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Systemic and Intestinal Antibody-Secreting Cell Responses and Correlates of Protective Immunity to Human Rotavirus in a Gnotobiotic Pig Model of Disease†

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Neonatal gnotobiotic pigs orally inoculated with virulent (intestinal-suspension) Wa strain human rotavirus (which mimics human natural infection) developed diarrhea, and most pigs which recovered (87% protection rate) were immune to disease upon homologous virulent virus challenge at postinoculation day (PID) 21. Pigs inoculated with cell culture-attenuated Wa rotavirus (which mimics live oral vaccines) developed subclinical infections and seroconverted but were only partially protected against challenge (33% protection rate). Isotype-specific antibody-secreting cells (ASC) were enumerated at selected PID in intestinal (duodenal and ileal lamina propria and mesenteric lymph node [MLN]) and systemic (spleen and blood) lymphoid tissues by using enzyme-linked immunospot assays. At challenge (PID 21), the numbers of virus-specific immunoglobulin A (IgA) ASC, but not IgG ASC, in intestines and blood were significantly greater in virulent-Wa rotavirus-inoculated pigs than in attenuated-Wa rotavirus-inoculated pigs and were correlated (correlation coefficients: for duodenum and ileum, 0.9; for MLN, 0.8; for blood, 0.6) with the degree of protection induced. After challenge, the numbers of IgA and IgG virus-specific ASC and serum-neutralizing antibodies increased significantly in the attenuated-Wa rotavirus-inoculated pigs but not in the virulent-Wa rotavirus-inoculated pigs (except in the spleen and except for IgA ASC in the duodenum). The transient appearance of IgA ASC in the blood mirrored the IgA ASC responses in the gut, albeit at a lower level, suggesting that IgA ASC in the blood of humans could serve as an indicator for IgA ASC responses in the intestine after rotavirus infection. To our knowledge, this is the first report to study and identify intestinal IgA ASC as a correlate of protective active immunity in an animal model of human-rotavirus-induced disease.

Rotavirus is an important pathogen causing severe gastroenteritis in infants and young children, as well as other young animals (1, 15). The worldwide impact of this virus on public health has led to efforts to develop vaccines to control rotaviral disease. Rotaviruses replicate in the enterocytes of the small intestine, inducing villous atrophy and malabsorptive diarrhea (24). Local mucosal immunity is therefore a critical factor in protection against rotaviral diarrhea after natural infection, suggesting that highly effective vaccines must induce local immune responses (12, 41). Several heterologous (bovine and simian), homologous (M37 human), and animal-human reassortant live attenuated oral candidate rotavirus vaccines have been assessed for infants and young children, but with variable efficacy (21, 24). The variable responses to these vaccines underscore a need for a better understanding of the host immune response to virulent and attenuated strains of rotavirus in both the systemic and mucosal immune systems.

Several animal models have been used (mice, rabbits, calves, and pigs) to study rotavirus pathogenesis and immunity (6, 11, 22, 30, 39, 43, 44, 57). Gnotobiotic pigs present a number of important advantages over other animal models for investigating the pathogenesis and immune responses to human rotavirus: they closely resemble humans in gastrointestinal physiology and in the development of mucosal immunity (26, 38); they are susceptible until at least 6 weeks of age to infection and disease with several human rotavirus strains (40, 43, 44); they are born devoid of maternal antibodies but are immunocompetent, allowing assessment of true primary immune responses (26, 31, 38); and their gnotobiotic status assures that exposure to extraneous rotaviruses or other enteric pathogens is eliminated as a confounding variable. Thus, delineating the mucosal immune responses and identifying the correlates of protective immunity to human rotavirus infection in gnotobiotic pigs may enable us to define similar correlates of protective immunity to rotaviral infections in infants and to develop more effective vaccine strategies. The pig model has been used in our laboratory previously to investigate the mucosal and systemic antibody responses to porcine and human rotaviruses and to porcine coronaviruses (10, 43, 48), confirming their usefulness for evaluating approaches to mucosal immunization.

We selected a virulent (intestine-passaged) and the attenuated (cell culture-passaged) derivative strain of Wa (G1P1A [P8]) human rotavirus for our studies because G1/P1A rotaviruses belong to the most common G and P serotypes associated with rotaviral gastroenteritis in infants and children worldwide (3, 24, 27, 58). The pathogenicity of the virulent Wa rotavirus has been confirmed for gnotobiotic pigs, as has the attenuation of the cell culture-passaged strain, with the latter strain able to induce serum-neutralizing antibodies without disease or pathology (40, 43, 50, 56).

Our goal was to evaluate protection induced by natural exposure or vaccination, using the virulent Wa rotavirus as the challenge virus. The virulent Wa human rotavirus was used to mimic natural exposure, and the derivative attenuated Wa

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rotavirus mimicked live oral vaccine exposure. The immunoglobulin (Ig) isotypes of rotavirus-specific antibody-secreting cells (ASC) and total-Ig-secreting cells (IgSC) in intestinal and systemic lymphoid tissues after inoculation and challenge of the experimental pigs were enumerated by using an enzyme-linked immunospot (ELISPOT) assay. Correlates of protective active immunity were assessed in this animal model of human rotavirus disease.

MATERIALS AND METHODS

Human rotavirus. The virulent Wa human rotavirus strain (serotype G1P1A[P8]) consisted of a suspension of intestinal contents obtained by serial passage in a gnotobiotic pig of a Wa rotavirus-infected infant stool specimen as described previously (56). Intestinal contents from the 16th gnotobiotic pig passage were used as the virulent Wa rotavirus inoculum (50). The cell culture-attenuated Wa human rotavirus was adapted to growth in cell culture (56), cloned six times by limiting dilution, and passaged at least 27 times in fetal rhesus monkey kidney cells (MA104) (50). The attenuated Wa rotavirus inoculum consisted of clarified Wa rotavirus-infected MA104 cell lysates. The virulent and attenuated Wa rotaviruses were diluted in minimal essential medium (Gibco Life Technologies, Grand Island, N.Y.) containing 100 IU of penicillin per ml, 0.1 mg of dihydrostreptomycin per ml, and 0.14% (wt/vol) $NaHCO_3$. The in vitro infectivity titers of each inoculum were determined by a cell culture immunofluorescence (CCIF) assay (7).

Inoculation and challenge of gnotobiotic pigs. Near-term pigs were delivered and maintained asceptically in sterile isolation units as described previously (33). At 3 to 5 days of age, gnotobiotic pigs were inoculated orally with $\sim 10^5$ fluorescent focus units (FFU) of virulent Wa rotavirus (group 1) representing $\sim 10^5$ 50% infectious doses (ID_{50}) (the 50% diarrheal dose was approximately equal to the ID_{50}) or $\sim 2 \times 10^7$ FFU of live attenuated Wa rotavirus (group 2) approximately 10 min after being fed 5 ml of 100 mM sodium bicarbonate (19). Pigs receiving attenuated Wa rotavirus were reinoculated with the same dose 10 days later (postinoculation day [PID] 10). Pigs given equal volumes of diluent served as sham-inoculated controls. Three weeks after the first inoculation (PID 21), virus-inoculated and sham-inoculated pigs were challenged orally with $\sim 10^6$ FFU (10^6 ID_{50}) of virulent Wa rotavirus. The pigs were examined daily for severity of diarrhea (fecal scores: 0 = normal [no diarrhea]; 1 = pasty [mild diarrhea]; 2 = semiliquid [moderate diarrhea]; and 3 = liquid [severe diarrhea]). Rectal swabs were collected daily, and blood samples were collected weekly from all pigs. The microbial sterility of pigs was tested by routine bacteriological tests (8). A subset of each group of pigs (n = 4 to 6 at each time point) were euthanized at 0, 8, 21, 25, and 28 PID (equivalent to −21, −13, 0, 4, and 7 postchallenge days [PCD]). Because of the difficulty of maintaining large pigs in the isolators, only six pigs were used for investigating longer-term (6 weeks) protective immunity after inoculation with virulent Wa rotavirus (hereafter called "longer-term pigs"). Two pigs inoculated with virulent Wa rotavirus as described above were euthanized at PID 43 (PCD 0), two were euthanized at PID 50 (PCD 8), and the other two, which were sham inoculated, were euthanized at PID 49 (PCD 7). The small intestines (duodenum and ileum), mesenteric lymph nodes (MLN), spleens, and blood samples were collected at euthanasia for isolation of mononuclear cells (MNC).

CCIF assay. The CCIF assay was performed as described previously (7). Briefly, rectal swabs and intestinal contents were diluted 1:25 by wt/vol and vol/vol, respectively, in minimal essential medium. All samples were vortexed and then clarified by centrifugation. The samples were serially diluted (10-fold), and each dilution was assayed in duplicate on MA104 cell monolayers in 96-well plates (100 μl per well). Fluorescent foci within wells were visualized by fluorescent microscopy and enumerated, and the infectivity titers were expressed in fluorescent focus units per milliliter.

ELISA. An antigen-capture sandwich enzyme-linked immunosorbent assay (ELISA) using hyperimmune antiserum to group A rotavirus was done as previously described to detect Wa rotavirus antigen in rectal swab fluids, feces, or intestinal contents (42). Fecal samples from sham-inoculated gnotobiotic pigs were used as negative controls. A sample was positive if the mean absorbance of replicates was greater than the mean absorbance of the negative controls plus 3 standard deviations.

Plaque reduction VN assay. A plaque reduction virus neutralization (VN) assay was performed as previously described, using the cell culture-passaged strain of Wa rotavirus to detect the titer of neutralizing antibodies to Wa rotavirus in serum (44). Titers were expressed as the reciprocal of the serum dilution that reduced the number of plaques by 80%.

Isolation of MNC. The MNC from spleen, MLN, lamina propria (duodenum and ileum), and blood were isolated by using modifications of previously published methods (10, 47, 48). The lymphoid tissues were collected aseptically and placed in ice-cold wash medium (RPMI 1640 with 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 200 μg of gentamicin per ml, and 20 μg of ampicillin per ml.) The MNC from spleen and MLN were pressed through stainless steel 80-mesh screens of a cell collector (Cellecter; E-C Apparatus Corp., St. Petersburg, Fla.) to obtain single-cell suspensions. Segments of duodenum and ileum were rinsed twice each with wash medium and Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (Gibco BRL, Gaithersburg, Md.) and mechanically rotated and vortexed to dislodge epithelial cells and intraepithelial lymphocytes. After removal of epithelial cells, the segments were minced, suspended in RPMI 1640 containing 10% fetal bovine serum and 300 U of type II collagenase (Sigma Chemical Co., St. Louis, Mo.) per ml, and digested for 30 min at 37°C with gentle shaking. The digest supernatants (containing lamina propria lymphocytes) were collected, and the remaining tissues were pressed through 80-mesh screens to obtain single-cell suspensions. The single-cell suspensions and digest supernatants were pooled. The cell suspensions from spleen, MLN, duodenum, and ileum were suspended in a 30% Percoll solution (Sigma Chemical Co.) and centrifuged at 1200 × *g* for 30 min at 4°C. Cell pellets were resuspended in 43% Percoll, underlaid with 70% Percoll, and centrifuged at 1200 × *g* for 30 min at 4°C. The MNC were collected from the 43-to-70% interface and washed once with wash medium. Blood was collected in 30% (vol/vol) acid citrate glucose, and peripheral blood lymphocytes (PBL) were obtained by Ficoll-Hypaque (Ficoll-Paque 1.077; Sigma Chemical Co.) density gradient centrifugation. Cells at the interface were collected and washed twice in Hanks balanced salt solution. The purified MNC were suspended in RPMI 1640 enriched with 8% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 20 mM HEPES, 100 μg of gentamicin per ml, and 10 μg of ampicillin per ml (E-RPMI). The viability of each MNC preparation was determined by trypan blue exclusion (all were >95%). The number of MNC per 100 μl was adjusted to 5×10^5, 5×10^4, and 5×10^3 in E-RPMI.

ELISPOT for Wa rotavirus-specific ASC. (i) Fixed cell plates. Confluent monolayers of MA104 cells in 96-well plates (Corning Glass Works, Corning, N.Y.) were inoculated with 100 μl of cell culture-passaged Wa rotavirus per well ($\sim 1.25 \times 10^6$ FFU/ml) containing 0.05% pancreatin. After incubation for 18 h at 37°C in 5% CO_2, the cells were fixed with 80% acetone. This procedure produced ≥80% infected cells without loss of monolayers. The plates were stored at −20°C until use. Mock-infected MA104 cell monolayers were prepared similarly as controls.

(ii) Semipurified antigen-coated plates. Cell culture-passaged Wa rotavirus was partially purified by centrifugation through a 40% (wt/vol) sucrose cushion as previously described (10). The lysates from mock-infected MA104 cell cultures were processed similarly as a control antigen. The Wa rotavirus antigen and control antigen were diluted in 50 mM carbonate buffer (pH 9.6), and 100 μl per well was added to 96-well plates (Nunc-Immuno, Roskilde, Denmark). The plates were incubated for 30 min at 37°C and then for 16 h at 4°C. Optimization of viral antigen coating in the ELISPOT assay was determined as described previously (10) by selecting the virus antigen dilution that produced the greatest number of specific ASC (chromogenic colored spots) with hybridoma cell lines RG25A10 (secreting VP6 broadly-reactive group A monoclonal antibody) and Common 60 (secreting VP7 broadly-reactive group A monoclonal antibody) (23, 34).

The ELISPOT assay was adapted from our previously described laboratory procedures (10, 47, 48). Briefly, the fixed-cell plates were thawed and rehydrated by washing five times with deionized water (dH_2O), and antigen-coated plates were washed in dH_2O once prior to use in the ELISPOT assay. Each MNC suspension (5×10^5, 5×10^4, and 5×10^3 cells per well) was added to duplicate wells. Common 60 hybridoma cells were used on each plate as positive ASC controls. All plates were incubated for ~12 h at 37°C in 5% CO_2 and then washed six times in phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% (vol/vol) Tween 20 (PBS-Tw) to remove cells. The detector antibodies consisted of 100 μl of biotinylated mouse monoclonal antibody (purified ascites fluids) to pig IgG (derived from hybridoma 3H7, 0.03 μg/ml), pig IgA (derived from hybridoma 6D11, 0.04 μg/ml), or pig IgM (derived from hybridoma 5C9, 0.35 μg/ml) (hybridomas provided by P. Paul, Iowa State University, Ames) (36) which were diluted in PBS-Tw, added to each well, and incubated for 2 h at room temperature. Biotinylated F (antibody′)2 goat anti-mouse IgG, A, M (Cappel, Organon Teknika Corp., West Chester, Pa.) was used (1:7,000 dilution) as the detector antibody for positive control hybridoma cells. The plates were washed with PBS-Tw, and 100 μl of horseradish peroxidase-conjugated streptavidin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md. [KPL Inc.]) was added (diluted 1:40,000). After incubation for 1 h at room temperature, the plates were washed in PBS-Tw, and tetramethylbenzidine with H_2O_2 peroxidase substrate system (KPL Inc.) was added (50 μl per well) for 1 h at room temperature to develop spots. The MNC were assessed for de novo antibody synthesis by treatment of the cells with cycloheximide (50 μg/ml in E-RPMI) during the 12-h incubation.

ELISPOT for total IgSC. Affinity-purified goat anti-pig IgM [25 μg/ml; KPL Inc.), goat anti-pig IgA (30 μg/ml; Bethyl Laboratories Inc., Montgomery, Tex.), and goat anti-pig IgG (5 μg/ml; KPL Inc.) were used as isotype-specific capture antibodies to quantitate total IgSC in the ELISPOT assay. The capture antibodies were diluted in 50 mM carbonate buffer (pH 9.6), and 100 μl per well was added to 96-well plates (Nunc-Immuno). The plates were incubated for 30 min at 37°C and then for 16 h at 4°C. Wells coated with only carbonate buffer were used as controls. The optimal coating concentrations of each capture antibody were determined by checkerboard titration, using pig IgSC in the ELISPOT assay and selecting the coating concentrations that gave the highest numbers of IgSC for each isotype. Coated plates were blocked with 1% bovine serum albu-

TABLE 1. Summary of data for diarrhea, virus shedding, serum VN antibody titers, and seroconversion after primary inoculation and challenge of gnotobiotic pigs with Wa human rotavirus

Wa rotavirus inoculum group[a]	Result[b] after:						
	Primary inoculation				Challenge		
	Diarrhea[c]	Virus shedding[d]	Seroconversion[e]	VN GMT[e]	Diarrhea	Virus shedding	VN GMT
Group 1, virulent	95 (41/43)	100 (43/43)	100 (40/40)	69^{Ae} ($n = 40$)	11 (2/18)	0 (0/18)	107^{A} ($n = 23$)
Group 2, attenuated	12 (6/51)	6 (3/51)	96 (40/42)	45^{A} ($n = 42$)	56 (9/16)	81 (13/16)	296^{B} ($n = 16$)
Control	13 (2/15)	0 (0/15)	0 (0/8)	$<4^{B}$ ($n = 8$)	83 (10/12)	100 (12/12)	9^{C} ($n = 11$)

[a] Gnotobiotic pigs were orally inoculated with virulent (1 dose, $\sim 10^5$ FFU) or attenuated (2 doses, $\sim 2 \times 10^7$ FFU) Wa human rotavirus and orally challenged with virulent Wa human rotavirus ($\sim 10^6$ FFU). Controls were inoculated with diluent on primary inoculation and with virulent Wa human rotavirus on challenge.

[b] Data are percentages (rates of diarrhea and virus shedding) and number of pigs affected (or seroconverted for seroconversion) per total number of pigs (in parentheses).

[c] Diarrhea present if daily fecal consistency score was ≥2 during the first postinoculation or postchallenge week. Fecal consistency was scored as follows: 0 = normal, 1 = pasty, 2 = semiliquid, 3 = liquid.

[d] Virus shedding determined by CCIF and ELISA.

[e] Seroconversion and VN antibody GMTs determined at PID 19 to 21 and PCD 3 to 9 by plaque reduction VN assay. Values across rows or in columns with different superscripts (A, B, and C) differ significantly ($P < 0.05$).

min fraction V in PBS (pH 7.4) (Miles Laboratories Inc.) for 1 h and then rinsed in dH_2O once prior to use in the ELISPOT assay. Each dilution (100 μl) of MNC suspension (5×10^5, 5×10^4, and 5×10^3 cells per well) was added to duplicate wells, and the plates were incubated for ~12 h at 37°C in 5% CO_2. The plates were washed and spots were developed as described for Wa rotavirus-specific ASC.

The numbers of ASC and total IgSC were determined by counting blue spots in the wells. Counts were averaged from the duplicate wells at the dilution which yielded less than 40 spots per well and were reported as the number of virus-specific ASC or total IgSC per 5×10^5 MNC. Spots were rarely found on fixed mock-infected cell plates or control antigen-coated plates and, if present, were subtracted from specific-ASC or total IgSC numbers.

Statistical analyses. The mean ASC numbers were calculated for each treatment group at the various assay time intervals (PID 8 [PCD −13], PID 21 [PCD 0], PID 25 [PCD 4], and PID 28 [PCD 7]). Significant differences between groups in numbers of virus-specific ASC and total IgSC in each tissue type at each time point were determined by Student's *t* test. Significant differences within a group in numbers of virus-specific ASC in each tissue type between different time points were determined by two-way analysis of variance with the Statistical Analysis Systems (SAS Institute Inc., Cary, N.C.). One-way analysis of variance was used to determine if serum VN antibody titers differed between groups. The correlation between protection from challenge and numbers of virus-specific ASC at PID 21 (PCD 0) were determined by Spearman's correlation. Statistical significance was assessed at $P < 0.05$.

RESULTS

Clinical signs, virus shedding, and VN antibody responses in serum. The temporal appearance of clinical signs, virus shedding, and pathological changes in Wa rotavirus-inoculated pigs were described in detail elsewhere (50, 51). As summarized in Table 1, pigs initially exposed to virulent Wa rotavirus were 100% protected from virus shedding, and the protection rate against diarrhea was 87%. Attenuated-Wa rotavirus-inoculated pigs were partially protected from virus shedding (19% protection rate) and diarrhea (33% protection rate). Diarrheal incidence of virulent-Wa rotavirus-inoculated pigs postchallenge (11%) was similar to that observed in controls and attenuated-Wa rotavirus-inoculated pigs after primary inoculation (13 and 12%, respectively; Table 1). When the two longer-term pigs were challenged at 6 weeks after inoculation, they were also completely protected from shedding and diarrhea. The two sham-inoculated longer-term pigs shed virus and developed severe diarrhea upon challenge with virulent Wa rotavirus. Diarrheal incidence in uninfected age-matched animals at PID 21 to 28 was similar to that of the sham-inoculated pigs at PID 1 to 7 (51).

After inoculation with 1 dose of virulent rotavirus or 2 doses of attenuated rotavirus, 100 and 96% of the pigs, respectively, seroconverted with VN antibodies to Wa rotavirus by challenge (PID 21) as determined by a plaque reduction VN assay (Table 1). The geometric mean titer (GMT) for virulent-rotavirus-inoculated pigs was higher than that for attenuated-rotavirus-inoculated pigs, although not significantly (69 versus 45) (Table 1). By PCD 3 to 9, most pigs (81%) exposed to attenuated Wa rotavirus showed increased serum antibody titers, and this groups' GMT was significantly higher (~6.6-fold) than that before challenge. The VN GMT of the virulent-rotavirus-inoculated pigs did not increase significantly postchallenge (Table 1).

Rotavirus-specific ASC responses. The isotype distribution of ASC on fixed rotavirus-infected cell plates and that on semipurified rotavirus antigen-coated plates were similar, but the overall numbers of ASC were higher on the former plates. Hence, only the results of the fixed cell plates are summarized in the figures (Fig. 1 and 2) and in Table 2. Following cycloheximide treatment, the number of spots decreased by 60 to 100%, indicating that antibody secretion was the result of de novo synthesis by ASC. No virus-specific ASC were detected at PID 0 from any pigs. In the virulent-rotavirus-inoculated group, IgM rotavirus-specific ASC predominated in all tissues at PID 8, with the greatest numbers in the ileum (Fig. 1, 45 ASC per 5×10^5 MNC). By PID 21, the numbers of IgM ASC had declined significantly in all tissues (5- to 12-fold decrease) (Fig. 1 and 2). In the attenuated-Wa rotavirus-inoculated group, significantly lower mean numbers of IgM ASC were observed in all tissues (except PBL) compared with those observed with the virulent-rotavirus-inoculated pigs at PID 8 (Fig. 1 and 2). After challenge, few virus-specific IgM ASC were detected in the tissues from either virus-inoculated group (Fig. 1 and 2).

At challenge (PID 21 [PCD 0]), the numbers of virus-specific IgA and IgG ASC were 5- to 19-fold greater in the small intestinal lymphoid tissues than in the systemic lymphoid tissues for both groups (Fig. 1 and 2 and Table 2). The numbers of virus-specific IgA ASC in the intestinal lymphoid tissues and blood and the numbers of IgG ASC in the MLN of the virulent-Wa rotavirus-inoculated pigs were significantly greater (5- to 11-fold) than those of the attenuated-Wa rotavirus-inoculated pigs (Fig. 1 and 2 and Table 2).

After challenge, in pigs inoculated with virulent rotavirus, only small (≤2-fold), transient, or no increases in the numbers of virus-specific IgA and IgG ASC and serum VN antibody GMT occurred (Fig. 1 and 2; Tables 1 and 2). In contrast, in attenuated-Wa rotavirus-inoculated pigs, numbers of ASC in intestinal lymphoid tissues increased significantly (>7-fold for IgA and >3.7-fold for IgG) at PCD 4. Even greater increases

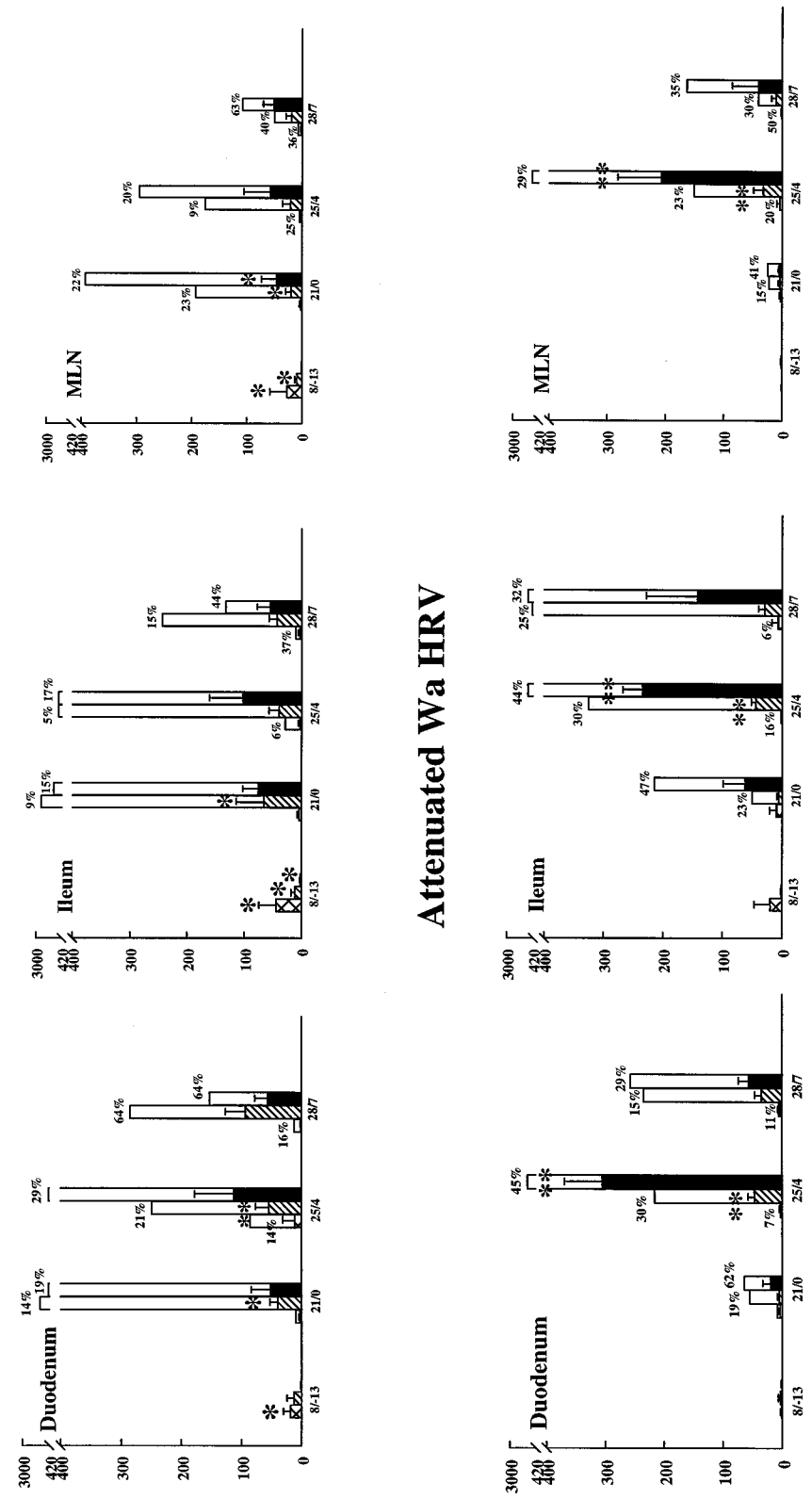

FIG. 1. Isotype-specific ASC to Wa human rotavirus strain (Wa HRV) in gnotobiotic pigs following oral inoculation and challenge with Wa HRV. MNC from duodena, ilea, and MLN of pigs were collected and assayed by ELISPOT on PID 8, 21, 25, and 28 (PCD −13, 0, 4, and 7, respectively). Data are the mean numbers of Wa HRV-specific ASC and total IgSC per 5×10^5 MNC for three to six pigs at each time point (except the total IgSC at PID 8, which were not tested). Percentages above total-IgSC data are the averages of Wa HRV-specific ASC/total IgSC (×100) at the time point. An asterisk denotes significant difference ($P < 0.05$) in numbers of ASC between virulent- and attenuated-Wa rotavirus-inoculated pigs at the same PID-PCD. Two asterisks denote significant difference ($P < 0.05$) in numbers of ASC before challenge (PID 21 [PCD 0]) and after challenge (PID 25 [PCD 4]) in the same group. ▩, IgM; ▨, IgA; ■, IgG; □, total IgSC.

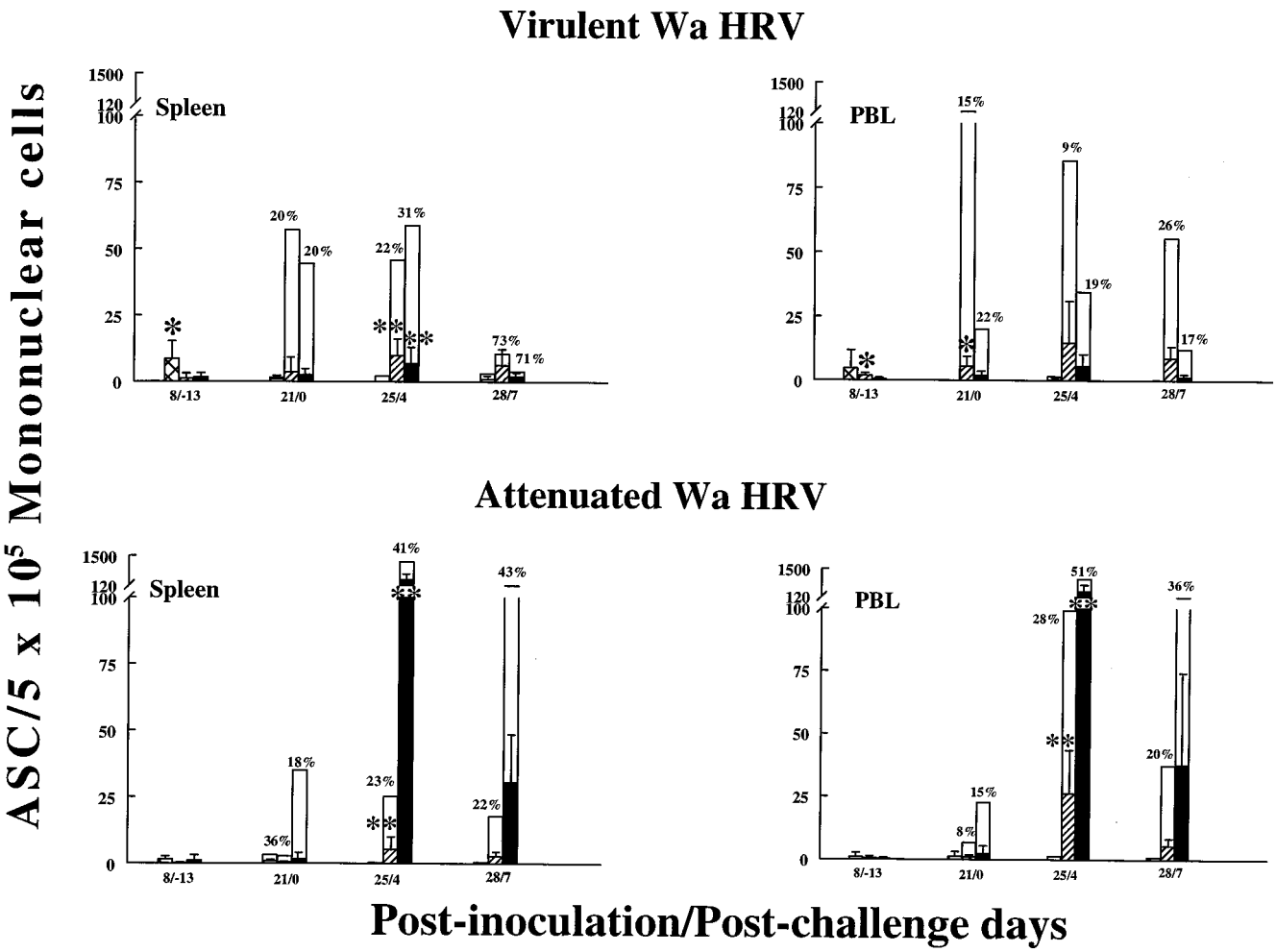

FIG. 2. Isotype-specific ASC to Wa human rotavirus strain (Wa HRV) in gnotobiotic pigs following oral inoculation and challenge with Wa HRV. MNC from spleens and PBL of pigs were collected and assayed by ELISPOT on PID 8, 21, 25, and 28 (PCD −13, 0, 4, and 7, respectively). Data are the mean numbers of Wa HRV-specific ASC and total IgSC per 5×10^5 MNC for three to six pigs at each time point (except the total IgSC at PID 8, which were not tested). Percentages above total-IgSC data are the averages of Wa HRV-specific ASC/total IgSC (×100) at the time point. An asterisk denotes significant difference ($P < 0.05$) in numbers of ASC between virulent- and attenuated-Wa rotavirus-inoculated pigs at the same PID-PCD. Two asterisks denote significant difference ($P < 0.05$) in numbers of ASC before challenge (PID 21 [PCD 0]) and after challenge (PID 25 [PCD 4]) in the same group. ▩, IgM; ▨, IgA; ■, IgG; □, total IgSC.

in the numbers of ASC were observed in the systemic lymphoid tissues, especially in blood (IgG ASC increased about 200-fold, and IgA increased 26-fold). The intestinal IgG/IgA ASC ratios for pigs given attenuated Wa rotavirus were higher than those for pigs given virulent Wa rotavirus (range, 4 to 10.3 and 1.2 to 2.6, respectively) (Table 2). The IgG/IgA ratios for the spleens of the attenuated-rotavirus-inoculated pigs were 110-fold (77 versus 0.7) higher than in virulent-rotavirus-inoculated pigs at PCD 4. In the blood of virulent-rotavirus-inoculated pigs, IgA ASC were predominant at all time points tested, with the blood IgG/IgA ratios less than 1.0 (range, 0.16 to 0.4). By comparison, blood IgG/IgA ratios for attenuated-rotavirus-inoculated pigs ranged from 0.7 to 2.0 before challenge and 7 to 15.6 after challenge (Table 2).

Protection from disease correlated positively with the numbers of IgA ASC present in the small intestine at the time of challenge (PID 21 [PCD 0]) (Spearman's rank correlation test; $P < 0.05$). The correlation coefficients were 0.9 for duodenum and ileum, 0.8 for MLN, 0.6 for blood, and 0.5 for spleen. In addition, the numbers of IgA, but not IgG, ASC in the small intestine correlated with the numbers of IgA ASC in blood at PID 21. The correlation coefficients were 0.7 for ileum, 0.6 for duodenum and MLN, and 0.48 for spleen.

In longer-term pigs inoculated with virulent Wa rotavirus, numbers of virus-specific IgA ASC in the small intestine and blood at PID 43 (PCD 0) (112 and 63 ASC per 5×10^5 MNC, respectively) and PID 50 (PCD 8) (249 and 44 ASC per 5×10^5 MNC, respectively) were greater (~1.7- to 10.5-fold) than those of pigs inoculated with virulent Wa rotavirus (group 1) at PID 21 (PCD 0) (53 and 6 ASC per 5×10^5 MNC, respectively) and PID 25 (PCD 4) (47 and 15 ASC per 5×10^5 MNC, respectively) (Table 2). In the two sham-inoculated pigs, at PCD 7, the numbers of virus-specific IgA ASC in the small intestines and blood were much lower than those of the two virus-inoculated pigs at PID 50 (PCD 8) (26 versus 249 and 17 versus 44 per 5×10^5 MNC, respectively) but were greater than those of group 1 pigs at PID 7 (26 versus 12 and 17 versus 2 per 5×10^5 MNC, respectively).

Total IgSC responses. The total IgSC responses at PID 8 were not tested (insufficient numbers of MNC were obtained). At PID 21, the numbers of total IgG and IgA IgSC in the intestines of the virulent-Wa rotavirus-inoculated pigs were

TABLE 2. Peak isotype-specific ASC responses to rotavirus after inoculation and challenge of gnotobiotic pigs with Wa human rotavirus

Wa rotavirus inoculum group[a] and ASC isotype	No. of ASC (mean ± SEM)/5 × 10^5 MNC[b]									
	Duodenum		Ileum		MLN		Spleen		PBL	
	PID 21	PCD 4	PID 21	PCD 4	PID 21	PCD 4	PID 21	PCD 4	PID 21	PCD 4
Virulent										
IgG	52 ± 31	113 ± 65	76 ± 27	103 ± 60	46 ± 28	58 ± 55	3 ± 3	7 ± 5	2 ± 1	6 ± 4
IgA	40 ± 18	55 ± 25	66 ± 45	39 ± 17	20 ± 10	21 ± 16	4 ± 5	10 ± 6	6 ± 5	15 ± 16
G/A[c]	1.3	2.1	1.2	2.6	2.3	2.8	0.8	0.7	0.3	0.4
Attenuated										
IgG	20 ± 13	305 ± 6	62 ± 46	234 ± 48	4 ± 2	207 ± 77	2 ± 2	385 ± 19	2 ± 3	405 ± 23
IgA	5 ± 5	47 ± 14	6 ± 4	44 ± 7	4 ± 2	32 ± 21	1 ± 0.7	5 ± 6	1 ± 0.8	26 ± 17
G/A	4.0	6.5	10.3	5.3	1.0	6.5	2.0	77.0	2.0	15.6

[a] Data represent four to six pigs for each tissue at each time point.
[b] Numbers of Wa rotavirus-specific ASC were assessed by ELISPOT at PID 21 (PCD 0) and PCD 4 (PID 25) in duodenum, ileum, mesenteric lymph nodes (MLN), spleen, and PBL.
[c] G/A = ratio of IgG to IgA based on numbers of ASC per 5 × 10^5 MNC.

greatest and significantly higher than those of the attenuated-rotavirus-inoculated pigs (Fig. 1 and 2). Numbers of total IgA and IgG IgSC in spleens and blood were also greater (but not significantly greater, compared with those of pigs inoculated with attenuated Wa rotavirus) in virulent-Wa rotavirus-inoculated pigs at this time. After challenge, numbers of total IgA and IgG IgSC in attenuated-Wa rotavirus-inoculated pigs increased markedly in all tissues (3- to 12-fold for total IgA IgSC and 5- to 42-fold for total IgG IgSC). The virus-specific IgG and IgA ASC constituted 5 to 73% of the numbers of total IgG and IgA IgSC for the virus-inoculated pigs (Fig. 1 and 2).

DISCUSSION

Our laboratory and others have documented the usefulness of the gnotobiotic pig as a model for studies of human rotavirus infection, disease, pathogenesis, and passive immunity (6, 22, 40, 43, 44, 50, 51). To our knowledge, this is the first report to identify intestinal IgA ASC as a correlate of protective active immunity in an animal model of human-rotavirus-induced disease. A single exposure of pigs to the virulent Wa rotavirus produced significant virus-specific ASC responses in intestinal lymphoid tissues of pigs and conferred 100% protection against rotavirus infection (shedding) and 87% protection against disease upon challenge with homologous virus (Table 1). The preliminary longer-term experiment showed that this protective immunity was present at least 6 weeks after inoculation and that IgA ASC were present in large numbers in the intestinal lamina propria. Our results concur with studies indicating that natural symptomatic and asymptomatic rotavirus infections in infants in the first year of life confer a high level of protection (81 to 93% protection rates) against rotavirus-associated diarrhea upon subsequent reexposure (4, 52). Similarly, others have shown that inoculation of various species (mice, rabbits, and pigs) with virulent rotaviruses of homologous host origin induced protection against challenge with the corresponding virulent rotaviruses (9, 10, 11, 54). Thus, humans and animals that recover from infection with virulent rotaviruses develop a high level of protective immunity at least against the infecting serotype.

The inoculation of pigs with attenuated Wa human rotavirus mimics the Jennerian approach to rotavirus vaccines based on the use of live attenuated heterologous rotaviruses given twice orally to human infants (12, 21, 24, 49). In our study, two doses of live attenuated Wa rotavirus induced smaller numbers of virus-specific ASC in intestinal and systemic lymphoid tissues than a single dose of virulent Wa rotavirus. In particular, significantly smaller numbers of intestinal IgA ASC in attenuated-Wa rotavirus-inoculated pigs coincided with only partial protection from infection and disease. Our results for attenuated-Wa rotavirus-inoculated pigs (33% protection rate against disease) are comparable to results for asymptomatic infections of human newborns with an unusual G9P11 (common bovine type P11) rotavirus which conferred a 46% protection rate against subsequent rotavirus-associated diarrhea (5). Various rates of protection (60 to 93%) against severe diarrhea have also been reported with the use of live oral rotavirus vaccines of heterologous bovine or rhesus origin in clinical trials (49).

The failure of the attenuated Wa rotavirus to elicit complete protection against virulent Wa rotavirus challenge in gnotobiotic pigs may relate to the limited replication of the attenuated virus in the small intestine as evidenced by only 6% of pigs shedding rotavirus after inoculation with attenuated virus compared with 100% of pigs shedding rotavirus after inoculation with virulent rotavirus (Table 1) and lack of detection of rotavirus antigen in the intestines of attenuated-Wa rotavirus-inoculated pigs (50). In an adult mouse model, mice were inoculated with a series of reassortants between murine rotavirus EDIM (epidemic diarrhea of infant mice) and heterologous rhesus rotavirus strains (29). The protection against virulent murine rotavirus challenge (virus shedding) was correlated with the extent of primary intestinal replication of virus as determined by fecal shedding. Such data suggest that the efficacy of a live attenuated rotavirus strain in an oral vaccine may depend on the virus's ability to replicate sufficiently within the intestine. However, if virus replication cannot be enhanced without a concomitant increase in virulence, then the use of higher doses of such viruses in oral vaccines in combination with mucosal adjuvants and/or microencapsulation procedures may result in better clinical protection in future vaccine trials.

There was a strong correlation (0.8 to 0.9) between the degree of protection and the numbers of the small-intestinal virus-specific IgA ASC at the time of challenge (PID 21). This finding is consistent with results from naturally infected infants and experimentally infected animals, indicating an association between protection against rotavirus infection or disease and levels of intestinal and/or fecal rotavirus-specific IgA antibodies. In children with natural rotavirus exposure, preexposure titers of fecal IgA antibodies to rotavirus in uninfected children were significantly higher than in symptomatic infected chil-

dren, and they were also higher in asymptomatic infected children than in symptomatic infected children (13, 14, 28). In studies of homologous rotavirus infection in mice, protection against virulent homologous rotavirus challenge correlated with the presence of fecal or intestinal IgA antibodies to rotavirus (9, 18).

Although titers of VN and IgG antibodies to rotavirus in serum have been correlated with protection against infection or disease in humans in some studies (35, 53), our results and those of other studies with children, calves, and mice did not show a correlation between high levels of protection and serum VN antibody titers and intestinal or systemic rotavirus-specific IgG ASC responses (4, 18, 54, 55). A correlation between serum rotavirus IgA antibody titers and protection against rotavirus infection and disease in mice (29) and in infants with natural rotavirus infection (35) has been reported. In our study, the numbers of virus-specific IgA ASC, but not IgG ASC, in blood were significantly greater at challenge (PID 21 [PCD 0]) in those pigs which were subsequently protected (previously exposed to virulent rotavirus). Although numbers of virus-specific IgA ASC in the blood were significantly smaller than those in the intestine, there was a correlation (0.6) between numbers of rotavirus-specific IgA ASC in the blood and protection and between numbers of IgA ASC in the blood and in the intestine (0.6 to 0.7). The presence of the virus-specific IgA ASC circulating in peripheral blood after oral administration of virulent rotavirus may reflect their origin in intestinal lymphoid tissues and their brief trafficking in the peripheral blood in transit from the MLN back to the intestinal lamina propria (2, 16, 37). Thus, the numbers of rotavirus-specific IgA ASC in blood, albeit transient, may thus be more reliable correlates of protective immunity to rotavirus than serum VN antibody titers for evaluation of candidate human rotavirus vaccines. An association between serum and fecal IgA antibody and IgA secretion in the duodenum in young children has been reported (20). Determination of the titers of virus-specific serum and fecal IgM, IgA, and IgG antibodies to Wa human rotavirus in the test pigs is in progress in our laboratory (46). These results will permit analysis of the correlation between the numbers of IgM, IgA, and IgG ASC in the blood and intestine and the isotype-specific antibody responses in serum and feces.

Several investigations have shown that after rotavirus or coronavirus enteric infections, virus-specific IgA ASC prevailed over IgG ASC in the intestinal lamina propria (10, 25, 32, 47). In mice inoculated with simian rotavirus, the magnitude of the intestinal IgA ASC response was about 10 times greater than the IgG ASC response (32). The numbers of rotavirus-specific IgA ASC in the duodena (40 ± 18) and ilea (66 ± 45) of pigs inoculated with human rotavirus in this study (Table 2) are similar to the numbers of IgA ASC from porcine-rotavirus-inoculated pigs (83 ± 38 in duodenum, 63 ± 22 in ileum; reported by Chen et al. [10]) at PID 21. However, our data showed larger numbers of virus-specific IgG ASC in all tissues examined. Following inoculation with virulent Wa rotavirus, the numbers of IgG ASC were comparable to those of IgA ASC in the intestinal lamina propria, resulting in an IgG/IgA ratio of ~1.2 at PID 21 (Table 2). In contrast, the IgG/IgA ratios for tissues from pigs given attenuated Wa rotavirus were higher (range, 1 to 77) at PID 21, reflecting the greater predominance of IgG over IgA ASC. The greatest differences in the IgG/IgA ratios between the virulent- and attenuated-rotavirus-inoculated pigs were observed for the systemic lymphoid tissues of pigs after challenge (Table 2). After a virulent enteric coronavirus challenge, VanCott et al. (47) also observed smaller numbers of IgA ASC and larger numbers of IgG ASC in the intestines of pigs which had been previously inoculated with an attenuated respiratory coronavirus but a predominance of IgA ASC in systemic and intestinal tissues of pigs previously inoculated with a virulent enteric coronavirus. Explanations for the greater IgG ASC responses to attenuated Wa rotavirus are uncertain but raise questions as to a possible extraintestinal antigenic stimulation by this virus.

After homologous challenge of the virulent-Wa rotavirus-inoculated pigs, the numbers of rotavirus-specific IgA and IgG ASC (except in the spleen and except for IgA ASC in the duodenum) and serum VN antibody GMTs were not significantly boosted, and pigs were completely protected from rotavirus shedding (Tables 1 and 2). Our results agree with observations made with pigs (10), mice (9), and rabbits (11), inoculated and challenged with homologous rotaviruses, showing that the absence of significant increases in serum or intestinal antibody responses to rotavirus upon homologous rotavirus challenge is associated with the presence of protective immunity.

An analysis of relatively short-term protection (3 to 6 weeks) may be a limitation of this model, relative to human vaccine trials. However, to date, there is no other animal model susceptible to infection and disease with human rotavirus, even for this limited period. From the few longer-term pigs inoculated with virulent Wa human rotavirus, we found that the numbers of virus-specific IgA ASC in intestines and blood were increased (1.7- to 10.5-fold) at PID 43 and postchallenge compared with the corresponding responses of the group 1 pigs at PID 21 and postchallenge. In children, rotavirus-specific IgA antibody titers in the duodenum and serum were significantly elevated in convalescent-phase serum compared with those in acute-phase serum (17). In a mouse model, virus-specific IgA ASC were present for more than 1 year (45). The persistence of virus-specific IgA and IgG ASC in pigs and their correlation with long-term protective immunity need further investigation.

The total-IgSC responses in our study paralleled the virus-specific ASC responses of the different tissues at different PID-PCD, and the IgG/IgA ratios for total IgSC (data not shown) closely mirrored the rotavirus-specific IgG/IgA ASC ratios. The numbers of IgA IgSC were largest in the intestinal lamina propria of virulent-Wa rotavirus-inoculated pigs, reflecting the predominance of IgA plasma cells in the intestines (2, 41). The percentages of IgA and IgG rotavirus-specific ASC to total IgSC (5 to 73%) in this study were comparable to those obtained from investigations with mice (25, 32). In mice inoculated with a heterologous simian rotavirus, virus-specific IgA ASC constituted ~14% (24) or ~50% (32) of total IgA IgSC in the intestine. In the ilea of virulent-Wa rotavirus-inoculated pigs, the smaller percentages (9% at PID 21 [PCD 0]; 5% at PID 25 [PCD 4]) of IgA rotavirus-specific ASC reflected the increased magnitude of total IgA IgSC (largest numbers among all tissues). Increased numbers of IgA IgSC in the intestine may reflect nonspecific triggering of IgA B cells due to exposure to dietary antigens after extensive villous atrophy (especially severe in the ileum) following infection with virulent Wa rotavirus (6, 50). By comparison, the overall greater percentages of virus-specific IgA ASC relative to total IgA IgSC (15 to 30%) in the intestines of the attenuated-Wa rotavirus-inoculated pigs may be due to virus being the main antigenic stimulus to the intestinal immune system of these pigs, because no intestinal disruption (villous atrophy) was seen following inoculation with attenuated Wa human rotavirus (50).

In summary, we used the neonatal gnotobiotic pig model of rotavirus disease to compare the induction of active immunity to a virulent human rotavirus with that to an orally adminis-

tered live attenuated human rotavirus in an attempt to identify correlates of protective immunity. Our findings have important implications for developing and evaluating new rotavirus vaccination strategies. In addition, this model provides an in vivo challenge system to examine new approaches (microencapsulation, mucosal adjuvants, etc.) for enhancing immune responses to candidate rotavirus vaccines.

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