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Development of Mucosal and Systemic Lymphoproliferative Responses and Protective Immunity to Human Group A Rotaviruses in a Gnotobiotic Pig Model†

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Gnotobiotic pigs were orally inoculated with virulent Wa strain (G1P1A[8]) human rotavirus (group 1), attenuated Wa rotavirus (group 2) or diluent (controls) and were challenged with virulent Wa rotavirus 21 days later. On various postinoculation or postchallenge days, virus-specific responses of systemic (blood and spleen) and intestinal (mesenteric lymph node and ileal lamina propria) mononuclear cells (MNC) were assessed by lymphoproliferative assays (LPA). After inoculation, 100% of group 1 pigs and 6% of group 2 pigs shed virus. Diarrhea occurred in 95, 12, and 13% of group 1, group 2, and control pigs, respectively. Only groups 1 and 2 developed virus-specific LPA responses prior to challenge. Group 1 developed significantly greater mean virus-specific LPA responses prior to challenge and showed no significant changes in tissue mean LPA responses postchallenge, and 100% were protected against virulent virus challenge. By comparison, both group 2 and controls had significantly lower LPA responses at challenge and both groups showed significant increases in mean LPA responses postchallenge. Eighty-one percent of group 2 and 100% of control pigs shed challenge virus, and both groups developed diarrhea that was similar in severity postchallenge. The virus-specific LPA responses of blood MNC mirrored those of intestinal MNC, albeit at a reduced level and only at early times postinoculation or postchallenge in all pigs. In a separate study evaluating antibody-secreting-cell responses of these pigs (L. Yuan, L. A. Ward, B. I. Rosen, T. L. To, and L. J. Saif, J. Virol. 70:3075–3083, 1996), we found that the magnitude of a tissue's LPA response positively correlated with the numbers of virus-specific antibody-secreting cells for that tissue, supporting the hypothesis that the LPA assesses T-helper-cell function. The magnitude of LPA responses in systemic and intestinal tissues also strongly correlated with the degree of protective immunity elicited by the inoculum ($\rho = 0.81$). We conclude that blood may provide a temporary "window" for monitoring intestinal T cells and that the LPA can be used to assess protective immunity to human rotaviruses.

Rotaviruses are the single most important cause of diarrhea in children younger than 5 years of age in both developed and developing countries, resulting in some 870,000 deaths and several million cases of severe diarrhea in this age group annually (12, 19, 21). A vaccine, targeted for use in early infancy, may be the most effective means of controlling rotavirus diarrhea (12, 19, 21). The current goal of rotavirus vaccination is to induce immunity which is at least as efficacious as that elicited by naturally acquired infections (20). Naturally occurring rotavirus infections in infancy prevent severe diarrhea following subsequent infections (3, 4). Hence, a successful rotavirus vaccine will reduce the occurrence of severe dehydrating diarrhea that leads to death, thereby eliminating the major threat posed by rotavirus infections in young children. Unfortunately, all candidate rotavirus vaccines to date have failed in one or more aspects of safety, immunogenicity, or efficacy testing in infants, although recent trials utilizing a quadrivalent human rotavirus vaccine hold promise (12, 19, 21). The failure of candidate vaccines to provide the desired level of protection underscores the need for a better understanding of both humoral and cellular immune responses to human rotavirus infections and identification of the host factors involved in protection against disease.

Because of the limitations imposed on investigators studying the natural host of the human rotaviruses (infants and young children), animal models have been used to study and define mucosal immune responses and to assess development of protective immunity to these viruses. Mice serve as useful models for the evaluation of rotavirus-specific immunity but not protection from disease, which is the current goal for human rotavirus vaccination. Inoculation of neonatal mice (<14 days old) with rotaviruses from other animal species (heterologous viruses) can result in subclinical infection, but expression of disease appears to require administration of high virus doses (10, 13, 14, 42). Furthermore, mice that are >14 days old are refractory to disease after both homologous (murine) and heterologous rotavirus inoculation, regardless of dose (10, 13, 14, 42), thereby restricting assessment of protective immunity to prevention of shedding only. In addition, only G3 human rotavirus serotypes have been reported to infect and cause disease in neonatal mice, and then only at high doses ($>10^6$ PFU) compared with murine strains (<10 PFU) (10, 13, 14, 42). Rabbits pose similar problems in being refractory to rotavirus-associated disease at an early age after homologous rotavirus infection (11, 16) and develop no evidence of either infection or disease following oral inoculation with human rotavirus (23).

Although the gnotobiotic pig model of rotavirus infection

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and disease is limited in use because of the nature of the model (expense, requirement of special facilities, etc.), it offers several important advantages over mouse and rabbit models. Because there is no placental transfer of maternal antibodies in swine, the true primary immune response to human rotavirus infection may be defined first and the influence of other factors (such as maternal antibodies) may be determined later (25). The gnotobiotic environment ensures that extraneous rotaviruses and enteric pathogens are eliminated as confounding variables. Human rotavirus strains belonging to each of the four major G serotypes (G1 to G4) associated with disease in young children appear to infect gnotobiotic pigs (20, 33a, 35, 37, 41, 45, 48). Gnotobiotic pigs remain susceptible to rotavirus-associated disease for as long as 6 weeks after oral inoculation with human (G1 and G3) as well as porcine (G3, G4, G5, and G11) rotavirus strains (15, 20, 35, 36, 41, 45). Neonatal pigs and human infants also share many similarities in their gastrointestinal physiology, milk diets, and immune development (22, 25, 30). Thus, rotavirus-specific immune responses of neonatal pigs after oral inoculation with human rotaviruses may be predictive of the immune response in infants after natural rotavirus infection or oral vaccine administration.

Although large numbers of T cells exist within the intestinal lymphoid tissues, at both inductive and effector immune sites, relatively little is known about their role in protection against human rotavirus-associated disease. Therefore, the development of intestinal and systemic T-cell responses to human group A rotavirus and identification of the cellular correlates of protection to homologous rotavirus challenge were investigated in this study by using the gnotobiotic pig model of human rotavirus-associated disease. The Wa strain (G1P1A[8]) of human rotavirus was selected for these studies because G1 is the most frequent G serotype associated with diarrheal disease in infants and young children worldwide (2, 24) and gnotobiotic pigs are susceptible to Wa rotavirus for an extended time period, thereby allowing assessment of protective immunity in terms of both shedding and diarrhea (35, 36, 41). In addition, a derivative of the virulent strain of Wa rotavirus has been attenuated for the gnotobiotic pig through serial cell culture passage (41, 45). The availability of attenuated and virulent human virus strains permits comparative immunologic studies to be performed by using the attenuated Wa rotavirus to mimic live oral vaccine exposure and the virulent Wa rotavirus to mimic natural exposure.

MATERIALS AND METHODS

Cells and viruses. The Wa strain (G1P1A[8]) of human rotavirus from a diarrheic infant stool specimen (obtained from R. G. Wyatt, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.) was passaged in gnotobiotic pigs (36, 45). A pool of intestinal contents from the 16th gnotobiotic pig passage of Wa rotavirus was prepared in minimal essential medium (MEM; Gibco, Life Technologies, Grand Island, N.Y.) for use as the virulent Wa rotavirus inoculum. The virulent Wa rotavirus was adapted to cell culture after 11 passages in gnotobiotic pigs (45). A cell lysate pool obtained from the 27th passage of the cell culture-adapted Wa rotavirus in fetal rhesus monkey kidney cells (MA104 cells) was used as the attenuated Wa rotavirus inoculum. The in vitro infectivity titers of the Wa rotavirus inocula were determined by cell culture immunofluorescence (CCIF) assays (5). The in vivo infectivity of each Wa rotavirus inoculum in gnotobiotic pigs was determined as the lowest dose of virulent Wa rotavirus that caused virus shedding and diarrhea in 50% of inoculated pigs (median infectious dose [ID_{50}]) or the lowest dose of attenuated Wa rotavirus (administered twice 10 days apart) that elicited virus-neutralizing (VN) serum antibodies by post-primary inoculation day (PID) 21 in 50% of inoculated pigs (median seroconversion dose [SD_{50}]). The ID_{50} of the virulent Wa rotavirus inoculum was at least 1 fluorescence focus-forming unit (FFU), whereas the SD_{50} of the attenuated Wa rotavirus inoculum was $\sim 1.3 \times 10^6$ FFU (41).

Gnotobiotic pigs and experimental design. Near-term pigs were derived and maintained in gnotobiotic isolator units as described elsewhere (26). At 3 to 5 days of age, the pigs were fed 5 ml of 100 mM sodium bicarbonate to reduce gastric acidity (15) and were orally inoculated 10 min later with $\sim 10^5$ FFU ($\sim 10^5$ ID_{50}) of virulent Wa rotavirus (group 1), with $\sim 2 \times 10^7$ FFU (~ 10 SD_{50}) of attenuated Wa rotavirus (group 2), or with an equal volume of MEM diluent (controls). Pigs receiving attenuated Wa rotavirus were reinoculated with the same dose ($\sim 2 \times 10^7$ FFU) 10 days later. At PID 21, pigs from each group were challenged with $\sim 10^6$ FFU ($\sim 10^6$ ID_{50}) of virulent Wa rotavirus. A total of 6 to 13 pigs from each virus-inoculated group and 2 to 3 control pigs were euthanized at the following intervals: PID 5 to 9 and 19 to 21 and postchallenge days (PCD) 3 to 4 and 7 to 9. Because of the difficulties associated with the maintenance of large pigs in the gnotobiotic isolator units, a single group of 6 pigs was used for the study of protective immunity to Wa rotavirus at ~7 weeks of age, consisting of 4 pigs inoculated with $\sim 10^5$ FFU of virulent Wa rotavirus and 2 pigs sham inoculated with MEM as controls at 5 days of age. Two of the virulent Wa rotavirus-inoculated longer-term pigs were euthanized at PID 43 (not challenged), and 2 pigs were challenged with virulent Wa rotavirus (5×10^6 FFU) at PID 42 and were euthanized 8 days later (PCD 8). The 2 sham-inoculated longer-term control pigs were also challenged with virulent Wa rotavirus (5×10^6 FFU) at PID 42 and were euthanized at PCD 7. Weekly blood samples and daily rectal swabs were collected from all pigs after inoculation and challenge. The consistency of each pig's feces was scored as follows and recorded daily: 0, normal (no diarrhea); 1, pasty or mucoid (mild diarrhea); 2, semiliquid (moderate diarrhea); and 3, liquid, (severe diarrhea). Blood, spleens, small intestines (ilea), and mesenteric lymph nodes (MLN) were collected from all pigs at euthanasia for isolation of mononuclear cells (MNC).

Isolation of MNC from blood and tissues. Blood was collected aseptically in ~25% acid citrate glucose (vol/vol), and peripheral blood lymphocytes were obtained by density gradient centrifugation (Ficoll-Paque 1.077 density; Sigma Chemical Co., St. Louis, Mo.) as described by Boyum (6). The MNC at the interface were collected, washed in Ca^{2+} Mg^{2+}-free Hanks' balanced salt solution, and suspended in RPMI 1640 (Gibco) enriched with 10% fetal bovine serum, 5.5 mM glutamine, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 10 μg of ampicillin and 0.5 mg of gentamicin per ml (enriched medium).

Splenic and MLN MNC were obtained by a modification of the procedure described by VanCott et al. (40). Tissues were collected and single cell suspensions were obtained by mechanical disruption of tissues through stainless steel wire mesh screens (Cell-Selector; E-C Apparatus Corp., St. Petersburg, Fla.) as described elsewhere (40). Cell suspensions were subjected to discontinuous Percoll (Sigma) gradient centrifugation ($1,800 \times g$, 30 min, 4°C). The MNC were collected from the 43%/70% interface, washed, and suspended in enriched medium.

Isolation of MNC from the small intestinal (ileum) lamina propria was done by a modification of published procedures (33, 40). Approximately 20 g of ileum was collected as described elsewhere (40), placed in 200 ml of wash solution containing 1 mM dithiothreitol and 10 mM EDTA, and vigorously shaken for 30 min (37°C) to dislodge epithelial cells. After removal of epithelial cells, the tissues were finely minced into 1-mm pieces, suspended in 100 ml of enriched medium containing 300 U of type II collagenase (Sigma C-2139) per ml, and subjected to two 15-min digests (37°C) with gentle shaking. Digest supernatants were saved, and undigested tissues were forced through stainless steel wire mesh screens to obtain single cell suspensions (34). The digest supernatants and cell suspensions were pooled and centrifuged ($1,000 \times g$, 20 min, 4°C), and cell pellets were subjected to Percoll gradient centrifugation as described for splenic MNC.

LPA for detection of Wa rotavirus-specific T cells. The lymphoproliferative assay (LPA) was adapted from the method described by Brim et al. (7) with semipurified attenuated Wa rotavirus as antigen (Wa antigen). The Wa antigen was obtained by differential centrifugation of a pool of Wa rotavirus-infected MA104 cell lysates through 40% sucrose (concentrated ~50 times; titer prior to concentration was 10^7 FFU/ml). Control antigen was prepared similarly from mock-infected MA104 cell lysates. Optimum antigenic stimulation was obtained after ~110 h of incubation (37°C, 5% CO_2) by using 5×10^5 lymphocytes per well and 7.5 μg of Wa antigen per ml (final concentration). All MNC were stimulated in quadruplicate with Wa antigen, control antigen, and phytohemagglutinin (PHA [Gibco]) at a final concentration of 5 μg/ml on 96-well plates. Each well was labeled with 1 μCi of [^{3}H]thymidine (ICN Biomedicals, Irvine, Calif.) for 18 h prior to harvesting. The stimulation index (SI) of each MNC assayed was calculated as SI = cpm of Wa antigen-stimulated wells/mean cpm of control antigen-stimulated wells, where cpm is counts per minute. The mean counts per minute for each pig's MNC populations were adjusted by subtracting background counts per minute (mean cpm of Wa antigen-stimulated wells − mean cpm of control antigen-stimulated wells). The adjusted counts-per-minute value for each tissue from an individual pig was used for calculating the group mean count per minute for a given tissue, and all reported LPA data represent each group's mean counts per minute for a given lymphoid tissue at each PID or PCD.

Plaque reduction neutralization assay for detection of VN antibodies. The plaque reduction virus neutralization assay was done as described elsewhere (36). VN antibody titers in serum were calculated as the reciprocal of the serum dilution which reduced the number of plaques by >80%. Titers of <4 were assigned a value of 2 for calculation of the group geometric mean titer (GMT).

CCIF assay for detection of infectious Wa rotavirus. Diluted (~1:25) fecal suspensions were prepared in MEM from the daily rectal swabs collected from

TABLE 1. Summary of fecal virus shedding and clinical disease in gnotobiotic pigs after oral inoculation with live virulent Wa rotavirus (group 1), live attenuated Wa rotavirus (group 2), or diluent (controls)[a]

Pig group (*n*)	Value for virus shedding[b]			Value for diarrhea[c]			
	Total (%)	Mean onset (days)	Mean duration (days)	Total (%)	Mean onset (days)	Mean duration (days)	Avg additive score
1 (43)	100^{A}	1.9 (0.1)^{A}	6.0 (0.3)^{A}	95^{A}	1.9 (0.2)^{A}	4.1 (0.4)^{A}	10.4 (0.5)^{A}
2 (51)	6^{B}	2.5 (1.7)^{A}	1.5 (0.3)^{B}	12^{B}	2.5 (0.5)^{A}	0.7 (0.1)^{B}	3.8 (0.4)^{B}
Controls (18)	NA	NA	NA	13^{B}	3.0 (1.4)^{A}	0.5 (0.2)^{B}	4.0 (1.0)^{B}

[a] Pigs were inoculated at 3 to 5 days of age. These data have been presented in part by Yuan et al. (46). Values in columns with different superscript letters differ significantly ($P < 0.05$); NA, not applicable. Numbers in parentheses are ± 1 SEM.
[b] Determined by antigen-capture ELISA.
[c] Onset and duration of moderate to severe diarrhea (fecal consistency score of ≥2); average additive score = (daily fecal scores from PID 1 to 8)/*n*.

each pig and were clarified by centrifugation prior to assay (800 × *g*, 20 min, 4°C). The CCIF assay was done with the prepared fecal suspensions as described above by using MA104 cell monolayers in 96-well plates, group A rotavirus-specific fluorescein isothiocyanate-conjugated pig antiserum, and fluorescence microscopy (5).

ELISA for detection of Wa rotavirus antigen in feces. Prepared fecal suspensions were evaluated in duplicate by antigen-capture enzyme-linked immunosorbent assay (ELISA) as described elsewhere (18, 34). Rotavirus-negative and -positive fecal controls were included on each plate. A sample was positive if the mean absorbance of replicate samples on rotavirus antibody-coated wells was greater than the mean absorbance of replicate samples on negative control wells ± 3 standard deviations. This ELISA detected ~10^2 FFU of attenuated Wa rotavirus per ml suspended in MEM and was hence similar in sensitivity to the CCIF assay.

Statistical analyses. Analyses were performed pre- and postchallenge for the following treatment groups: group 1 (virulent Wa rotavirus), group 2 (attenuated Wa rotavirus), and control pigs (diluent). Kruskal-Wallis nonparametric analysis of variance was used to identify differences in virus shedding and diarrhea between treatment groups; the Wilcoxon-Mann-Whitney test was used to determine the significance of differences identified. To establish the significance of each individual MNC LPA response, each tissue's SI was tested by Student's *t* test. Comparisons between LPA responses by groups, tissues, and days were done by one-way analysis of variance after rank transformation of the adjusted counts-per-minute data. All tissue counts per minute data obtained from each group at euthanasia intervals PID 5 to 9, 19 to 21 (PCD 0), 24 to 25 (PCD 3 to 4), and 28 to 30 (PCD 7 to 9) were analyzed collectively and are reported as the group mean counts per minute ± standard error of the mean (± SEM) at PID (PCD) 8 (−13), 21 (0), 25 (4), and 28 (7). Serum VN antibody GMTs for each group were compared by one-way analysis of variance. The rate ratio (RR) and Fisher's exact test were used to determine whether protection was conferred by an inoculum. The correlation between tissue LPA responses at challenge (PCD 0) and protection from challenge was established by Spearman's measure of correlation (ρ). Significant differences in this report are recognized as *P* values of <0.05 unless indicated otherwise.

RESULTS

Clinical responses after inoculation and virulent Wa rotavirus challenge. The clinical responses of pigs and Wa rotavirus inoculation and challenge have been briefly described in part by Yuan et al. (46). Detailed assessment of the clinical responses of each group after inoculation is given in Table 1. Moderate to severe diarrhea (fecal score of ≥2) and virus shedding were almost exclusively found in group 1 (virulent Wa rotavirus) pigs, with 95% developing moderate to severe diarrhea and 100% shedding rotavirus. Only 6 and 2% of group 2 (attenuated Wa rotavirus) pigs shed virus after their first and second inoculations, respectively, and the percentage of group 2 pigs which developed moderate diarrhea after primary inoculation was similar to that of controls (12 and 13%, respectively). In pigs that did shed virus following primary inoculation, the onset of shedding did not differ significantly between the Wa strains, although the duration of shedding was significantly longer in virulent Wa rotavirus-inoculated pigs. Group 1 pigs also developed more severe diarrhea postinoculation, as determined by average additive fecal scores during PID 1 to 8 compared with those for group 2 and control pigs. The mean onset of moderate to severe diarrhea in group 1 pigs was 1.9 days. The onset of moderate to severe diarrhea which developed in the few group 2 pigs and controls was slightly delayed compared with that for group 1 pigs (2.5 and 3.0 days versus 1.9 days, respectively) and the duration was significantly shortened.

A detailed summary of each group's clinical response to challenge is given in Table 2. Virus shedding was limited to controls and group 2 pigs. The duration of shedding following challenge was significantly reduced in group 2 pigs compared with controls, although the times of onset of virus shedding were similar. Following challenge, 100% of controls, 81% of group 2 pigs, and no group 1 pigs shed virus, whereas 83% of controls, 56% of group 2 pigs, and 11% of group 1 pigs developed moderate to severe diarrhea. Although fewer group 2 pigs than controls developed moderate to severe diarrhea, the scores for overall severity of diarrhea observed during PCD 1 to 7 (average additive fecal scores) did not differ significantly. Group 1 pigs developed significantly less diarrhea from PCD 1 to 7 than control and group 2 pigs. The percentage of group 1 pigs which developed diarrhea postchallenge (11%) was similar to those observed for control and group 2 pigs after primary inoculation (13 and 12%, respectively [Table 1]), and the over-

TABLE 2. Summary of fecal virus shedding and clinical disease after oral challenge with live virulent Wa human rotavirus in gnotobiotic pigs previously inoculated with virulent Wa rotavirus (group 1), attenuated Wa rotavirus (group 2) or diluent (controls)[a]

Pig group (*n*)	Value for virus shedding[b]			Value for diarrhea[c]			
	Total (%)	Mean onset (days)	Mean duration (days)	Total (%)	Mean onset (days)	Mean duration (days)	Avg additive score
1 (18)	0^{A}	NA	NA	11^{A}	2.0 (0)^{A}	0.3 (0.1)^{A}	4.8 (0.4)^{A}
2 (16)	81^{B}	2.0 (0.2)^{A}	2.1 (0.3)^{A}	56^{B}	2.2 (0.5)^{A}	1.7 (0.4)^{B}	6.3 (1.1)^{AB}
Controls (12)	100^{B}	2.0 (0.3)^{A}	4.0 (0.4)^{B}	83^{B}	2.2 (0.4)^{A}	2.7 (0.6)^{B}	8.9 (1.2)^{B}

[a] These data have been presented in part by Yuan et al. (46). Values in columns with different superscript letters differ significantly ($P < 0.05$); NA, not applicable. Numbers in parentheses are ± 1 SEM.
[b] Determined by antigen-capture ELISA.
[c] Onset and duration of moderate to severe diarrhea only (daily fecal consistency score of ≥2); Average additive fecal score = (daily fecal scores from PCD 1 to 7)/*n*.

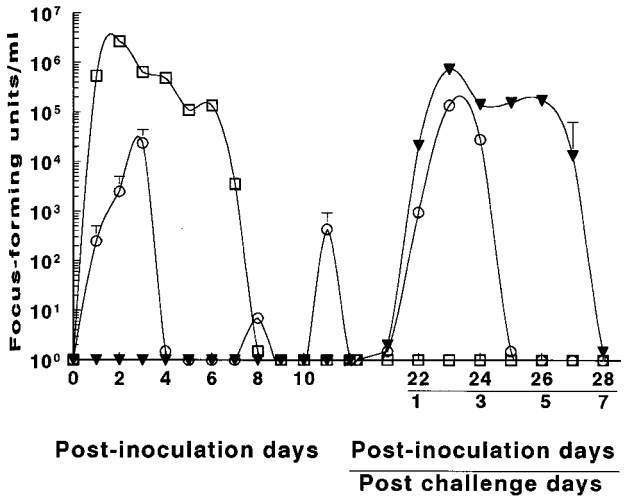

FIG. 1. Titers of rotavirus in feces of gnotobiotic pigs orally inoculated with virulent Wa rotavirus (□ [group 1]), attenuated Wa rotavirus (○ [group 2]), or diluent (▼ [controls]) at 3 to 5 days of age (PID 0) and challenged with virulent Wa rotavirus 21 days later. Data are average daily FFU per milliliter ± 1 SEM in fecal samples from 18 to 51 pigs per day (group 1), 16 to 51 pigs per day (group 2), and 12 to 18 pigs per day (controls) postinoculation and 12 to 18 pigs per day (all groups) postchallenge. SEM that are smaller than symbols are not shown. Data from samples with no detectable virus are indistinguishable from baseline.

all severity of diarrhea in group 1 pigs after challenge was similar to those for controls after inoculation with diluent (average additive scores of 4.8 [Table 2] versus 4.0 [Table 1], respectively) and four age-matched mock-challenged pigs (data not shown). The clinical responses of the two virulent Wa rotavirus-inoculated longer-term pigs after challenge at PID 42 were similar to that observed for group 1 pigs postchallenge, with no shedding and no significant diarrhea (average additive scores postchallenge, 4.0 versus 4.8 [Table 2]). By comparison, disease in the two sham-inoculated longer-term challenge controls differed slightly from that of younger challenged controls, with a shorter mean onset of diarrhea (1.0 versus 3.0 days [Table 2]), longer mean duration of diarrhea (5.5 versus 2.7 days [Table 2]), and more severe diarrhea postchallenge (average additive scores, 17.0 versus 8.9 [Table 2]). Virus shedding in the two sham-inoculated longer-term challenge controls also differed from that of younger challenged controls, with a shorter mean onset of virus shedding (1.0 versus 2.5 days [Table 2]) and longer mean duration of virus shedding (5.0 versus 2.7 days [Table 2]).

The progression of virus titer shed by each group after inoculation and challenge is depicted in Fig. 1. Group 1 pigs shed 100 to 10,000 times more rotavirus than group 2 pigs after inoculation. After challenge, group 1 pigs shed no detectable infectious virus whereas group 2 pigs shed infectious virus at titers similar to those for challenge controls (peak average daily titers were 1.4×10^5 and 7.2×10^5 FFU/ml for group 2 and control pigs, respectively). The peak average daily titer of virus shed by the two longer-term challenged control pigs was 5.0×10^4 FFU/ml. The lower peak titer shed by these longer-term pigs is probably a reflection of fecal dilution due to the more severe diarrhea observed.

Virulent Wa rotavirus inoculation conferred a protection rate of 87% against development of moderate to severe diarrhea after challenge with virulent Wa rotavirus, whereas attenuated Wa rotavirus inoculation conferred protection only at a rate of 33% (Table 2). To determine the significance of protection conferred by each inoculum, the odds or RRs of protection from disease were calculated and are as follows: diluent, 1; virulent Wa rotavirus inoculum, 5.33 (95% confidence interval [$1.49 < RR < 19.10$]); and attenuated Wa rotavirus inoculum, 2.63 (95% confidence interval [$0.66 < RR < 10.45$]) (Fig. 3). Thus, compared with diluent alone, inoculation with virulent Wa rotavirus significantly increased the probability of protec-

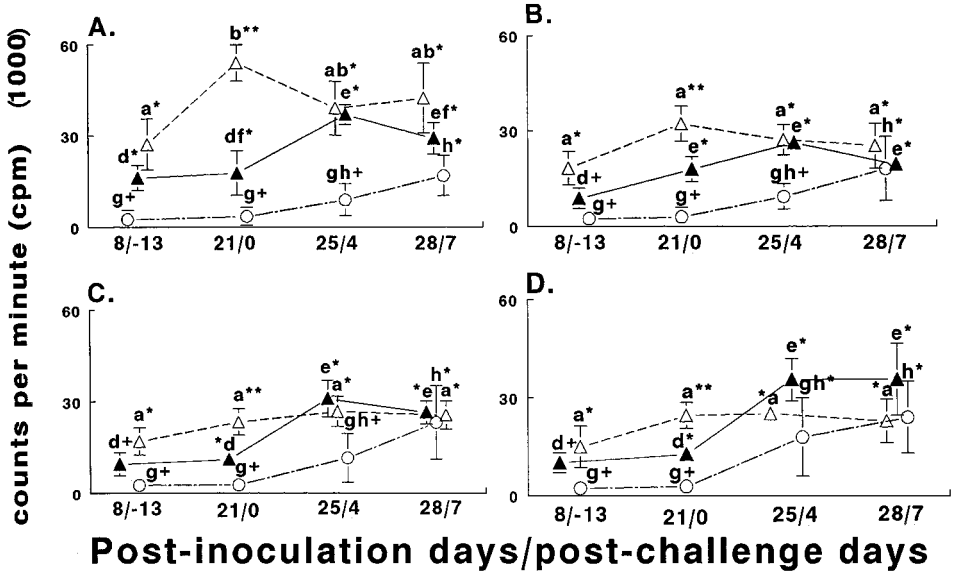

FIG. 2. Progression of rotavirus-specific lymphocyte proliferation in the MLN (A), small intestinal (ileum) lamina propria (B), blood (C), and spleen (D) from gnotobiotic pigs inoculated with live virulent Wa rotavirus (△ [group 1; $n = 5$ to 11]), live attenuated Wa rotavirus (▲ [group 2; $n = 5$ to 14]), or diluent (○ [controls; $n = 2$ to 4]) at 3 to 5 days of age and challenged with virulent Wa rotavirus 21 days later. Data are the mean virus-specific counts per minute ± 1 SEM (SEM smaller than the symbols are not shown). Comparisons of means within a group across time were made, and significant differences are denoted by the letters a, b, and c for group 1, d, e, and f for group 2, and g, h, and i for controls. Comparisons of means between groups at each time point were also made, and significant differences are indicated by +, *, and **.

TABLE 3. VN antibody responses in the serum of gnotobiotic pigs inoculated with live virulent Wa rotavirus (group 1), live attenuated Wa rotavirus (group 2), or diluent (controls) before and after challenge with live virulent Wa rotavirus[a]

Pig group	Prechallenge serum VN antibody response value[b]			Postchallenge serum VN antibody response value[c]		
	n	% Positive	GMT	*n*	% with ≥4-fold increase	GMT
1	40	100	69[AC]	23	17	107[C]
2	42	96	45[A]	16	81	296[D]
Controls	8	0	2[B]	11	64	9[E]

[a] These data have been presented in part by Yuan et al. (46). Values with different superscript letters differ significantly ($P < 0.05$).

[b] Prechallenge VN titers were determined at PID 19 to 21; positive VN titers were those >4; negative VN titers of <4 were assigned a value of 2 for determination of GMT.

[c] Postchallenge VN titers were determined at PCD 3 to 9.

tion from challenge ($P < 0.001$), whereas inoculation with attenuated Wa rotavirus did not ($P = 0.22$).

Serologic responses after inoculation and virulent Wa rotavirus challenge. The serological responses of each group have been briefly summarized by Yuan et al. (46) and are described in detail in Table 3. Between PID 19 and 21, 100% of group 1 and 96% of group 2 pigs had serum Wa VN antibodies. The serum VN GMT of group 2 was lower than that of group 1 pigs at PID 21, but not significantly. After challenge, 81% of group 2 but only 17% of group 1 pigs showed fourfold or greater increases in their serum VN titers; consequently, the serum VN GMT increased significantly in group 2 but not group 1 pigs after challenge. The serum VN GMT of controls also increased significantly postchallenge, with 64% demonstrating increases fourfold or greater in serum VN titers at PCD 3 to 9. The serum VN GMT of the four longer-term virulent Wa rotavirus-inoculated pigs at challenge (PCD 0 [PID 42]) was higher than that of group 1 pigs at challenge (PCD 0 [PID 21]) (69 versus 165 [Table 3]); however, as with group 1 pigs, only slight increases were observed postchallenge (PCD 8 [PID 50]). No serum VN antibodies were detected in the two sham-inoculated longer-term control pigs prior to challenge (serum VN titers of <4 at PCD 0 [PID 42]), and low serum VN titers were detected postchallenge (serum VN titers of 4.4 and 10 at PCD 7 [PID 49]).

Wa rotavirus-specific LPA responses after inoculation and virulent Wa rotavirus challenge. Blood, splenic, and MLN MNC were >98% viable, and ileal lamina propria MNC were >95% viable as measured by the trypan blue exclusion test at the time each LPA was set up. The mean background counts per minute of each tissue's MNC at any given PID or PCD were subtracted from the mean virus-specific counts per minute to correct for individual nonspecific lymphoproliferation prior to determination of the group mean counts per minute. All background counts per minute were <15,000. Each group's tissue LPA responses to Wa antigen at the various times postinoculation and postchallenge are depicted (Fig. 3). By PID 8, >90% of group 1 (virulent Wa rotavirus) pigs had developed significant Wa antigen-specific proliferation in all tissues examined, resulting in group mean counts per minute for all group 1 tissues significantly higher than those for controls. The MLN MNC from a majority (72%) of group 2 (attenuated Wa rotavirus) pigs responded strongly to Wa antigen stimulation at PID 8, resulting in significantly higher mean counts per minute for the MLN of group 2 pigs than those for diluent controls at PID 8. However, other tissues (ileal lamina propria, blood, and spleen) at PID 8 from group 2 pigs showed little to no stimulation with Wa antigen, and thus, the group 2 mean counts per minute for these tissues were similar to those for diluent controls. On the day of challenge (PCD 0 [PID 21]), group 1 pigs had significantly greater LPA responses in all tissues examined than both group 2 and control pigs. Group 2 pigs had lower LPA responses than group 1 pigs but significantly greater LPA responses than controls at PID 21. The LPA responses of the two longer-term virulent Wa rotavirus-inoculated pigs at PCD 0 (PID 43) were similar to or slightly greater than those of the younger group 1 pigs at PCD 0 (PID 21), with mean blood, splenic, ileal lamina propria, and MLN MNC values of 9,613 cpm (versus ~11,000 [Fig. 2]), 15,787 cpm (versus ~12,700 [Fig. 2]), 24,415 cpm (versus ~18,000 [Fig. 2]), and 21,771 cpm (versus ~17,800 [Fig. 2]).

After challenge at PID 21, the LPA responses of MNC from group 2 pigs increased significantly for blood, spleen, and MLN at PCD 4 (PID 25) compared with their respective responses at PCD 0 (PID 21), whereas all MNC LPA responses in controls increased significantly at PCD 7 (PID 28) compared with their responses at PCD 0 (PID 21). The LPA responses of MNC from group 1 pigs after challenge (PCD 4 [PID 25] and PCD 7 [PID 28]) were similar to their respective LPA responses at challenge (PCD 0 [PID 21]). After challenge of the two longer-term virulent Wa rotavirus-inoculated pigs, a marked increase in the blood (mean, 41,780 cpm) but not spleen (mean, 13,770 cpm) or ileal lamina propria (mean, 14,660 cpm) LPA responses and a slight increase in the MLN MNC (mean, 28,345 cpm) LPA responses were observed at PCD 8 (PID 50) compared with their respective responses at PCD 0 (PID 42) as described above.

Detailed evaluation of individual pig LPA responses at the earliest times postinoculation (during the first euthanasia interval [PID 5 to 9]) and postchallenge (PCD 3 to 4) showed that the magnitude of blood LPA responses correlated with the magnitude of MLN LPA responses (92% correlation) and lamina propria LPA responses (89% correlation). At later inter-

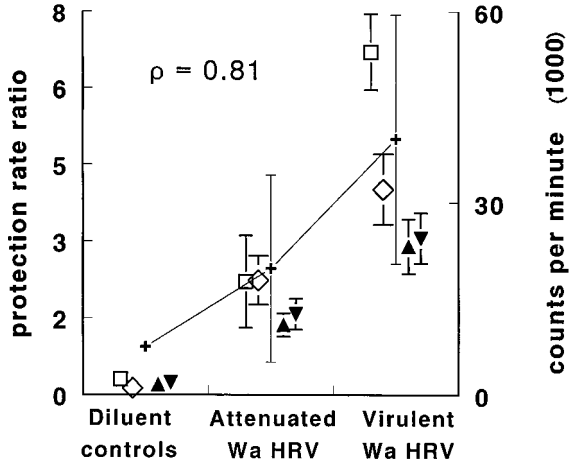

FIG. 3. Correlation between protection and tissue LPA responses at challenge in gnotobiotic pigs orally inoculated with virulent or attenuated Wa human rotavirus or diluent (controls). Tissues examined in each group included MLN (□), ileum lamina propria (◇), blood (▲), and spleen (▼). The virus-specific LPA responses are depicted as tissue mean counts per minute ± SEM at PID 21 (PCD 0). The ability of the virus inoculum to confer protection against moderate to severe diarrhea following virulent virus challenge is depicted as the group's RR ± SD (+). The magnitude of tissue LPA responses at challenge positively correlated with the protection afforded by the inoculum (ρ = 0.81).

TABLE 4. Mitogenic responses of MNC of gnotobiotic pigs orally inoculated with virulent (group 1) or attenuated (group 2) Wa rotavirus or diluent (controls)[a]

Treatment group	Age range (days)	*n*	cpm for the following MNC sources[b]:			
			Blood	Spleen	LP	MLN
Group 1 before challenge	7–25	25	98,085^{AB}	97,391^{AB}	19,924^{AB}	128,894^{AB}
Group 2 before challenge	8–24	21	72,701^{B}	101,381^{AB}	16,726^{B}	92,205^{B}
Controls before challenge	8–24	7	93,017^{AB}	74,299^{AB}	19,566^{AB}	93,648^{AB}
Group 1 after challenge	23–36	18	110,036^{A}	124,191^{A}	24,766^{A}	145,004^{A}
Group 2 after challenge	23–34	16	86,479^{AB}	73,772^{B}	20,284^{AB}	78,987^{B}
Controls after challenge	24–30	6	78,093^{AB}	118,748^{AB}	19,362^{AB}	124,581^{AB}

[a] Pigs were inoculated at 3 to 5 days of age and were challenged with virulent Wa rotavirus 21 days later. Mitogenic responses were assayed prechallenge at PID 5 to 21 and postchallenge at PCD 3 to 9. Values in columns with different superscripts are significantly different ($P < 0.05$).
[b] Data are the actual group mean counts per minute. LP, small intestine lamina propria.

vals postinoculation or postchallenge, the correlation between the magnitudes of the LPA responses in blood and intestinal MNC was poor (<55%).

Among tissues from group 1 pigs at PID 8, virus-specific LPA responses were highest in MLN, which was followed by responses in the ileum, blood, and spleen, with only splenic responses being significantly lower than MLN responses. Virus-specific LPA responses remained highest in the MLN of group 1 pigs at PID 21, after which came responses in the ileum, blood, and spleen, with both blood and splenic mean responses being significantly lower than the MLN mean response. At the time of challenge of the longer-term virulent Wa rotavirus-inoculated pigs (PCD 0 [PID 43]), the gut-associated lymphoid tissues had the highest LPA responses (similar to those for group 1 pigs at PCD 0 [PID 21]), except that the ileal lamina propria value was the highest and was followed closely by the MLN and then by the spleen and blood responses, with both blood and splenic mean responses being significantly lower than both the ileal lamina propria and MLN mean responses. The LPA responses of group 1 pigs did not differ among tissues after challenge at PID 21. Differences in the LPA responses of group 2 pigs did not differ significantly among tissues at PID 8 and 21, although the mean MLN responses were highest, after which came similar responses for the blood, spleen, and ileum. After challenge at PID 21, virus-specific LPA responses of group 2 pigs at PCD 4 (PID 25) were highest in the MLN, after which came those for the blood, spleen, and ileum, with the ileum responses being significantly lower than the MLN responses at this time. At PCD 7 (PID 28), no significant differences among tissues from the group 2 pigs were observed. Significant differences in LPA responses were not observed among tissues from controls at any time point before or after challenge at PID 21. After challenge of the longer-term pigs, the blood LPA responses were notably greater than those of other tissues from the virulent Wa rotavirus-inoculated pigs at PCD 8 (PID 50), whereas the LPA responses of the two longer-term challenged control pigs postchallenge (PCD 7 [PID 49]) were lower among tissues (mean range, ~6,200 to ~12,800 cpm), with splenic response values being approximately twice those for blood responses.

The correlation between each group's RR and their tissue LPA responses at challenge (PID 21) was determined by the Spearman rank correlation test and is depicted in Fig. 3. The magnitude of the LPA response in all tissues examined at PID 21 correlated positively with the degree of protection from homologous challenge ($\rho = 0.81$).

Mitogenic responses of tissue MNC after inoculation and virulent Wa rotavirus challenge. The mitogenic responses of MNC to PHA in each group before and after challenge are summarized (Table 4). The lamina propria MNC responses were significantly lower than those for all other tissues within each group. The primary inoculum had no significant effect on the responsiveness of a given MNC population to PHA before or after challenge. Examination of the mitogenic responses from 7 to 36 days of age showed similar responses within a given tissue, with the lamina propria MNC showing consistently lower responses than all of the other tissues across time. The mean mitogenic responses of the longer-term virulent Wa rotavirus-inoculated pigs before and after challenge at PID 42 were only slightly higher (1.2 to 1.7 times higher) than the PHA responses of tissues from the group 1 (virulent Wa rotavirus-inoculated) pigs before and after challenge at PID 21 (Table 4), whereas the mean PHA responses of the longer-term sham-inoculated challenged control pigs at PCD 8 (PID 50) were slightly lower (1.1 to 2.3 times lower) than those of younger challenged controls (Table 4). Also similar to those observed in younger pigs, the PHA responses of the lamina propria MNC were lowest among the tissues from the longer-term pigs both before and after challenge.

DISCUSSION

Using the gnotobiotic pig model, we compared inoculations with live attenuated and virulent human rotavirus followed by homologous virulent virus challenge to identify cellular correlates of protective immunity. Although the LPA does not distinguish between proliferating-cell phenotypes, rotavirus antigen-induced proliferation in vitro has been recognized as a property of $CD4^+$ (T helper or inducer) cell progeny in studies conducted with human and mouse MNC (9, 28, 38). Consequently, the LPA described in the present study most likely reflects rotavirus-specific T helper cell/inducer cell activity. Further supporting this hypothesis, we found that the magnitude of a tissue's LPA response (especially MLN and ileum) positively correlated ($\rho = 0.82$ and 0.89 for MLN and ileum, respectively) with the numbers of virus-specific immunoglobulin A antibody-secreting cells in that tissue, as determined in a separate study evaluating the antibody-secreting-cell responses of these same pigs (46). The correlation between the magnitude of the LPA response in all tissues with the degree of protection against homologous human rotavirus challenge suggests that T helper or inducer cells play a role in protection against human rotavirus-associated disease and that the LPA may be used to assess the development of this protective response.

In vivo depletion studies of calves showed that $CD4^+$ but not $CD8^+$ T cells were important in the generation of both muco-

sal and systemic antibody responses to rotavirus (29). The presence of rotavirus-specific T helper cells which recognize crucial rotavirus epitopes such as the neutralizing epitopes on VP4 and VP7 may be required for establishing a protective neutralizing antibody response at the mucosal surface. Further study is needed to delineate the role of T helper or inducer cells in immunity to human rotavirus. It is conceivable that $CD4^+$ T cells, like $CD8^+$ cytotoxic T cells (27), recognize a common epitope(s) among different rotavirus serotypes and thereby contribute to heterologous as well as homologous immunity.

The presence of clinical disease after inoculation of gnotobiotic pigs with virulent but not attenuated Wa rotavirus is consistent with the results of our earlier studies (35, 41) and has been attributed to infection of few enterocytes and/or reduced rotavirus replication efficiency by the attenuated virus (20, 39, 41). Despite the apparent absence of intestinal replication (as determined by the absence of detectable rotavirus shedding in 94% of group 2 pigs), most of the attenuated Wa rotavirus-inoculated pigs developed serum VN antibodies at challenge and possessed low but significant Wa rotavirus-specific LPA responses at challenge. The serum VN antibody titers at PID 19 to 21 did not correlate with any of the tissue LPA responses in the Wa rotavirus-inoculated groups at PID 21. Investigators evaluating rotavirus-specific LPA responses in human peripheral blood have similarly failed to establish a correlation between the serum antibody response and the subsequent LPA response of an individual (28, 38).

The immune responses elicited by the two Wa rotavirus strains differed markedly. All of the LPA responses of tissues from virulent Wa rotavirus-inoculated pigs were significantly greater than those from attenuated Wa rotavirus-inoculated pigs prior to challenge, and these greater responses correlated with effective disease prevention after challenge. The small longer-term pig study similarly showed high intestinal LPA responses at challenge and showed that protection was present for at least 6 weeks after primary inoculation. The absence of rotavirus shedding postchallenge in all of the virulent Wa rotavirus-inoculated pigs and the absence of significant increases in their serum VN titers and tissue LPA responses postchallenge suggest that the protection conferred by the virulent Wa rotavirus inoculation successfully prevented infection and replication. Inoculation with attenuated Wa rotavirus elicited significantly lower LPA responses in all tissues, compared with virulent Wa rotavirus, especially within intestinal tissues, and the responses elicited did not prevent the occurrence of disease in a majority of pigs (56% with moderate to severe diarrhea) after homologous rotavirus challenge at PID 21. Further, >4-fold increases in most serum VN antibody titers (81%) and significant increases in systemic but not intestinal LPA responses were observed for the attenuated Wa rotavirus-inoculated pigs following challenge. Increased (>4-fold) serum VN antibody titers occurred in 64% of the challenged controls by PCD 3 to 9, and all MNC from challenged controls showed significant virus-specific LPA responses at PCD 7, which was indicative of early primary immune responses.

The differences in the immune responses of the virus-inoculated groups pre- and postchallenge suggest that inherent differences may exist between virulent and attenuated Wa rotavirus in terms of generating an immune response. Both viruses elicited rotavirus-specific LPA responses and serum antibody responses, but only virulent Wa rotavirus elicited significantly greater local cellular immune responses (up to approximately 2 months of age) and protective immune responses. Therefore, although oral inoculation of gnotobiotic pigs with live attenuated heterologous (human) rotavirus elicits an immune response, the protective capacity and local magnitude of the immune responses generated, unlike heterologous rotavirus inoculations in mice (15), appear to be strongly related to virus virulence and intestinal replication efficiency.

The responses of intestinal lamina propria MNC to PHA in this study were significantly lower than those of other tissues. Similarly low intestinal lamina propria MNC responses to mitogen have been reported by others (8, 44). We found that the percentage of viable lamina propria MNC remaining in mitogen-stimulated wells after 4 days in culture (optimum time for antigen stimulation) was consistently lower than those of its antigen-stimulated counterpart and antigen- or mitogen-stimulated MNC from blood, spleen, and MLN, which would account in part for the lower average counts per minute obtained for these cells. Poor viability may be due to the absence of a specific cell phenotype essential for optimal in vitro stimulation (47), and phenotypic analyses of the MNC populations are under way. The harsh treatment of lamina propria MNC prior to culture (collagenase digested, etc.) could also contribute to poor viability, although similar processing of spleens from two mock-challenged control gnotobiotic pigs did not affect their splenic MNC mitogenic responses (40a). Consequently, the reason(s) for the low mitogenic responses of the intestinal lamina propria MNC in this study is unknown.

Evaluation of individual LPA responses at early times postinoculation (PID 5 to 9) and postchallenge (PCD 3 to 4) revealed that the blood MNC activity closely reflected the pig's intestinal MNC activity. Of the pigs with significant rotavirus-specific MLN responses in the LPA at these early times, >90% also showed rotavirus-specific blood and lamina propria responses but <20% showed splenic LPA responses. These observations, together with our current understanding of lymphocyte trafficking and homing (1, 31), suggest that intestinally derived T cells are present in blood for a very limited but definable time following infection with human rotavirus. Although studies assessing the cell phenotypes involved and the homing receptors and tissue adhesin molecules or vascular addressins present after Wa rotavirus infection will help to establish the significance of these observations, these findings lend credence to the use of blood MNC for evaluating intestinal T-cell immunity to human rotavirus.

Some investigators have suggested that lamina propria T cells are specialized as cytokine-secreting regulatory cells that proliferate poorly in response to antigen (32, 47). However, we found relatively high proliferative responses to Wa antigen in lamina propria MNC, especially in the virulent Wa rotavirus-inoculated pigs. We did not exclude Peyer's patches from the intestinal segments during isolation of the lamina propria MNC. Although murine lymphocytes originating from Peyer's patches were reported to proliferate poorly in response to rotavirus antigen in vitro (32), significant antigen-specific proliferative responses by both mouse and porcine Peyer's patch lymphocytes have been reported elsewhere (17, 43). The presence of Peyer's patch-derived lymphocytes in our lamina propria MNC isolates may therefore have contributed in some positive manner to the virus-specific LPA responses that we observed.

In conclusion, the LPA responses of both intestinal and systemic tissues from gnotobiotic pigs correlated with the degree of protection conferred against homologous virulent human rotavirus challenge for up to approximately 2 months of age, suggesting that T cells contribute to protective immunity against human rotavirus infection and disease in gnotobiotic pigs. In addition, the finding that peripheral blood MNC activity in the LPA transiently reflected that of intestinal MNC supports the use of blood as a temporary window to view

intestinal T-cell activity and provides a possible tool by which mucosal immunity may be studied indirectly in both animals and humans. The time at which a Wa rotavirus-specific T-cell response developed and the magnitude of that response within a tissue were dependent in some manner on the pathogenicity, virulence, or replication efficiency of the inoculating virus strain in the gnotobiotic pig model of human rotavirus-induced disease. Further studies are needed to identify the viral or host mechanisms responsible for the differences observed in the clinical and immunological responses of gnotobiotic pigs to attenuated or virulent human rotavirus strains. Understanding these mechanisms should aid human rotavirus vaccine development.

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