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Enumeration of isotype-specific antibody-secreting cells derived from gnotobiotic piglets inoculated with porcine rotaviruses

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Abstract

In order to evaluate mucosal antibody responses to rotavirus, an enzyme-linked immunospot (ELISPOT) assay was adapted to enumerate antibody-secreting cells (ASC) in the mesenteric lymph nodes (MLN), lamina propria (LP) of the small intestine and spleens of gnotobiotic pigs orally inoculated with porcine rotaviruses (SB1A and Gottfried). Rotavirus-specific IgM ASC occurred by post-inoculation day (PID) 3, and numbers peaked in spleen and MLN tissues by PID 7 and in intestinal LP by PID 7-14. Numbers of rotavirus specific IgA and IgG ASC in these tissues peaked at PID 14-21. Rotavirus specific IgA ASC were predominant in the gut and IgA to IgG rotavirus specific ASC ratios were highest for all rotavirus antigen coatings in the gut LP. However, the relative ratios of specific IgA to IgG ASC were lower (ratios of 5 to 7) against combined structural and nonstructural viral antigens (rotavirus-infected fixed cell ELISPOT plates) than ratios (13 to 46) against only viral structural antigens (rotavirus-coated ELISPOT plates), indicating that there were proportionately more specific IgG ASC to the nonstructural viral antigens in the LP, the tissue adjacent to the site of rotavirus replication in the intestine. In the node cells (spleen and MLN) rotavirus-specific IgA to IgG ASC ratios were lowest and against the various ELISPOT rotavirus coatings ranged from 0.7 to 4. Gnotobiotic piglets inoculated at different ages with porcine rotaviruses generally showed similar specific immunoglobulin (Ig) ASC responses to rotavirus infection, along with similar diar-

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rhea and virus shedding patterns in the different age groups. However, the numbers of specific IgA ASC in the MLN of 3-4 week old pigs were higher than those of 3-5 day old pigs. Although challenge of SB1A or Gottfried rotavirus-inoculated pigs with SB1A (G4P7) or Gottfried (G4P6) rotavirus revealed a high degree of protection from diarrhea and virus shedding, greater numbers of specific IgM ASC were observed in spleen after challenge of SB1A-inoculated pigs with Gottfried rotavirus (same G type, distinct P type). Thus, by using the ELISPOT technique, we successfully measured intestinal mucosal antibody-related responses to rotavirus in gnotobiotic pigs. Moreover, our results support the use of gnotobiotic piglets as an animal model to evaluate active antibody responses and protection against rotavirus infection and disease.

1. Introduction

Rotavirus is recognized worldwide as a major cause of infectious diarrhea in mammalian species, including infants and young children (Barnett, 1983; Cukor and Blacklow, 1984). Public health problems and economic losses attributed to rotavirus have stimulated research into the development of effective vaccines for humans and animals (Kapikian et al., 1986; Chanock et al., 1988; Estes and Cohen, 1989; Kapikian and Chanock, 1990). Candidate rotavirus vaccines have been tested in infants (Kapikian et al., 1986; Estes and Cohen, 1989; Kapikian and Chanock, 1990) and swine (Hoblet et al., 1986). Failures of many of these vaccines to provide sufficient protection against homologous and heterologous serotypes of rotavirus demonstrate a need for comprehensive studies of immunity to rotavirus and investigations of alternative approaches to rotavirus vaccine development.

The rotavirus outer capsid consists of proteins VP4 and VP7 which contain the antigenic determinants that induce protective immunity when evaluated in murine or porcine models of rotavirus infection (Offit et al., 1986a; Hoshino et al., 1988). In these and calf models of rotavirus infection, neutralizing local antibodies to VP4 and VP7 provided protection against rotavirus infection localized in the epithelium of the small intestine (Offit and Clark, 1985a,b; Saif, 1987; Kapikian and Chanock, 1990). Limited studies in mice have also demonstrated a role for cell-mediated immunity in rotavirus infections (Offit and Dudzik, 1989).

Merchant et al. (1991) and Shaw et al. (1991, 1992) characterized the mucosal antibody responses of mice to a heterologous infection with simian rotavirus (RRV) using an enzyme-linked immunospot (ELISPOT) assay to enumerate antibody-secreting cells (ASC) isolated from different tissues. In their study, ASC isolated from the small intestine lamina propria (LP) secreted predominantly rotavirus-specific immunoglobulin A (IgA). This extended previous findings that antibodies in fecal and nasal secretions of calves recovered from a bovine rotavirus infection were primarily IgA (Saif, 1987). Similar antibody responses were reported in rabbits orally inoculated with rabbit rotavirus (Connor et al., 1991). In addition, predominantly IgA ASC were identified in the small intestinal LP of pigs recovered from infection with the enteric coronavirus, trans-
missible gastroenteritis virus, but were detectable only in low numbers in the LP of pigs exposed to the antigenically related porcine respiratory coronavirus which replicates in the respiratory tract but not the gut (VanCott et al., 1993, 1994).

Suckling mice are a useful model for the evaluation of passive immunity to certain rotaviruses; however, active immunity to rotavirus cannot be studied because older mice are refractory to rotaviral disease (Offit and Clark, 1985a,b; Offit et al., 1986b; Ramig, 1988). The rabbit model suffers similar limitations in the ability to induce clinical disease (Conner et al., 1991). Pigs are monogastric and closely resemble humans physiologically (Phillips and Tumbleson, 1986). The porcine placenta prevents the transfer of maternal antibodies; hence, newborn pigs are agammaglobulinemic but immunocompetent (Porter, 1979). Thus, studies of rotavirus infection in gnotobiotic pigs permit basic investigations of the ontogeny of the immune response and evaluation of true primary antibody responses. In our laboratory, gnotobiotic pigs have been used to study the pathogenesis and immune responses to human rotaviruses (Wyatt et al., 1980; Schaller et al., 1992) as well as to homologous porcine rotaviruses (Bohl et al., 1984). Gnotobiotic pigs present advantages over mice and rabbits as models for rotavirus infection, mostly owing to their extended susceptibility (up to 6 weeks of age) to infection and disease with porcine (Bohl et al., 1984) and human rotaviruses (Wyatt et al., 1980; Schaller et al., 1992; Saif and Chen, unpublished data, 1992).

To improve our understanding of mucosal immune responses to rotaviral antigens and to further identify correlates of protective immunity to rotavirus, we have adapted the ELISPOT assay for the study of ASC to porcine rotavirus infections in gnotobiotic pigs. Thus, we can establish a basis upon which to evaluate novel approaches to mucosal immunization and rotavirus vaccine development in gnotobiotic pigs. In this paper we present data based on the ELISPOT assay to describe the isotype-specific mucosal-associated and systemic ASC response to homologous porcine rotavirus infections in gnotobiotic pigs.

2. Materials and methods

2.1. Virus strains

The porcine rotavirus strains, Gottfried (G4P6), OSU (G5P7) (Bohl et al., 1984), and SB1A (G4P7) (Hoshino et al., 1988), the latter a reassortant possessing gene 4 (VP4) from OSU and all other genes including gene 9 (VP7) from Gottfried, were grown in monolayers of fetal Rhesus monkey kidney cells (MA104) as described previously (Bohl et al., 1984). Stocks of Gottfried and SB1A were passaged in gnotobiotic pigs in order to maintain viral virulence (Bohl et al., 1984; Hoshino et al., 1988). Suspensions of intestinal contents from these pigs were used as rotavirus inocula for the experimental gnotobiotic pigs. The infectivity titers of these suspensions were determined by a cell culture immunofluorescence assay (CCIF) (Kang et al., 1989).
2.2. Virus purification

The lysates from the rotavirus-infected cell cultures were clarified by centrifugation at 8700×g for 20 min, and the supernatants were pelleted at 72 660×g for 90 min. The pellets were suspended in TBS–CaCl₂ (50 mM Tris–HCl, 100 mM NaCl, 2.0 mM CaCl₂ pH 7.5) and the viruses were partially purified (PP) by centrifuging on 40% w/v sucrose cushions, at 112 700×g for 2 h at 4°C. The viral pellets were resuspended at 25-fold concentration in TBS–CaCl₂ and stored at −70°C. Fluids from mock-infected MA104 cell cultures were processed in a similar manner.

Rotavirus purification by centrifugation in CsCl gradients was performed as previously described (Jiang et al., 1990). Briefly, the partially purified viral suspensions were layered on top of a CsCl solution (1.37 g ml⁻¹) and centrifuged to isopycnic equilibrium at 107 170×g for 16 h at 4°C. The viral bands were collected, and dialyzed in TBS–CaCl₂ for double-shelled (DS) particles or in TBS for single-shelled (SS) particles, followed by examination by immune electron microscopy (IEM) to verify the morphology (DS or SS) of the particles from the gradient bands. The purified viruses were stored at −70°C.

2.3. Gnotobiotic pig maintenance

Gnotobiotic pigs were delivered and maintained as described previously (Bohl et al., 1984). Pigs 3-5 days of age were inoculated orally with (2-5) × 10⁷ fluorescent focus units (FFU) of an intestinal contents suspension containing Gottfried or SB1A rotavirus previously shown to cause diarrhea in 100% of the inoculated pigs. The virus was diluted with MEM (Gibco, Life Technologies, Grand Island, NY) containing 100 U penicillin, 25 U mycostatin, 0.1 mg dihydrostreptomycin, and 0.14% w/v NaHCO₃ ml⁻¹. Pigs were challenged 3 weeks later with the same viral dose of Gottfried or SB1A porcine rotavirus. Groups of control pigs which did not receive an initial inoculum were challenged concurrently. Pigs were examined daily for clinical signs (anorexia and onset, duration and severity of diarrhea in terms of semi-liquid or watery stools) (Heckert et al., 1990). Rectal swabs were collected daily, processed and tested for virus shedding by CCIF (Kang et al., 1989) and fecal samples were examined by IEM (Saif et al., 1977). Pigs were slaughtered at approximately weekly intervals post-inoculation, and portions of the spleen, mesenteric lymph nodes (MLN) and small intestine (duodenum and ileum) were collected for isolation of mononuclear cells (MNC) (VanCott et al., 1993, 1994). The microbial sterility of gnotobiotic pigs was confirmed by bacteriologic testing of the rectal swabs (Bohl et al., 1984). A group of uninoculated pigs served as negative controls.

2.4. CCIF assay

The rotavirus inocula and the rectal swab fluid specimens were processed and diluted in MEM using fourfold serial dilutions as described previously (Bohl et
al., 1984; Kang et al., 1989). Briefly the diluted specimens were added to MA104 cell monolayers in 96-well plates, and incubated at 37°C for 18–20 h with 5% CO₂. The cells were fixed in 80% acetone for 15 min and stained with a hyper-immune gnotobiotic pig anti-group A rotavirus serum conjugated with fluorescein isothiocyanate (Bohl et al., 1984).

2.5. Isolation of mononuclear cells (MNC)

The preparation of MNC from spleen and MLN was described previously (VanCott et al., 1993, 1994). Briefly, single cell suspensions of MNC were obtained by pressing the tissues from spleen and MLN through stainless steel mesh screens of a cell collector (E-C Apparatus Corp., St Petersburg, FL). The MNC from spleen and lamina propria were treated with HEPES (Gibco, Life Technologies, Inc., Grant Island, NY) (25 mM, pH 7.4) buffered ammonium chloride (145 mM) to lyse splenic erythrocytes. The MNC preparations of spleen and MLN were isolated on Ficoll-Paque (Sigma, St. Louis, MO) after centrifugation at 400×g for 30 min at 4°C. The small intestinal lamina propria lymphocytes from the duodenum or ileum were isolated using modifications of techniques described previously by VanCott et al. (1994) and others (Wilson et al., 1986; Van der Heijden and Stok, 1987). Briefly, after rinsing the tissues with Hank’s medium (HBSS) (Gibco) containing 5 mM EDTA, the tissues were digested using 300 U ml⁻¹ collagenase (Sigma) and 0.25 mg ml⁻¹ DNase (Sigma) in RPMI 1640 at pH 7.2 with shaking at 37°C for a total of 30 min. Then, the suspensions containing MNC were combined, treated with the ammonium chloride buffer at 37°C for 7 min, washed with RPMI 1640 (Gibco) containing 5% fetal bovine serum, and purified by centrifugation (400×g 30 min) in a discontinuous Percoll (Sigma) gradient (40–60%). The MNC were collected from the gradient interface and diluted to 5×10⁶ cells ml⁻¹, 5×10⁵ cells ml⁻¹ and 5×10⁴ cells ml⁻¹ with enriched RPMI 1640 containing 8% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol, 20 mM HEPES (pH 7.2), ampicillin (10 μg ml⁻¹), and gentamicin (100 μg ml⁻¹).

2.6. Preparation of antigen-coated plates for ELISPOT

Infected fixed-cell monolayers.
MA104 cell monolayers in 96-well tissue culture plates were inoculated with 0.1 ml per well of cell culture-passaged rotavirus inoculum containing approximately 1.5×10⁶ FFU ml⁻¹ to yield over 80% infected cell monolayers. The infected fixed-cell plates were stored for up to 4 weeks at −20°C.

Viral antigen coating.
Partially purified (PP) virus particles, purified SS particles, DS particles, and recombinant OSU VP4 (P7) or Gottfried VP4 (P6) expressed by a baculovirus vector (supplied as baculovirus infected cell lysates by Dr. M. Gorziglia, NIAID,
NIH, Bethesda, MD) were diluted in 50 mM carbonate–bicarbonate buffer (pH 9.6), and 0.1 ml per well was added to 96-well plates (Dynatech Laboratories Inc., Chantilly, VA). The coated plates were incubated for 30 min at 37°C, then for 16 h at 4°C or 4 h at 25°C prior to being used. Additionally, 0.1 ml per well of goat anti-mouse-IgG (1.0 μg ml⁻¹) or -IgM (0.8 μg ml⁻¹) (Boehringer Mannheim, Indianapolis, IN) was used to coat plates for comparison with the viral antigen-coated plates to standardize the specificity and sensitivity of the ELISPOT assay. The optimal coating concentration of each antigen we used in the ELISPOT assay was determined by using the hybridoma cells in the standardization ELISPOT assay with serially diluted antigen coatings and selecting those antigen coating concentrations which gave the highest numbers of ASC.

2.7. Hybridoma cells

Anti-rotavirus-specific hybridoma cells were used for standardization of the ELISPOT assay (Table 1). These included VP6 (RG25A10; Kang et al., 1989) and VP7 (Common 60; Offit et al., 1986b) broadly-reactive group A monoclonal antibodies (mAbs), VP4 type specific mAbs (RG24B9 and RO3F1, Kang et al., 1989), and as a control, a group C rotavirus VP6-specific mAb (RC15B7) (Ojeh et al., 1991). Rotavirus antibody secretion by the hybridoma cells was verified by testing the hybridoma cell culture supernatants using the CCIF assay (Kang et al., 1989) as well as a rabbit anti-mouse-IgG or -IgM capture ELISPOT assay (Merchant et al., 1991; Shaw et al., 1991).

2.8. ELISPOT assay

The ELISPOT assay was modified from published methods (Sedgwick and Holt, 1983; Czerkinsky et al., 1983; Merchant et al., 1991; Shaw et al., 1991) and adapted for the analysis of porcine ASC (VanCott et al., 1993, 1994). Briefly, the rotavirus-infected or mock-infected fixed-cell plates were thawed and rehydrated with deionized water (dH₂O) and the virus antigen-coated or mock antigen-coated plates or the rabbit anti-mouse-IgG or -IgM-coated plates were rinsed once in dH₂O. The MLN cells at dilutions of 5×10⁵, 5×10⁴, and 5×10³ MNC per well and the serially diluted hybridoma cells were plated in duplicate wells. The plates were incubated for 6–7 h at 37°C in 5% CO₂ and then were washed six times in PBS containing 0.05% v/v Tween 20 (PBS-T) to remove the cells. Biotinylated mouse monoclonal anti-porcine-IgG, IgA or IgM, derived from hybridomas 3H2 and 3H7 to porcine IgG, 3H5 and 3D11 to porcine IgA, 5C9 and 1A11 to porcine IgM (hybridomas provided by Dr. P. Paul, Iowa State University, Ames) (Paul et al., 1989), were added and the plates were incubated for 2 h at 25°C or 16 h at 4°C. In the case of the ELISPOT using hybridoma cells, biotinylated F(ab')2 goat anti-mouse-IgG, A, M (Cappel, Organon Teknika Corp., West Chester, PA) was added. Following the addition of horseradish peroxidase-conjugated streptavidin (KPL Inc., Gaithersburg, MD), the plates were washed thoroughly with PBS-T. The spots were developed by the tetramethylbenzidine (TMB) with H₂O₂
Table 1
Standardization of the ELISPOT assay for detecting hybridomas secreting IgG monoclonal antibodies to rotavirus

| Hybridomas            | Rotavirus G/P serotype | Ig Class | Protein antigen specificity | Antigen specificity: average number of spots (ratios) |
|-----------------------|------------------------|----------|-----------------------------|------------------------------------------------------|
|                       |                        |          |                             | Gottb/IgG | OSUb/IgG | SB1Ab/IgG | (ss)c/IgG | VP4(G)d/IgG | VP4(O)d/IgG |
| Gottfried (G4/P6)     |                        |          |                             | G2a/VP4/P6 | 28.5/29 | 0/29 | 0/29 | 0/29 | 28.5/29 | 0/29 |
| RG24B9                | G2a                    |          |                             | Human 1-4 | (0.98)  | (0)  | (0)  | (0)  | (0.98)  | (0)  |
| RG25A10               | G1                     |          | VP6/- all Gp A              | 45/39.5 | 34.5/39.5 | 37/39.5 | 42.5/39.5 | (0.87)  | (0.94)  | (1.08)  | (0)  |
| OSU(G5/P7)            |                        | G1       | VP4/P7                      | 0/14    | 14.5/14 | 12.5/14 | 0/14 | 0/14 | 11/14 |
| R03F1                 |                        |          |                             | (0)     | (1.04)  | (0.89) | (0) | (0) | (0.78) |
| Control               | G1                     |          | VP7/Gp A                    | 11/12   | 12/12   | 12.5/12 | 0/12 | 0/12 | 0/12 |
| Cowden (GpC)          |                        | G1       | VP6/- Gp C only             | 0/21.5  | 0/21.5  | 0/21.5 | 0/21.5 | 0/21.5 | 0/21.5 |

* The spots were generated by adding similar hybridoma cell numbers, based on serial dilutions of each hybridoma, to duplicate wells. Each hybridoma ELISPOT assay was performed separately, but was the same antigen coating conditions.

b Ratio of the mean number of spots from the double-shelled viral particle-coated plates compared with the mean number from goat anti-mouse IgG coated plates. Data from the partially purified particles and fixed infected-cell plates presented similar ELISPOT numbers and results. Porcine rotaviruses Gottfried (Gott), OSU and SB1A strains. SB1A possesses the VP4 gene of OSU (P type 7) and VP7 gene of Gottfried (G type 4).

c ss, single-shelled Gottfried viral particles purified by CsCl gradient ultracentrifugation.

d VP4(G) and VP4(O) represent the supernatants of cell lysates from baculovirus vectors expressing the Gottfried VP4(G) or the OSU VP4 (O), respectively.

e Represents hybridoma cells secreting VP7 MAb broadly reactive with most Gp A rotavirus serotypes, supplied by Dr. H.B. Greenberg, Stanford University School of Medicine, CA (Offit et al., 1986b).

f Represents hybridoma cell secreting mAb which recognizes the VP6 counterpart of group C rotaviruses only (Ojeh et al., 1991).
membrane peroxidase substrate system (KPL Inc.). Counts were averaged from the duplicate wells at the dilutions that yielded less than 40 spots per well and were recorded as numbers of virus-specific ASC per $5 \times 10^5$ MNC.

3. Results

3.1. Susceptibility of gnotobiotic pigs to rotavirus infection

Pigs initially inoculated with porcine rotavirus SB1A or Gottfried typically exhibited watery diarrhea at post-inoculation days (PID) 1-3, lasting for 3-7 days (Table 2). Rectal virus shedding was detected by CCIF or IEM as early as 18 h post-inoculation. A comparison of the different inoculation groups in terms of diarrhea and virus shedding duration is shown in Table 2. Generally, pigs inoculated at 3-5 days of age exhibited diarrhea 1-2 days earlier (data not shown) and diarrhea persisted 1-2 days longer than the corresponding pigs inoculated at 25-27 days of age. There was little difference in the length of virus shedding, however, between the two groups. For observing development of protective active immunity to rotavirus infection in piglets, we challenged the pigs with the porcine rotavirus strains, SB1A or Gottfried, that share the same serotype-specific VP7 (G4) but a serotypically distinct VP4 (P7 for SB1A; P6 for Gottfried). As shown in Table 2, pigs initially inoculated with the SB1A strain demonstrated no diarrhea or viral shedding when challenged with SB1A. In the case of Gottfried infection and challenge, pigs exhibited similar results, but one of the 4 pigs ini-

| No. of pigs | Inoculum strain | Clinical responses |
|-------------|-----------------|--------------------|
|             | Primary\(^b\)\n(3-5 days) | Challenge\(^b\)\n(25-27 days) | Diarrhea (mean no. days ± SEM) | Virus shedding (mean no. days ± SEM)\(^a\) |
|             |                 |                    | Primary | Challenge | Primary | Challenge |
| 12          | SB1A            | NC\(^d\)           | 5.2 ± 0.5 | -         | 5.1 ± 0.2 | -         |
| 13          | Gott            | NC\(^d\)           | 5.5 ± 0.5 | -         | 5.5 ± 0.5 | -         |
| 2           | NI\(^e\)        | SB1A               | -       | 4.5 ± 0.5 | -         | 5.5 ± 0.5 |
| 2           | NI\(^e\)        | Gott               | -       | 3.5 ± 0.5 | -         | 5.0 ± 0.0 |
| 4           | SB1A            | SB1A               | 6.5 ± 0.4 | 0         | 5.5 ± 0.4 | 0         |
| 1           | Gott            | Gott               | 7.0     | 0         | 5.0       | 0         |
| 4           | SB1A            | Gott               | 4.8 ± 0.6 | 0.5 ± 0.3 | 5.8 ± 0.9 | 0.3 ± 0.3 |

\(^{a}\) Rectal virus shedding determined by CCIF assay.
\(^{b}\) Pigs were orally inoculated at 3-5 days of age.
\(^{c}\) Pigs were orally challenged at 25-27 days of age.
\(^{d}\) NC, not challenged.
\(^{e}\) NI, not inoculated.
tially inoculated with SB1A and then challenged with Gottfried, demonstrated a mild case of diarrhea with minimal (1 day) viral shedding.

3.2. Standardization of ELISPOT assay for porcine rotavirus antigens using hybridomas secreting rotavirus antibodies

The efficiency and specificity of the different antigen coatings used for the ELISPOT assay were tested with hybridoma ELISPOT assays and are illustrated in Table 1. The tests were performed with IgG-secreting hybridomas, whose specificities were examined with both goat-anti-mouse IgM and goat-anti-mouse IgG coated plates. No spots were generated in the anti-IgM wells (data not shown). By incubating hybridomas secreting antibodies specific to group A rotavirus VP6 and VP7, as well as Gottfried and OSU VP4 in wells coated with either rotavirus antigens or anti-mouse IgG, the effectiveness and specificity of the antigen coating was verified. The ratios of spots observed in the antigen-coated wells to spots in the anti-IgG wells were similar and ranged from 0.78 to 1.14, demonstrating that all hybridomas secreting IgG could be measured on the appropriate antigen-coated plates. For example, hybridomas secreting antibodies specific to VP4, VP6 and VP7 reacted with the corresponding antigens in plates coated with virus-infected acetone-fixed cells (viral structural proteins including VP4, 6, 7 and nonstructural proteins), purified double-shelled viral particles (DS) containing structural proteins VP4, 7 and also VP6 exposed on any broken particles (Shaw et al., 1991), and partially purified (PP) viral particles (structural proteins including VP4, 6, 7). The anti-VP6 hybridoma reacted with the DS viral preparations of all three group A rotavirus strains. However, the purified single-shelled particles (SS) (structural proteins including VP6, but lacking VP4, VP7) of Gottfried, SB1A or OSU (data not shown) reacted only with the anti-VP6 secreting hybridoma (RG25A10). Baculovirus-expressed Gottfried or OSU VP4 reacted only with the hybridomas (RG24B9 and RO3F1, respectively) secreting antibody to the corresponding antigen, although the capture ability of the OSU VP4 protein coating for hybridoma RO3F1 was only about 80% (0.78/1.04) of that detected using the purified OSU viral particles. The ELISPOT results from a test performed with IgG-secreting hybridoma cells, RC15B7 specific to group C rotavirus (Ojeh et al., 1991), were negative (Table 1).

3.3. Kinetics of Gottfried rotavirus-specific ASC responses following primary inoculation

The MNC were isolated from the MLN, spleen and LP of the small intestine (duodenum and ileum) of Gottfried rotavirus-inoculated pigs at various intervals between PID 0 and 29 to study the kinetics of the ASC responses (Fig. 1). The MNC were assayed for numbers of ASC on plates containing Gottfried rotavirus-infected fixed cells. Numbers of pigs tested at each time point for the different tissues varied from n = 1 (3 PID) to n = 5 (14 PID) for the MLN and spleen. At least two to three pigs were tested at all other time points for all tissues.
Fig. 1. Specific ASC to rotavirus per 5 x 10⁵ mononuclear cells in lymphoid tissues of rotavirus-inoculated gnotobiotic piglets. The pigs were inoculated with the Gottfried strain of porcine rotavirus and were slaughtered at 3–29 days after inoculation. The ELISPOT assay was performed with plates containing Gottfried rotavirus-infected fixed cells. MLN, mesenteric lymph nodes; Duod, duodenum; n, number of pigs. Bar, mean ± SEM.

Reasons for this variability in pig numbers included the difficulty in isolating the MNC from the LP of pigs in our initial experiments which used MNC from MLN and spleen at peak response days (PID 7 and 14) for standardization of the ELISPOPOT. Rotavirus-specific ASC of each isotype were detected by the ELISPOT from PID 7 through 29 in all four tissues (Fig. 1). In the negative control pigs, no viral-specific ASC were detected from the LP, MLN and spleens examined at 21 days of age (data not shown). Also, no spots were evident in uninfected control wells examined at each time point for each tissue and antibody isotype.

Rotavirus-specific IgM ASC appeared earlier in the MLN (PID 3) than in spleens (PID 3 was not tested for the LP because of the severe pathological damage to the small intestine at this time made it impossible to isolate the MNC numbers needed for the ELISPOT). Numbers of virus-specific IgM ASC peaked earliest in the MLN and spleen at PID 7, but in the LP of small intestine at PID 7 to 14. By PID 7, the numbers of specific IgM ASC were predominant in all tissues, except the duodenum (Fig. 1). The IgM ASC response was transient and declined in all tissues by PID 21. The specific IgA ASC numbers peaked in the MLN, LP and spleens by 14 to 21 PID. In the LP, the IgA ASC were predominant from PID 14 to 29 and were higher than in MLN and spleens. The virus-specific IgA and IgG ASC responses were similar in MLN and spleens. Overall, the numbers of virus-specific IgG ASC were highest in the spleen and duodenum at PID 14, but the IgG ASC made up a greater proportion of the ASC response in the spleen and
MLN versus the LP. In contrast, in the LP, the numbers of virus-specific IgA ASC remained higher than IgG or IgM ASC after PID 14. Moreover, the ASC responses in the spleen were the most transient and declined sharply by PID 21.

3.4. Primary SB1A rotavirus-specific ASC responses assayed using different viral coating preparations

In order to examine the relative contribution of different viral antigens, i.e. structural and nonstructural antigens as well as outer capsid antigens (VP7 and VP4) in eliciting ASC responses in different tissues, plates were coated with the different viral preparations indicated in Table 3. We employed the plates coated with the virus-infected fixed cells containing both structural and nonstructural rotavirus antigens; the viral DS particles having outer shell proteins VP7 and VP4 that surround the SS particles; and the partially purified viral particles (PP) possessing a mixture of DS and SS particles. This test was performed using gnotobiotic pigs (n = 3) from the same litter, inoculated with SB1A rotavirus.

The MNC isolated from the various tissues of the SB1A rotavirus-infected pigs were tested against each of the rotavirus coatings (Table 3). In the negative control uninoculated pigs, the ELISPOT specificity of the MNC isolated from the MLN and spleen was tested on plates with fixed SB1A-infected cells (data not shown) and plates coated with partially purified SB1A rotavirus (Table 4); no spots were generated.

The data for the SB1A rotavirus-specific IgG and IgA ASC from PID 14 (n = 2) and 21 (n = 1) were pooled, because numbers of IgG or IgA ASC were similar and peaked at these two time points. Table 3 can be summarized as follows. (i) The numbers of virus-specific IgG ASC generated were dramatically greater on the rotavirus-infected fixed-cell plates than on the other rotavirus coatings. The numbers of IgA ASC detected on the fixed cells also were higher than against the other coatings. (ii) The numbers of specific ASC that were detected using the PP and SS rotavirus antigen coatings were similar within a tissue type, whereas the numbers of specific ASC against the DS coating were more variable. (iii) The highest number of rotavirus-specific IgA ASC were in the LP of the small intestine as measured against any of the virus coatings. For example, on the rotavirus-infected fixed-cell plates the number of specific IgA ASC in the duodenum reached 83.3 ± 37.7 per 5 × 10^5 MNC; and the ratios of IgA/IgG ASC in the duodenum and ileum were much higher than those in the spleen or MLN. According to data from the fixed-cell coating, the number of IgA ASC in ileum was about 8-fold (63.3/7.7) greater than the number in the MLN, whereas the IgA ASC numbers in the duodenum were about 14-fold (83.3/6.0) greater than the number in the spleen. These results are in agreement with the data shown in Fig. 1.

Interestingly, the IgA to IgG ASC ratios against the structural viral antigens (PP, DS or SS) ranged from 13 to 46 for the gut LP, whereas these ratios were only 1 to 4 for the lymph node cells (spleen and MLN). However, the data for the infected fixed-cells (structural and nonstructural viral proteins) showed lower ratios of IgA to IgG ASC in the LP of the duodenum and ileum of 7 and 5, re-
| Tissue                  | ASC isotype | Spots detected in plates coated with viral antigens$^a$ |
|------------------------|-------------|------------------------------------------------------|
|                        |             | Fixed cells (A/G)$^b$ | P$^c$ (A/G) | DS$^c$ (A/G) | SS$^c$ (A/G) | VP4$^d$ (A/G) |
| Spleen                 | IgA         | 6.0 ± 4.5 (0.7)      | 4.2 ± 3.9 (2) | 5.5 ± 4.1 (4) | 4.2 ± 3.1 (1.4) | 0.2 ± 0.2 ND$^e$ |
|                        | IgG         | 8.8 ± 5.6            | 2.2 ± 0.7     | 1.3 ± 0.9     | 3.0 ± 1.8     | 0             |
| Mesenteric lymph nodes | IgA         | 7.7 ± 3.4 (1)        | 2.8 ± 2.1 (1) | 3.3 ± 1.4 (2) | 3.7 ± 1.4 (2) | 0             | ND$^e$        |
|                        | IgG         | 7.3 ± 2.8            | 2.2 ± 0.3     | 1.8 ± 0.4     | 1.7 ± 0.3     | 0             |
| Duodenum               | IgA         | 83.3 ± 37.7 (7)      | 61.2 ± 30.5 (41) | 39.2 ± 22.9 (33) | 77.8 ± 39.4 (46) | 0.8 ± 0.8 (4.0) |
|                        | IgG         | 11.7 ± 6.7           | 1.5 ± 1.5     | 1.2 ± 0.7     | 1.7 ± 1.2     | 0.2 ± 0.1     |
| Ileum                  | IgA         | 63.3 ± 21.9 (5)      | 41.3 ± 22.8 (24) | 26.5 ± 12.2 (13) | 33.2 ± 18.5 (18) | 0.2 ± 0.2 ND$^e$ |
|                        | IgG         | 14.1 ± 4.5           | 1.7 ± 0.3     | 2.0 ± 0.6     | 1.8 ± 0.9     | 0             |

$^a$ Mean ± SEM (5 x 10$^4$ mononuclear cells).

$^b$ Ratio of IgA- to IgG-ASC.

$^c$ PP, partially purified particles; DS, double-shelled particles; SS, single-shelled particles; rotavirus strain SB1A.

$^d$ Supernatant of cytolysates containing OSU rotavirus VP4 expressed in a baculovirus expression system.

$^e$ ND, not determined.
Table 4

Isotypic-specific ASC responses to rotavirus after inoculation and/or challenge of gnotobiotic pigs with SBIA or Gottfried porcine rotaviruses

| No. of pigs | Inoculation at 3-5 days of age (PIDs) | Challenge at 25-27 days (PCDs) | 25-27 days post-challenge day (PCD)d | Mesenteric lymph nodes IgG (mean ± SEM) | Spleen IgG (mean ± SEM) | Mesenteric lymph nodes IgM (mean ± SEM) | Spleen IgM (mean ± SEM) | Mesenteric lymph nodes IgA (mean ± SEM) | Spleen IgA (mean ± SEM) |
|-------------|--------------------------------------|-------------------------------|---------------------------------|---------------------------------|-----------------|---------------------------------|-----------------|---------------------------------|-----------------|
| SBIA(7)     | 1.0 ± 0.2                            | < 1                           | 1.0 ± 0.2                       | 1.0 ± 0.2                       | 1.0 ± 0.2       | 1.0 ± 0.2                       | 1.0 ± 0.2       | 1.0 ± 0.2                       | 1.0 ± 0.2       |
| Gott(7)     | 2.0 ± 0.4                            | 1.0 ± 0.2                      | 2.0 ± 0.4                       | 2.0 ± 0.4                       | 2.0 ± 0.4       | 2.0 ± 0.4                       | 2.0 ± 0.4       | 2.0 ± 0.4                       | 2.0 ± 0.4       |
| SBIA(29)    | 1.0 ± 0.2                            | 1.0 ± 0.2                      | 1.0 ± 0.2                       | 1.0 ± 0.2                       | 1.0 ± 0.2       | 1.0 ± 0.2                       | 1.0 ± 0.2       | 1.0 ± 0.2                       | 1.0 ± 0.2       |
| Gott(29)    | 2.0 ± 0.4                            | 2.0 ± 0.4                      | 2.0 ± 0.4                       | 2.0 ± 0.4                       | 2.0 ± 0.4       | 2.0 ± 0.4                       | 2.0 ± 0.4       | 2.0 ± 0.4                       | 2.0 ± 0.4       |
| SBIA(31)    | 1.0 ± 0.2                            | 1.0 ± 0.2                      | 1.0 ± 0.2                       | 1.0 ± 0.2                       | 1.0 ± 0.2       | 1.0 ± 0.2                       | 1.0 ± 0.2       | 1.0 ± 0.2                       | 1.0 ± 0.2       |
| Gott(31)    | 2.0 ± 0.4                            | 2.0 ± 0.4                      | 2.0 ± 0.4                       | 2.0 ± 0.4                       | 2.0 ± 0.4       | 2.0 ± 0.4                       | 2.0 ± 0.4       | 2.0 ± 0.4                       | 2.0 ± 0.4       |

a Data from the ELISPOT assay performed on plates coated with the corresponding partially purified viral particles.

b The inoculation was at 3-5 days of age.

c The challenge was at 21 days post-primary inoculation or at 25-27 days of age.

d Post-inoculation day (PID) or post-challenge day (PCD).

NI' Not challenged.

Not inoculated.

9 Control pigs were not inoculated with rotavirus and were slaughtered at 21 PID.

[322x6]<l>17.2±6.6 14.6±4.5 34.6±4.5 19.5±6.0 9.5±8.5 4.4±2.0 44.4±1.5 11.0±1.5 25.1±19.0 8.6±3.0
322x-4>11.9±78.8 37.9±20.6 66.5±20.6 19.5±6.0 1.9±1.5 24.3±15.7 24.3±15.7 1.9±1.5 24.3±15.7 0
322x-14>11.3±3.0 4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 19.5±3.0 19.5±3.0 19.5±3.0 0
322x-24>10.7±3.8 10.3±1.3 16.4±3.6 8.6±3.0 10.8±10.8 0
322x-34>14.8±13.7 14.8±13.7 14.8±13.7 14.8±13.7 18.0±18.0 0
322x-44>1.9±0.8 1.9±0.8 1.9±0.8 1.9±0.8 1.9±0.8 0
322x-54>12.6±4.5 7.3±0.8 11.0±1.5 25.1±19.0 8.6±3.0 0
322x-64>10.8±10.8 10.8±10.8 10.8±10.8 10.8±10.8 10.8±10.8 0

[325x-4]10.3±1.3 16.4±3.6 8.6±3.0 10.8±10.8 0
325x-34>14.8±13.7 14.8±13.7 14.8±13.7 14.8±13.7 18.0±18.0 0
325x-44>1.9±0.8 1.9±0.8 1.9±0.8 1.9±0.8 1.9±0.8 0
325x-54>12.6±4.5 7.3±0.8 11.0±1.5 25.1±19.0 8.6±3.0 0
325x-64>10.8±10.8 10.8±10.8 10.8±10.8 10.8±10.8 10.8±10.8 0

[394x-8]10.3±1.3 16.4±3.6 8.6±3.0 10.8±10.8 0
394x-14>14.8±13.7 14.8±13.7 14.8±13.7 14.8±13.7 18.0±18.0 0
394x-24>1.9±0.8 1.9±0.8 1.9±0.8 1.9±0.8 1.9±0.8 0
394x-34>12.6±4.5 7.3±0.8 11.0±1.5 25.1±19.0 8.6±3.0 0
394x-44>10.8±10.8 10.8±10.8 10.8±10.8 10.8±10.8 10.8±10.8 0

[450x-1]10.3±1.3 16.4±3.6 8.6±3.0 10.8±10.8 0
450x-14>14.8±13.7 14.8±13.7 14.8±13.7 14.8±13.7 18.0±18.0 0
450x-24>1.9±0.8 1.9±0.8 1.9±0.8 1.9±0.8 1.9±0.8 0
450x-34>12.6±4.5 7.3±0.8 11.0±1.5 25.1±19.0 8.6±3.0 0
450x-44>10.8±10.8 10.8±10.8 10.8±10.8 10.8±10.8 10.8±10.8 0

[504x-1]4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0
504x-14>4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0
504x-24>4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0
504x-34>4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0
504x-44>4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0

[564x-1]4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0
564x-14>4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0
564x-24>4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0
564x-34>4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0
564x-44>4.4±2.0 9.5±3.0 24.3±15.7 11.3±3.0 19.5±3.0 0

[69x-81]a Data from the ELISPOT assay performed on plates coated with the corresponding partially purified viral particles.

b The inoculation was at 3-5 days of age.

c The challenge was at 21 days post-primary inoculation or at 25-27 days of age.

d Post-inoculation day (PID) or post-challenge day (PCD).
spectively; and in the spleen and MLN where these ratios were only 0.7 and 1, respectively.

By coating OSU VP4 protein to capture the SB1A VP4-specific antibody from ASC, VP4-specific IgA ASC were detected only in the spleen, duodenum and ileum in very low numbers. VP4-specific IgG ASC occurred only in the duodenum in low numbers. These data (Table 3) indicate that at PID 14 to 21 the VP4-specific ASC is not over 5% (0.2/4.2) of the total structural (PP) antigen-specific ASC in the spleen and not over 1.3% (0.8/61.2) to 4.8% (0.2/41.3) of the corresponding ASC in the duodenum and ileum, respectively.

3.5. Specific ASC responses of young pigs at different ages after inoculation and challenge with rotavirus

We performed the ELISPOT assay to evaluate and compare the primary and secondary immune response to rotavirus inoculation using plates coated with the partially purified viral particles (structural viral proteins) (Table 4). Although the duration of diarrhea and virus shedding in the two groups of pigs that were initially inoculated with the same dose of SB1A rotavirus at 3-5 and 25-27 days of age was similar (Table 2), we observed some differences at PID 7 in the immune responses of gnotobiotic piglets of different ages. Our results (Table 4) indicate that the virus-specific ASC responses in pigs inoculated at different ages with SB1A rotavirus exhibited similar patterns in spleen and MLN, with regard to the numbers of specific IgM ASC which were much higher than IgG or IgA ASC. Results after primary infection with Gottfried rotavirus were similar (Table 4). However, the data show that in the MLN, specific IgA ASC responses occurred in the older pigs (2.2-9.5 IgA ASC per $5 \times 10^5$ MNC), but not in the younger pigs (less than 1 IgA ASC per $5 \times 10^5$ MNC) at PID 7.

In the case of the challenge groups, the specific ASC responses to the homotypic challenge (SB1A/SB1A), at post-challenge day (PCD) 6 (PID 27), were similar to the primary responses to SB1A at PID 29, but with higher numbers of IgM ASC (7.3 vs. 3.2) in MLN and IgA ASC (24.3 vs. 9.5) in spleen (Table 4). For both SB1A/SB1A and SB1A (PID 29) groups, the numbers of specific IgA ASC were greater than the numbers of IgM ASC. However, the ASC responses to the heterotypic challenge (SB1A/Gottfried), at either PCD 6 or PCD 10 showed that the heterologous rotavirus challenge elicited a higher increase in the IgM ASC in the spleen (7.9 or 21.0) versus the homologous response (1.8), whereas the IgM ASC responses in the MLN were more similar between heterologous and homologous virus challenges (14.8 or 10.8 vs. 7.3). Similar trends were not as apparent in the IgG or IgA ASC responses, but these responses were difficult to evaluate because of the small numbers of pigs and the variability observed.

4. Discussion

The present study provides evidence for rotavirus-specific ASC immune responses in gnotobiotic pigs inoculated with virulent porcine rotaviruses, focusing
especially on the small intestinal mucosa and draining lymph node (MLN) adjacent to the site of rotavirus replication (the gut). Mucosal immunity is important for protection against enteric pathogens (McGhee et al., 1992). However, investigations of protective mechanisms against rotavirus infections often focused on serum antibody responses which may not correlate with enteric protection; only a few researchers investigated the role of coproantibodies in recovery from rotavirus infection (Riepenhoff-Talty et al., 1981; Saif, 1987; Grimwood et al., 1988; Conner et al., 1991). Research groups have employed various techniques to study the local enteric mucosal antibody response to rotavirus infection. Those techniques included using ELISA to detect antibody isotypes in feces from humans (Riepenhoff-Talty et al., 1981; Grimwood et al., 1988), calves (Saif, 1987) and rabbits (Conner et al., 1991), identification of specific antibody-producing cells in intestinal tissue sections by immunohistochemistry in a mouse model (Dharakul et al., 1988), and counting the specific ASC isolated from mouse lamina propria (LP) using an ELISPOT assay (Merchant et al., 1991; Shaw et al., 1991, 1992). All of these studies confirmed the predominance of specific IgA antibody responses in the mucosal secretions or intestinal tissues. Our ASC data in pigs exposed to porcine rotaviruses corroborate and extend these earlier findings.

Because of the small numbers of gnotobiotic pigs available at each time point and the degree of variability in ASC numbers between pigs, it was not feasible to statistically evaluate our data. However, the following trends were evident. The data shown in Table 3 and Figs. 1 (C) and 1 (D) demonstrate that rotavirus-specific IgA ASC in the small intestine LP, especially the duodenum, represent the highest response (the mean number of spots was 65 IgA ASC per 5×10^5 MNC, n=6, in Fig. 1 (C) by PID 14–21. Further, in the intestinal LP of pigs, the ratio of the numbers of structural viral antigen-specific IgA ASC to those of specific IgG ASC ranged from 13 to 46 (Table 3). Although this ratio was higher than the LP ASC data (IgA/IgG = 10) from the mouse model employed by Merchant et al. (1991), their results were based on a heterologous simian rotavirus infection in mice. However, their findings concur that specific IgA ASC represent the greatest population of ASC in the LP of the small intestine. In our studies, the magnitude of the IgA ASC response in the gut LP to the structural and nonstructural antigens of rotavirus was about 8–14 times greater than for the MLN and spleen (Table 3). Although the ASC numbers we detected in gnotobiotic pigs were less than the ASC numbers reported in mice exposed to rotavirus by Merchant et al. (1991), it is possible that the immune stimulus induced by the normal gut flora in conventional animals in comparison with gnotobiotic ones may play a role in the overall magnitude of the ASC numbers. A similar phenomenon (reduced numbers of ASC or Ig synthesis) was observed in the nasal mucosa or Peyer's patches of gnotobiotic versus conventional mice (McClelland, 1976; Ichimiya et al., 1991).

In gnotobiotic pigs, the kinetics of the specific IgM ASC response showed a different pattern from the IgA ASC response. The specific IgM ASC were detected
in MLN as early as PID 3. The highest numbers of virus-specific IgM ASC (mean 49.8 IgM ASC per $5 \times 10^5$ MNC, $n=4$) were measured in the spleen at PID 7 (Fig. 1(B)). Similar results (82 IgM ASC per $10^6$ MNC) were reported by Kaila et al. (1992) using ELISPOT to measure ASC among circulating blood lymphocytes from children infected with human rotavirus. Our data demonstrate the specific IgM ASC reached a peak and were predominant in the MLN and spleen by PID 7 (Figs. 1(A) and 1(B)). The IgM ASC peaked in the duodenum and ileum by PID 14 but were fewer in number than IgA ASC (Figs. 1(C) and 1(D)).

By PID 14, the specific IgM ASC decreased in the MLN and spleen similar to the pattern for ASC in human blood observed by Kaila et al. (1992). Specific IgG ASC and IgA ASC in both MLN and spleen peaked at 14 PID. In the intestinal LP, the IgA ASC were predominant at all time points except PID 7 in ileum. We also observed an interesting difference in the immune responses in the LP to antigens of rotavirus (Table 3). The numbers of specific IgA ASC in the duodenum to total (structural and nonstructural) viral antigens (the rotavirus-infected fixed cells) were about seven times greater than the numbers of IgG ASC; in contrast, the IgA ASC numbers in the duodenum to only viral structural antigens (PP, DS or SS virus) were approximately 40 times higher than the corresponding IgG ASC numbers. Also, the IgA ASC response in ileum to total structural and nonstructural viral antigens was about five-fold higher than the IgG ASC response, and the average ratio of IgA to IgG in the ileum against the viral structural antigens was about 18. This phenomenon suggests a proportionately greater contribution of IgG ASC responses to the nonstructural rotaviral antigens adjacent to the site of rotavirus replication in the small intestine.

VP4 is a component of the outer shell of rotavirus and elicits neutralizing antibodies (Offit et al., 1986a,b; Hoshino et al., 1988; Ijzas et al., 1991). Using the ELISPOT assay, we detected very few positive ASC reactive with baculovirus-expressed VP4 in each tissue tested. Because VP4 constitutes only about 1.5% of the mass of the viral particle, it is not surprising that the specific ASC response to VP4 is very low. Our data showed that numbers of VP4-specific ASC generally constitute about 1.3–4.8% of the total virus-specific ASC; moreover, VP4-specific IgA ASC outnumbered IgG ASC in the tissues examined, especially in the LP of the duodenum. This is in agreement with observations for mice by Shaw et al. (1991, 1992), whose data showed that the VP4-specific ASC were less than 2% of rotavirus-specific ASC response in mice infected with a heterologous simian SA11 rotavirus. In gnotobiotic pigs, we previously found partial protection of SB1A-inoculated pigs from challenge with OSU porcine rotavirus (common VP4 type (P7), distinct VP7 type (G4 for SB1A, G5 for OSU)) (Hoshino et al., 1988). Although the VP4-specific ASC responses occurred at relatively low levels, evidence of protection against OSU challenge in pigs suggests that the VP4-specific immune responses still play an important role in intestinal mucosal immunity to rotavirus infection. However, such protection may not be as efficient as that induced by the other major outer capsid neutralizing antigen, VP7.

Our ELISPOT assay conducted using hybridoma cells secreting rotavirus-specific antibodies showed that VP6 specific hybridoma cells were able to efficiently
detect the corresponding antigen of the three group A rotaviruses based on the viral antigen coatings of partially purified viral particles and fixed infected-cells, as well as SS particles. However, we also found that the VP6 specific hybridoma cells reacted with DS viral preparations (Table 1). This result confirms the same phenomenon observed by Shaw et al. (1991) and suggests that DS viral particles used for coating the plates became partially degraded, resulting in exposure of VP6 on the surface of such particles. As also reported by Shaw et al. (1991), generally our data confirm that the various preparations of rotavirus structural antigens (PP, DS and SS) for the coatings generated similar numbers of ASC within a tissue type except for lower numbers of IgA ASC in the duodenum against the DS coating (Table 3). Because VP6 is the predominant protein in rotavirus particles, it is not unexpected that the specific ASC responses to other structural proteins such as VP4 can be eclipsed by the large quantity of VP6 specific ASC. Therefore, the positive ASC responses detected in DS coated plates probably included the VP6-specific ASC as well as the ASC elicited by the outer shell proteins, VP4 and VP7. Although 96-well plates have been used routinely for ELISPOT assays including the enumeration of specific isotypes of ASC in most tissues, when VP4 ASC are measured which constitute a very small proportion of the total ASC population, such plates may be not efficient enough for more accurate assessment of the more limited numbers of MNC secreting antibodies to VP4.

Table 2 shows that the susceptibility of gnotobiotic pigs to rotavirus (both clinical signs and virus shedding) lasted at least through 25 days of age. Further, generally similar response patterns were noted for the specific ASC detected at PID 7 when the pigs were initially inoculated at 3-5 or 25-27 days of age (Table 4), illustrating that humoral immune responses are induced in newborn pigs and supporting the concept that pigs are immunocompetent at birth (Porter, 1979). However, we observed that in the older pigs inoculated with either SB1A or Gottfried rotavirus at 25-27 days of age, the specific IgA ASC responses in the MLN at PID 7 were higher than in the younger pigs (Table 4), suggesting a possible delay in the maturation of the IgA antibody responses. Moreover, our data demonstrate that most piglets exposed to SB1A (G4P7) rotavirus are protected from diarrhea and virus shedding when challenged with heterotypic Gottfried (G4P6) rotavirus (Table 4), in agreement with earlier observations by Hoshino et al. (1988). However, we observed that one of four SB1A-inoculated pigs developed transient (1 day) diarrhea and virus shedding after challenge with heterotypic Gottfried rotavirus (Table 2), and that the numbers of virus specific IgM ASC in the spleen after the challenge of SB1A virus-inoculated pigs with heterotypic Gottfried rotavirus were higher than after challenge with homotypic SB1A rotavirus (Table 4). It is possible that limited viral replication in the gut of pigs challenged with the heterotypic rotavirus induced these specific IgM ASC.

Because pigs are monogastric and closely resemble humans physiologically (Phillips and Tumbleson, 1986), previous studies have suggested that the pig can be developed as an appropriate model for enteric infections in humans (Wyatt et al., 1980; Schaller et al., 1997). Equally important, the use of gnotobiotic pigs assures that exposure to extraneous rotaviruses or other enteric pathogens is
eliminated as a confounding variable. Moreover, we have found that clinical signs in gnotobiotic pigs infected with various heterologous human rotavirus strains resemble those seen in pigs after infection with rotaviruses of host origin (Wyatt et al., 1980; Schaller et al., 1992; Saif and Chen, unpublished data, 1992). Our results suggest that the gnotobiotic pig maybe have important advantages over mice and rabbits as an animal model to study rotavirus infections, particularly in studies designed to evaluate active antibody responses and protection against infection and disease.

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