	13 (65.0)	40.8–84.6	9 (47.4)	24.4–71.1	4 (40.0)	12.2–73.8	4 (44.4)	13.7–78.8	1 (9.1)	0.2–41.3	0	0.0–28.5
Sneezing	12 (60.0)	36.1–80.9	12 (66.7)	41.0–86.7	15 (75.0)	50.9–91.3	11 (57.9)	33.5–79.7	4 (40.0)	12.2–73.8	2 (22.2)	2.8–60.0	3 (27.3)	6.0–61.0	3 (27.3)	6.0–61.0
Blood tinged nasal mucus	2 (10.0)	1.2–31.7	1 (5.6)	0.1–27.3	4 (20.0)	5.7–43.7	3 (15.8)	3.4–39.6	0	0.0–30.8	0	0.0–33.6	1 (9.1)	0.2–41.3	0	0.0–28.5
Nasal bleed	0	0.0–16.8	1 (5.6)	0.1–27.3	1 (5.0)	0.1–24.9	0	0.0–17.6	0	0.0–30.8	0	0.0–33.6	0	0.0–28.5	0	0.0–28.5
Any local	19 (95.0)	75.1–99.9	15 (83.3)	58.6–96.4	19 (95.0)	75.1–99.9	18 (94.7)	74.0–99.9	10 (100.0)	69.2–10.0	8 (88.9)	51.8–99.7	8 (72.7)	39.0–94.0	3 (27.3)	6.0–61.0
Any symptom	19 (95.0)	75.1–99.9	15 (83.3)	58.6–96.4	19 (95.0)	75.1–99.9	19 (100.0)	82.4–10.0	10 (100.0)	69.2–10.0	8 (88.9)	51.8–99.7	8 (72.7)	39.0–94.0	3 (27.3)	6.0–61.0

NOTE. V1 and V2 are vaccinations 1 and 2, respectively. CI, confidence interval; MPL, monophosphoryl lipid A.

Table 2. Response Rates of Norwalk Virus-Like Particle–Specific Immunoglobulin A (IgA) Antibody Secreting Cells (ASCs) and ASC Geometric Mean (GM), by Group

Dose	IgA ASC response rate			IgA ASC GM of cells per 10^6 PBMC		
	Day 7	Day 21	Day 28	Day 7	Day 21	Day 28
Study 1						
5 µg of Norwalk vaccine	0/3 (0)	0/5 (0)	1/5 (20)	0.5 (−)	0.36 (−)	0.9 (−)
15 µg of Norwalk vaccine	0/5 (0)	0/5 (0)	4/5 (80)	0.6 (−)	0.35 (−)	12.1
50 µg of Norwalk vaccine	7/10 (70)	1/10 (10)	5/9 (56)	9.2	1.50	8.0
MPL plus chitosan control	0/8 (0)	0/8 (0)	0/8 (0)	0.2 (−)	0.19 (−)	0.4 (−)
Study 2						
50 µg of Norwalk Vaccine	5/5 (100)	0/5 (0)	5/5 (100)	50.2	0.3 (−)	16.5
100 µg of Norwalk vaccine	5/5 (100)	1/5 (20)	5/5 (100)	138.3	1.6	71.1
MPL plus chitosan control	0/3 (0)	0/3 (0)	0/3 (0)	0.1 (−)	0.1 (−)	0.1 (−)
Placebo control	0/2 (0)	0/2 (0)	0/2 (0)	0.1 (−)	0.1 (−)	0.1 (−)

NOTE. Response rates are presented as the proportion (%) of participants, unless otherwise indicated. The response rate and GM number of ASCs per 10^6 peripheral blood mononuclear cells (PBMCs) were assessed before each immunization (days 0 and 21) and 7 days later (days 7 and 28). These subjects were enrolled at the University of Maryland Center for Vaccine Development. A positive response was defined as a postvaccination ASC count ≥8 spots per 10^6 PBMCs and was ≥3 standard deviations above the mean prevaccination count for all subjects. The GM preadministration (day 0) ASC responses were all ≤1. The minus sign (−) indicates a negative response. MPL, monophosphoryl lipid A.

events were collected through study day 56 (35 days after the second dose). Serious adverse events and new medically significant conditions were collected for 180 days. Study 2 was amended to assess fibrinogen levels before and 1 and 7 days after each vaccination of the first 12 subjects (9 vaccinees and 3 control participants).

Immune Responses

Serum antibodies. Serum samples were collected on days 0, 7, 21, 28, 56, and 180 after intranasal administration. Norwalk VLP-specific immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) were measured by enzyme-linked immunosorbent assay (ELISA) [5, 20]. Geometric mean titers (GMTs), geometric mean of fold rises, and seroresponse rates (≥4-fold rises) were determined.

Antibody secreting cells. Norwalk VLP-specific IgG and IgA antibody secreting cells (ASCs) were measured in peripheral blood mononuclear cells (PBMCs) on days 0, 7, 21, and 28 [5]. The response rate and mean number of ASCs per 10^6 PBMCs were assessed. A positive response was defined as a postvaccination ASC count ≥8 spots per 10^6 PBMCs and at least 3 standard deviations above the mean prevaccination count for all subjects.

Norovirus hemagglutination inhibition assay. Red blood cells were purified from type O peripheral blood of healthy adult volunteers and maintained at 4°C in a 10% stock in

Table 3. Norwalk Virus-Like Particle–Specific Immunoglobulin G (IgG) and Immunoglobulin A (IgA) Antibody Seroresponse Rates (percent of subjects with ≥4-fold rise) and Geometric Mean of Fold Rise (GMFR), by Group by Study at Day 56 (35 days after Vaccination 2) Compared with Prevaccination

Dose	Serum IgG ≥4-fold rise	Serum IgG GMFR (95% CI)	Serum IgA≥ 4-fold rise	Serum IgA GMFR (95% CI)
Study 1				
5 µg of Norwalk vaccine	0/5 (0)	0.9	0/5 (0)	1.2
15 µg of Norwalk vaccine	2/5 (40)	1.9	2/5 (40)	2.5
50 µg of Norwalk vaccine	7/9 (78)	4.7	5/9 (56)	4.5
MPL plus chitosan control	1/8 (13)	1.0	0/8	1.0
Study 2				
50 µg of Norwalk vaccine	10/18 (56)	4.6 (2.5–8.6)	13/18 (72)	7.6 (4.2–13.8)
100 µg of Norwalk vaccine	12/19 (63)	4.8 (3.2–7.1)	15/19 (79)	9.1 (4.7–17.6)
MPL plus chitosan control	0/9 (0)	1.1 (0.9–1.4)	0/9 (0)	1.0 (0.8–1.3)
Placebo control	0/11 (0)	0.9 (0.8–1.1)	0/11 (0)	1.2 (0.9–1.5)

NOTE. Seroresponse rates (≥4-fold rise) are presented as the proportion (%) of participants, unless otherwise indicated. CI, confidence interval; MPL, monophosphoryl lipid A.
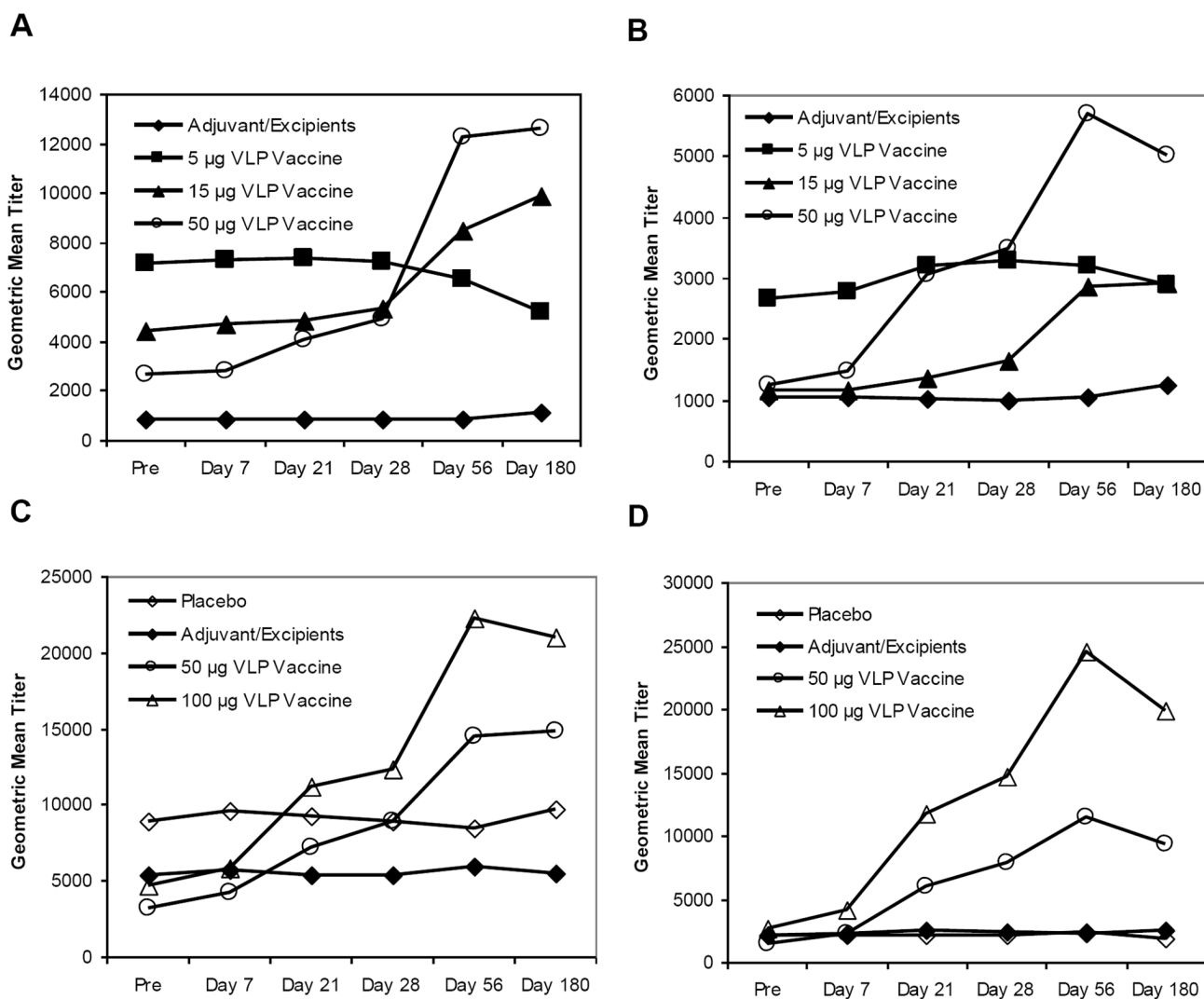
Figure 1. Norwalk virus-like particle (VLP)–specific immunoglobulin G (IgG) and immunoglobulin A (IgA) geometric mean antibody titers, by group in studies 1 and 2. In study 1, 28 adult subjects were randomized sequentially by group to receive 2 doses of (1) 5 μg of Norwalk VLP vaccine (5 subjects, *squares*) or adjuvant control (2 subjects, *diamonds*), (2) 15 μg of Norwalk VLP vaccine (5 subjects, *triangles*) or adjuvant control (2 subjects, *diamonds*), or (3) 50 μg of Norwalk VLP vaccine (10 subjects, *circles*) or adjuvant control (4 subjects, *diamonds*). *A,* Serum IgG geometric mean titers from study 1. *B,* Serum IgA geometric mean titers from study 1. In study 2, 61 healthy adult subjects were enrolled at 4 sites and randomized 2:2:1:1, respectively, to receive either 2 doses of (1) 50 μg of Norwalk VLP vaccine (20 subjects, *circles*), (2) 100 μg of Norwalk VLP vaccine (20 subjects, *triangles*); (3) adjuvant control (10 subjects, *filled diamonds*), or (4) true placebo (11 subjects, *open diamonds*) consisting of a puff of air (no dry powder). *C,* Serum IgG geometric mean titers from study 2. *D,* Serum IgA geometric mean titers from study 2. All doses were delivered intranasally, and the 2-dose regimen was separated by 21 days.

Alsever buffer; subsequent dilutions were made in 0.01 mol/L phosphate-buffered saline without Ca2+ or Mg2+ at pH 7.2 (Invitrogen). Serially diluted Norwalk VLPs (50 μL) were incubated with an equal volume of a 0.5% human red blood cell suspension in 96-well V bottom plates for 90 min at 4°C. The amount of Norwalk VLP antigen corresponding to 4 hemagglutination units was determined and confirmed by back titration. Test serum samples were heat inactivated at 56°C for 30 min and treated (in a 1:5 ratio) with freshly prepared 25% Kaolin suspension (Sigma). To eliminate serum inhibitors, test

samples were also preadsorbed with red blood cells. The hemagglutination inhibition (HAI) assay was performed as follows: pretreated serum samples (diluted 2-fold in phosphate-buffered saline, pH 5.5) were added to 96 well V-plates (Costar) and incubated with an equal volume of Norwalk VLP antigen (LigoCyte, lot 45–06002) containing 4 hemagglutination units, for 30 min at 4°C. A suspension of 0.5% red blood cells was added to each well; plates were incubated for an additional 90 min at 4°C, checking for agglutination every 30 min. Wells containing only phosphate-buffered saline or antigen without

Table 4. Norwalk Virus-Like Particle–Specific Hemagglutination Inhibition Antibody Geometric Mean Titers (GMTs), Geometric Mean of Fold Rises (GMFR), and Seroresponse Rates by Group in Study 2

| | Baseline (Prevaccination) | | Day 21 (Prevaccination 2) | | | |
Group	No.	GMT (95% CI)	No.	GMT (95% CI)	GMFR (95% CI)	%≥ 4-Fold Rise (95% CI)
50 μg of Norwalk Vaccine	18	13.2 (8.6–20.1)	18	32.0 (14.9–68.9)	2.4 (1.2–4.8)	33.3 (13.3–59.0)
100 μg of Norwalk vaccine	19	25.7 (14.7–44.9)	19	111.9 (41.7–300.5)	4.4 (2.1–9.0)	63.2 (38.4–83.7)
MPL plus chitosan	9	6.9 (4.1–11.5)	9	9.3 (5.2–16.7)	1.4 (1.0–1.8)	0.0 (0.0–33.6)
Placebo control	11	12.4 (5.7–27.3)	11	19.3 (7.8–48.0)	1.6 (0.9–2.7)	9.1 (0.2–41.3)

NOTE. MPL, monophosphoryl lipid A.

serum served as negative and positive controls, respectively. HAI titers were calculated as the inverse of the highest dilution that inhibited hemagglutination, with a compact negative red blood cell pattern (button of red blood cells).

Expression of homing molecules by Norwalk-specific ASCs. Fresh PBMCs were stained with monoclonal antibodies to CD19-PE-Cy7 (clone J3–119, Beckman Coulter), CD27-PE-Cy5 (Clone 1A4CD27, Beckman Coulter), CD62L-PE (L-selectin, Clone Dreg-56, B-D Biosciences), and integrin $\alpha 4\beta 7$ (Clone ACT-1) conjugated to Alexa 488 using an Alexa labeling kit (Molecular Probes). Cells were then simultaneously sorted into 4 populations: (N/MB) B naive (CD19$^+$ CD27$^-$), (P) B memory (B$_M$, CD19$^+$CD27$^+$) expressing CD62L but not integrin α_4/β_7 (CD62L$^+$ α_4/β_7^-), (M) B$_M$ expressing α_4/β_7 but not CD62L (CD62L$^-$ α_4/β_7^+), or (M/P) B$_M$ expressing both integrin α_4/β_7 and CD62L (CD62L$^+$ α_4/β_7^+). Four-way sorting was performed in a MoFlo flow cytometer/cell sorter system (Beckman-Coulter). Purities of the sorted populations were 84.1%–94%. The presence of Norwalk VLP-specific IgG and IgA ASCs in each population was measured as described above.

RESULTS

Safety
The safety data for study 2 are presented in Table 1. The safety data for study 1 (data not shown) were similar to those of study 2. After dose 1 or dose 2 of the 100-μg dosage of vaccine, local nasal symptoms were reported by 19 of 20 subjects and 18 of 20 subjects, respectively (Table 1). Likewise in the MPL plus chitosan control group without Norwalk antigen, 10 of 10 subjects and 8 of 10 subjects reported local nasal symptoms after each dose, respectively. In the true placebo group, 8 (73%) of 11 subjects and 3 (27%) of 11 subjects who received a puff of air (no dry powder) reported local nasal symptoms after dose 1 or dose 2, respectively. Local nasal symptoms were primarily mild (grade 1)—nasal stuffiness, nasal discharge, or sneezing. Two (5%) of 40 vaccinees reported mild (grade 1) nasal bleeds on a single day: 1 subject after dose 1 (100-μg vaccinee) and 1 subject after dose 2 (50 -μg vaccinee). No severe (grade 3) local nasal or systemic events were reported in the 7 days after

either dose of vaccine or control. None of the 61 subjects in study 2 reported fever in the 7 days after either nasal administration. Headache and malaise were the most common systemic symptoms observed and were not statistically different between groups.

A mild (grade 1) elevation in fibrinogen was observed in 1 of 9 vaccinees and in none of the 3 control subjects 1 day after dose 1 that returned to normal by day 7. No elevation in fibrinogen was observed at 1 day or 7 days after dose 2. Clinical laboratory abnormalities were infrequent and observed with similar frequency across study groups. Severe (grade 3) hematologic abnormalities were not observed. Two severe (grade 3) chemistry abnormalities were observed; an elevated aspartate aminotransferase in a recipient of the 50-μg dosage of vaccine and a decreased glucose in a placebo recipient. One serious adverse event not related to the vaccine was reported in the 180-day safety period, a hospitalization for appendectomy 111 days after the second dose of vaccine. No new onset, medically significant medical conditions were reported in the 180-day safety period.

Immune Responses
ASCs. Norwalk VLP-specific IgG and IgA circulating ASCs were detected at day 7, waned at day 21 (immediately prior to dose 2), and reappeared at day 28, 7 days after dose 2 (Table 2). In study 1, 7 (39%) of 18 subjects who were evaluated and received any vaccine dosage developed rises in specific IgA ASCs at day 7, and 10 (53%) of 19 subjects had ASC responses at day 28. In study 2, all 10 subjects evaluated (100%) who received 50 or 100 μg of vaccine developed IgA ASCs at days 7 and 28.

Serum antibodies. Norwalk VLP-specific IgG and IgA seroresponse rates and geometric mean of fold rises are presented in Table 3, and the kinetics of antibody production (geometric mean titers before and after vaccination) are presented in Figure 1. In study 1, the seroresponse rates showed a dose-dependent response with increased titers as the dosage of vaccine antigen increased; a logistic regression with dose as a continuous variable results in a χ^2 P value <.01 for IgG seroresponse rates and

Table 4. *(Continued.)*

	Day 56 (35 days after vaccination 2)				Day 180 (159 days after vaccination 2)		
No.	GMT (95% CI)	GMFR (95% CI)	%⩾ 4-Fold Rise (95% CI)	No.	GMT (95% CI)	GMFR (95% CI)	%⩾ 4-Fold Rise (95% CI)
18	52.8 (25.8–108.2)	4.0 (2.0–7.9)	38.9 (17.3–64.3)	17	54.4 (25.4–116.4)	4.0 (1.9–8.3)	47.1 (23.0–72.2)
19	234.9 (79.7–692.8)	9.1 (4.0–20.7)	73.7 (48.8–90.9)	19	151.6 (56.9–403.9)	5.9 (2.9–12.1)	57.9 (33.5–79.7)
9	9.3 (4.7–18.7)	1.4 (1.0–1.8)	0.0 (0.0–33.6)	9	8.6 (4.9–15.2)	1.3 (0.9–1.8)	11.1 (0.3–48.2)
11	21.9 (8.2–58.7)	1.8 (0.9–3.5)	9.1 (0.2–41.3)	10	19.7 (7.7–50.2)	1.7 (1.0–2.9)	10.0 (0.3–44.5)

a χ^2 *P* value <.02 for IgA seroresponse rates. In study 2, 12 (63%) of 19 subjects in the 100-μg group seroresponded for IgG antibodies, and 15 (79%) of 19 subjects seroresponded for IgA antibodies at day 56. The 100-μg group developed higher titers than did the 50-μg group, but the differences were not statistically significant. Both vaccine groups developed higher serum IgG and IgA responses than those of the 2 control groups; a logistic regression with dose as a continuous variable results in a χ^2 *P* value <.001 for IgG and IgA seroresponse rates. No subjects developed ⩾4-fold rises in serum IgM antibody (data not shown).

Vaccine-induced antibodies were also examined in their capacity to inhibit hemagglutination of O-type human red blood cells by Norwalk VLP. HAI titers (geometric mean titers, geometric mean of fold rises, and ⩾4-fold rises) are presented in Table 4, and the geometric mean titers are presented in Figure 2. Among subjects who received the 100-μg dosage of vaccine, the geometric mean HAI antibody titers peaked after the second dose, with a geometric mean of fold rises of 9.1 (95% confidence interval [CI], 4.0–20.7); seroresponse occurred in 73.7% of these subjects. The IgG and HAI data had a 72% agreement in 4-fold rise responses for day 56 (95% CI, 58%–83%). The IgA and HAI data had a 75% agreement in 4-fold rise responses for day 56 (95% CI, 62%–86%).

Homing potential of Norwalk-specific ASCs. To investigate the expression of homing molecules known to direct their migration to mucosal and peripheral lymphoid tissues, PBMCs from 5 subjects were stained, sorted simultaneously into 4 defined subsets, and assessed for their ability to secrete Norwalk IgG and IgA as described above (Table 5). The majority of IgA ASCs were observed in 2 main subsets: CD19$^+$ CD27$^+$ CD62L$^+$, integrin α_4/β_7^+, that is, expressing both peripheral lymphoid tissue and mucosal homing molecules (~700–10,700 ASCs per 10^6 sorted cells); and CD19$^+$ CD27$^+$ CD62L$^-$ integrin α_4/β_7^+, that is, expressing exclusively mucosal homing molecules (~2500–6700 ASCs per 10^6 sorted cells). The latter was observed in 3 of 4 vaccinees.

In contrast, the IgG ASCs, with the exception of 1 vaccinee, were of a single phenotype: CD19$^+$ CD27$^+$ CD62L$^+$ integrin α_4/β_7^+, and the frequencies (~150–670 ASCs per 10^6 sorted cells) were considerably lower than those observed for IgA ASCs. One vaccinee whose dosage was at the 50-μg level exhibited IgG ASC subsets bearing both mucosal and peripheral lymphoid tissues homing receptors.

No IgG or IgA ASCs exhibited a phenotype associated with either naive B cells (CD19$^+$, CD27$^-$) or B$_M$ (CD19$^+$, CD27$^+$) cells expressing CD62L in the absence of integrin α_4/β_7, which would presumably home exclusively to peripheral lymphoid tissues.

DISCUSSION

The intranasal monovalent adjuvanted Norwalk VLP vaccine was generally well tolerated and immunogenic. A second dose
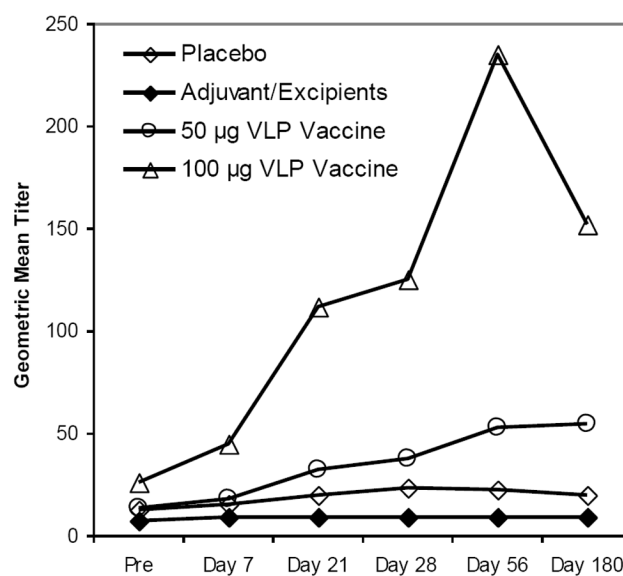

Figure 2. Norwalk virus-like particle (VLP)–specific hemagglutination inhibition antibody geometric mean titers, by group in study 2. Sixty-one healthy adult subjects were enrolled at 4 sites and randomized 2:2:1:1, respectively, to receive either 2 doses of (1) 50 μg of Norwalk VLP vaccine (20 subjects, *circles*), (2) 100 μg of Norwalk VLP vaccine (20 subjects, *triangles*), (3) adjuvant control (10 subjects, *filled diamonds*), or (4) true placebo (11 subjects, *open diamonds*) consisting of a puff of air (no dry powder). All doses were delivered intranasally, and the 2-dose regimen was separated by 21 days.

Table 5. Norwalk Virus-Like Particle–Specific Immunoglobulin G (IgA) and Immunoglobulin G (IgG) Cell Surface Receptor Homing Molecules in Study 2

Group	P*	N/MB*	M/P*	M*
	IgA (total no. of cells/10^6)			
Placebo	0	0	0	4
50 μg of vaccine	0	2	1000	5197
100 μg of vaccine	95	14	1999	2666
100 μg of vaccine	33	0	717	0
100 μg of vaccine	0	15	10,739	6668
	IgG (total no. of cells/10^6)			
Placebo	0	0	0	0
50 μg of vaccine	0	0	450	742
100 μg of vaccine	0	0	157	0
100 μg of vaccine	0	0	282	0
100 μg of vaccine	0	0	667	0
	Sorted subpopulation phenotype			
P*	CD19$^+$	CD19$^+$	CD19$^+$	CD19$^+$
N/MB*	CD27$^+$	CD27$^-$	CD27$^+$	CD27$^+$
M/P*	CD62L$^+$		CD62L$^+$	CD62L$^-$
M*	$\alpha4\beta7^-$		$\alpha4\beta7^+$	$\alpha4\beta7^+$

NOTE. M*, mucosal homing memory B/plasma cell; M/P*, mucosal and peripheral lymphoid tissues homing memory B/plasma cell; N/MB*, naive mature B cell; P*, peripheral lymphoid tissues homing memory B/plasma cell.

of vaccine provided increased serologic antibody responses, whereas the peak increase in ASC responses occurred after dose 1. HAI antibody (a functional measurement) increased only at the highest dosage tested, and the fold increase (9-fold) was similar to that of the serum IgA. Mucosal priming by the nasal mucosa was supported by the presence of high frequencies of IgA and IgG ASCs in peripheral blood. ASCs appear transiently in the circulation after naive B lymphocytes at an inductive site are exposed to a foreign antigen (eg, vaccine-primed B cells at the nasal associated lymphoid tissue return to the mucosa as immune effector cells). Norwalk IgA-specific ASC responses were observed 7 days after the first immunization in all 5 subjects tested that received the 100-μg vaccine dose, with a geometric mean of 138 cells per 10^6 PBMCs. The ASC numbers reported in this study are higher than what has been previously observed after administration of oral nonadjuvanted Norwalk VLP vaccine or after ingestion of nonadjuvanted Norwalk VLP antigen in edible transgenic plants [5, 16]. These ASC counts are also generally higher than those induced by live oral vaccines or by wild-type challenges with enteric organisms [5, 21–24]. The correlates of protective immunity against Norwalk virus illness are not known; however, these mucosally primed ASCs in combination with the serum IgG and IgA antibodies may contribute to protection. Graham et al [25] reported a 35-fold mean increase in serum antibody titers in a population of 41

Norwalk virus–infected subjects after virus challenge. Gray et al [20] evaluated a subset of these serum samples for IgG and IgA by ELISA and observed Norwalk IgG peak titers of ∼15,900 from days 15 to 90 and IgA peak titers of ∼12,600 from days 24 and 90. A direct comparison of IgG and IgA between challenged and vaccinated subjects has not been performed but should be included in future studies.

We report for the first time to our knowledge the presence of circulating Norwalk-specific IgA and IgG ASCs after intranasal vaccination with adjuvanted VLPs. Hence, it was important to study the homing characteristics of these effector cells. It is widely accepted that CD62L is a key molecule implicated in the initial phase of migration through high endothelial venules in lymphoid tissues, including lymph nodes and Peyer patches, by binding to the peripheral lymph node addressins (PNad) and the mucosal addressin cell adhesion molecule (MAdCAM-1) present in the high endothelial venule vascular endothelium, which results in tethering and rolling [27–29]. In contrast, at mucosal effector sites, although a number of adhesion molecules are involved, vascular adhesion specificity is mediated by integrin α_4/β_7 interacting with the mucosal addressin cell adhesion molecule [27–29]. Thus, cells expressing integrin α_4/β_7 but not CD62L are destined to home to the gut mucosa, whereas cells expressing CD62L but not integrin α_4/β_7 are destined to home to peripheral and mesenteric lymph nodes. As an example, a previous study that evaluated B cell surface markers in response to acute rotavirus infections showed that cells homing to the gut mucosa were CD27$^+$ integrin α_4/β_7^+ CD62L$^{+/-}$ [26]. In contrast, although it is likely that cells coexpressing integrin α_4/β_7 and CD62L can home to peripheral lymphoid tissues [27], the homing potential and activity of these cells remains controversial.

In the present study we observed that intranasal immunization elicited circulating VLP-specific IgA and IgG ASCs with different homing potentials. IgA-specific ASCs exhibited homing receptors likely to endow them with the ability to home to both the gut mucosa (CD19$^+$ CD27$^+$ integrin α_4/β_7^+ CD62L$^-$) and peripheral lymphoid tissues (CD19$^+$ CD27$^+$ integrin α_4/β_7^+ CD62L$^+$), whereas IgG-ASCs expressed homing receptors that support homing to peripheral lymphoid tissues (CD19$^+$ CD27$^+$ integrin α_4/β_7^+ CD62L$^+$). The fact that intranasal immunization was able to elicit ASCs with such a diverse homing profile, including the gut mucosa, is noteworthy and demonstrates that this route has the capacity to induce potent systemic and mucosal immune responses, including effector cell activity at a distant site of infection (ie, the gastrointestinal tract in the case of Norwalk virus). However, intranasal immunization was not effective in inducing significant levels of specific IgG or IgA ASCs with the potential to home exclusively to peripheral lymphoid tissues [27].

This adjuvanted Norwalk VLP vaccine is one of only a few

vaccine candidates directed against an enteric pathogen that has been delivered to humans by the intranasal route. An intranasal vaccine against *Shigella flexneri* (Invaplex 50) was generally well tolerated, but the immune responses were suboptimal; subjects who underwent subsequent challenge with *S. flexneri* were not protected [30]. The heat-labile enterotoxin of *Escherichia coli* and its enzymatically inactive mutants have been given as intranasal adjuvants in human studies; however, the toxin has been associated with facial nerve palsy [31], and intranasal administration of this antigen and/or adjuvant has been abandoned.

VLPs have been a successful strategy for development of parenteral vaccines, such as those against hepatitis B and human papillomavirus [32, 33]. Our data support that mucosal and systemic immune responses after adjuvanted Norwalk VLPs delivered by the intranasal route support this approach as an important vaccine development strategy.

Nasal vaccination has several advantages, including ease of administration, stimulation of mucosal dendritic cells to initiate immune responses locally and systemically, and ease of storage at room temperature. Disadvantages include the requirement of a specialized delivery device, the rapid clearance of antigens from the nose by mucociliary action, and that the mucosal immune response may be limited [34]. The intranasal adjuvanted Norwalk VLP vaccine was formulated with both MPL and chitosan to address these disadvantages [17, 35, 36]. MPL is a potent adjuvant for adaptive immunity signaling through TLR-4, inducing a blended Th1- and Th2-type immune response. MPL was recently approved by the FDA as a new adjuvant in the United States as a component of GlaxoSmith-Kline's human papillomavirus vaccine, CERVARIX [37, 38]. Chitosan, a cationic polysaccharide derived from chitin, has been used for nasal delivery because of its biocompatibility, biodegradability, and bioadhesiveness [17, 39, 40]. Chitosan has also been shown in animals to have immunostimulatory activity [41].

How effective the observed immune responses might be in preventing Norwalk infection and illness remains unknown. The immune responses to the prototype vaccine described here may not be cross-reactive against other genotypes, and a future norovirus VLP vaccine should include VLPs that represent the most epidemiologically important genotypes [9]. Preparations of wild-type Norwalk viruses used in challenges in humans established their pathogenicity [25, 42]. Norwalk shedding and clinical symptoms after Norwalk challenge in humans using virus of known genetic makeup and purity has recently been reported elsewhere [43]. Because this model of infection in human volunteers is established, the efficacy of adjuvanted Norwalk VLP vaccine administered by the intranasal route should be assessed in the human challenge model.

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