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Species-specific but not genotype-specific primary and secondary isotype-specific NSP4 antibody responses in gnotobiotic calves and piglets infected with homologous host bovine (NSP4[A]) or porcine (NSP4[B]) rotavirus

Lijuan Yuan[1], Shinjiro Honma, Shin-Ichi Ishida[2], Xiao-Yi Yan[3], Albert Z. Kapikian, Yasutaka Hoshino*

Epidemiology Section, Laboratory of Infectious Disease, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, United States

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Abstract

Using recombinant baculoviruses expressing rotavirus NSP4 [A], [B], [C], and [D] genotypes of bovine, porcine, human, simian, or murine origin, we analyzed serum antibody responses to NSP4s in gnotobiotic calves and piglets infected by the oral/alimentary or intraamniotic route with bovine (NSP4[A]) (Wyatt, R.G., Mebus, C.A., Yolken, R.H., Kalica, A.R., James, H.D., Jr., Kapikian, A.Z., Chanock, R.M., 1979. Rotaviral immunity in gnotobiotic calves: heterologous resistance to human virus induced by bovine virus. Science 203(4380), 548–550) or porcine (NSP4[B]) (Hoshino, Y., Saif, L.J., Sereno, M.M., Chanock, R.M., Kapikian, A.Z., 1988. Infection immunity of piglets to either VP3 or VP7 outer capsid protein confers resistance to challenge with a virulent rotavirus bearing the corresponding antigen. J. Virol. 62(3), 744–748) rotaviruses. Following primary infection and challenge with virulent rotaviruses, the animals developed higher or significantly higher antibody titers to homologous host homotypic NSP4s than to heterologous host homotypic or heterologous host heterotypic NSP4s, indicating that antibody responses were species specific rather than genotype specific. Antibody responses to NSP4s corresponded closely with the phylogenetic relationships of NSP4s within a species-specific region of amino acids (aa) 131–141. In contrast, NSP4 genotypes determined by amino acid full-length sequence identity predicted poorly their "serotypes". In piglets, antibodies to NSP4 induced by previous oral infection failed to confer protection against challenge from a porcine rotavirus bearing serotypically different VP4 and VP7 but essentially identical NSP4 to the porcine rotavirus in primary infection. Thus, in an approach to immunization with a live oral rotavirus vaccine, the NSP4 protein does not appear to play an important role in protection against rotavirus disease and infection.
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Keywords: Rotavirus; NSP4; Antibody response; NSP4 genotype/serotype; Species specificity; Protective immunity; Gnotobiotic pigs/calves

* Corresponding author. Epidemiology Section, Laboratory of Infectious Disease, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 50 South Drive MSC 8026, Building 50, Room 6308, Bethesda, MD 20892-8026. Fax: +1 301 480-1387.

E-mail addresses: yuan.21@osu.edu (L. Yuan), thoshino@niaid.nih.gov (Y. Hoshino).

[1] Present address: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Department of Veterinary Preventive Medicine, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691-4096, United States.

[2] Present address: The Imamura Hospital, Shimoshinjyo Nakano, Akita, Japan.

[3] Present address: Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, United States.

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Introduction

Rotaviruses are the single most important etiological agent of severe diarrhea in infants and young children worldwide (Kapikian et al., 2001), and estimated to be responsible for up to 592 000 deaths annually in children <5 years old mostly in developing counties (Parashar et al., 2003). Therefore, development of a safe and effective rotavirus vaccine has been a high global public health priority. Understanding the mechanism and identifying the determinant of protective immunity in rotavirus infection and vaccination are crucial for the development of effective vaccination strategies.

The rotavirus genome consists of 11 segments of double-stranded RNA, which are surrounded by a triple-layered capsid. The 11 gene segments encode six structural (VP1–6) and six nonstructural proteins (NSP1–6), with the gene segment 11 encoding both NSP5 and 6 (Estes, 2001). Rotavirus nonstructural protein NSP4, encoded by gene segment 10, is a multifunctional 28-kDa glycoprotein of 175 amino acids (aa) (Estes, 2001). NSP4 was first recognized to play an important role in rotavirus pathogenesis based on studies in gnotobiotic pigs (Hoshino et al., 1995) and was later proposed to function as a viral enterotoxin based on studies in mice (Ball et al., 1996). In addition, the NSP4 114–135 peptide derived from simian SA11 rotavirus was shown to possess part of the enterotoxin functional domain. Furthermore, mouse pups born to dams immunized with the NSP4 114–135 peptide derived from SA11 were partially protected passively against diarrhea induced by SA11 virus (homotypic protection) (Ball et al., 1996). Mouse pups born to dams fed transgenic potato tuber expressing cholera toxin-murine rotavirus NSP4 (114–135) were protected partially against diarrhea induced by SA11 virus (heterotypic protection because murine and simian NSP4s belong to different NSP4 genotypes, however, they share 82% amino acid (aa) identity in this region) (Yu and Langridge, 2001). Zhang et al. (1998) reported that changes within the region of aa residues 131–140 of NSP4 were associated with differences in the pathogenesis of two porcine rotaviruses (OSU and Gottfried strains) in neonatal mice. Such observations have aroused renewed scientific interest on NSP4 gene structure and function. Sequence analyses of NSP4 genes from human and animal (mammalian and avian) rotaviruses have shown that there are five NSP4 genetic groups (genotypes) (NSP4[A]–[E]) among group A rotaviruses (Lin and Tian, 2003; Mori et al., 2002). Genotypes [A], [B], and [C] NSP4s have been detected in human rotaviruses whereas NSP4[D] and [E] have been found only in mice and in birds, respectively. The full-length NSP4-deduced aa sequence identity was at least 87% or higher among members of the same genotype, 78–87% among NSP4[A], [B], and [C] (Ciarlet et al., 2000), and 60–65% between NSP4[D] and NSP4[A], [B], or [C]. Avian rotavirus NSP4s share only 31–37% identities with those of mammalian rotavirus NSP4s (Mori et al., 2002). Of note, aa sequences at the proposed pathogenicity-altering region (aa 131–140) have a higher variability than the average identities of full-length NSP4s, with greatest sequence variations occurring between aa residues 135–141 (Horie et al., 1997). Mohan and Atreya (2000) referred to this region as the interspecies variable domain because this domain demonstrated a certain degree of intraspecies conservation while having sufficient interspecies variation based on comparison of deduced aa sequences of NSP4s from a number of human, simian, bovine, and porcine rotavirus strains. On the other hand, Borgan et al. (2003) suggested that the antigenic site containing aa 136–150 is widely conserved among a variety of rotaviruses based on results that a group of 11 monoclonal antibodies generated against this region of an avian rotavirus NSP4 cross-reacted with NSP4s of mammalian rotavirus strains with different NSP4 genotypes.

Although the NSP4 protein has been suggested to play a potentially important role in rotavirus immunity and protection (Estes, 2003; Estes et al., 2001), the relative importance of this protein in pathogenesis and immunity in humans (especially in children who are the targets of rotavirus infection) is unknown. Furthermore, an association between the genotypic phylogeny and antigenic relationships of NSP4 proteins has never been explored. In a previous study, we analyzed using an immunocytochemistry assay as used in this study and recombinant baculoviruses expressing rotavirus proteins serum isotype-specific antibody responses to rotavirus NSP4[A], [B], and [C] in infant vaccinees and reported that the magnitude of antibody responses to homotypic and heterotypic NSP4s was not significantly different with the various NSP4 genotypes of administered vaccine strains (Yuan et al., 2004). Our results concurred with a report evaluating serum antibody responses to truncated fusion peptides of NSP4 genotype [A], [B], or [C] in children naturally infected with rotaviruses in India (Ray et al., 2003). In both studies, a definitive analysis of the antigenic relationships of NSP4[A], [B], and [C] was not possible since many infants and children had preexisting rotavirus-specific serum IgA or IgG antibodies.

Neonatal gnotobiotic calves and piglets have been used as animal models for study of rotavirus pathogenesis and immunity for decades (Theil et al., 1978; Wyatt et al., 1979; Yuan and Saif, 2002). They are susceptible to homologous host as well as certain heterologous host rotavirus infection and disease up to 6–8 weeks of age (Kohara and Tsunemitsu, 2000; Saif et al., 1996), allowing assessment of protective immunity against disease upon reexposure. In addition, colostrum-deprived gnotobiotic calves and piglets are totally devoid of maternal antibodies due to the lack of the transfer of immunoglobulins via the placenta from mothers to their offsprings in those animal species (Goddeeris, 1998; Wagstrom et al., 2000). The gnotobiotic status ensures that the nature of true primary immune responses to a single rotavirus strain can be evaluated since the

confounding factors in human studies (e. g., maternal antibody interference and unknown exposure to wild-type rotaviruses) can be avoided.

Using the immunocytochemical staining assay and seven recombinant baculoviruses expressing NSP4 proteins (three NSP4[A]s, two NSP4[B]s, and one each of NSP4[C] and [D]), we evaluated primary and secondary isotype-specific serum antibody responses to homotypic and heterotypic NSP4s in colostrum-deprived gnotobiotic calves and piglets infected orally with bovine (NSP4[A]) (Wyatt et al., 1979) or porcine (NSP4[B]) (Hoshino et al., 1988) rotavirus strains, respectively. In addition, we analyzed associations between antigenic (antibody geometric mean titer [GMT] ratios) and phylogenetic (sequence identity) relationships of homologous host homotypic, heterologous host homotypic, as well as heterologous host heterotypic NSP4s. Furthermore, we examined in piglets the role of antibodies to NSP4 on subsequent susceptibility or resistance to challenge with a porcine rotavirus bearing serotypically different VP4 and VP7 but essentially identical NSP4 to the porcine rotavirus used in primary infection.

Results

IgG and IgA antibody responses to NSP4s in gnotobiotic calves

No IgG or IgA antibodies to NSP4 were detected in cord blood samples of any calves before infection with bovine rotavirus NCDV. High levels of homotypic as well as varying degrees of heterotypic IgG antibodies were detected in all the calves infected in utero (group 1), or postnatally at 1 day old (group 2), or infected in utero initially then challenged postnatally at 1 day old (group 3) with NCDV (Fig. 1), indicating the cross-reactive nature of NSP4 protein. Homotypic response is defined as the antibody response to detector NSP4 antigen belonging to the same genotype as the NSP4 of the rotavirus strain used to infect the animals. Heterotypic response is defined as the antibody response to the NSP4 antigen belonging to the genotype different from the NSP4 of the infecting rotavirus strain. When the detector NSP4 and the NSP4 of infecting rotavirus belonging to the same genotype are derived from the same animal species, the antibody response is defined as homologous host homotypic response. When the two NSP4s belonging to the same genotype are derived from the different animal species, the antibody response is defined as heterologous host homotypic response.

Although the magnitude of the IgG antibody responses differed among three groups with group 3 calves having overall higher IgG antibody titers than group 1 and group 2 calves, the pattern of antibody responses was similar in all three groups. The highest IgG antibody titers were to the homologous host homotypic NCDV (NSP4[A]) in all groups. In group 1 calves, IgG antibody titers to NCDV (NSP4[A]) were significantly higher ($P < 0.05$) than those to the heterologous host homotypic DS-1 and SA11 (NSP4[A]) and heterologous host heterotypic RRV (NSP4[C]) and EB (NSP4[D]) (Fig. 1A). Higher levels of antibodies observed in calves infected in utero (versus calves infected postnatally) may be explained by prolonged exposure of the fetus to viral antigens. In group 2 calves, the IgG antibody titers to NCDV (NSP4[A]) were significantly higher ($P < 0.05$) than those to any of the other homotypic and heterotypic NSP4s (Fig. 1B). In group 3 calves, the small number of samples ($n = 2$) prevented statistical analysis. However, the pattern of IgG antibody responses in this group was basically identical to that of group 2 calves; even though the overall magnitude of IgG antibody responses in group 3 (anamnestic responses) was more than 20-fold higher than that in group 2 calves (primary responses) (Fig. 1C). Surprisingly, the IgG antibody titers to homotypic SA11[A] NSP4 were lower than those to heterotypic Wa[B] or SB1A[B] NSP4 in all three groups. These data suggested that (i) the NSP4-specific antibody responses were not genotype specific, and (ii) the antigenic relationship between homotypic NCDV and SA11 NSP4s was more distantly related than that between heterotypic NCDV and SB1A NSP4s.

Very low or no NSP4 IgA antibody titers were detected (GMTs ranging from <1:4 to 1:10) in any calves infected with NCDV in any group (data not shown).

IgG and IgA antibody responses to NSP4 in gnotobiotic piglets

To further evaluate homotypic versus heterotypic as well as possible species-specific antibody responses to NSP4, antibody titers were measured using a panel of the same seven recombinant NSP4s in serum samples from gnotobiotic piglets infected and then challenged with virulent porcine rotaviruses. The NSP4 of porcine rotavirus SB1A strain used as a detector antigen in this assay shares more than 97% aa identity with that of OSU or Gottfried strain. Because the serum samples were from a previous study (Hoshino et al., 1988), only a subset of samples from each group (Table 1) was available for the present study. There were no significant differences in the magnitude of IgG antibody responses to NSP4 among piglets in groups 1–4 at either PID (post infection day) 21 or PID 37/PCD (post challenge day) 16 (data not shown). The greatest differences of NSP4 IgG GMTs were less than 3.5-fold. Piglets in groups 1–4 were therefore combined for analysis of homotypic and heterotypic NSP4 IgG and IgA antibody responses. Eleven pairs of serum samples (combining all piglets from groups 1 to 4) were available for measuring IgG antibody titers to NSP4s pre- and postchallenge. Only 4 pre- and 13 postchallenge serum samples from groups 1–4 had sufficient amounts of serum available for measuring IgA antibody titers to NSP4. Because the serum IgA, but not IgG, antibody responses are closely related to the intestinal antibody responses (Yuan and Saif, 2002), the IgA results

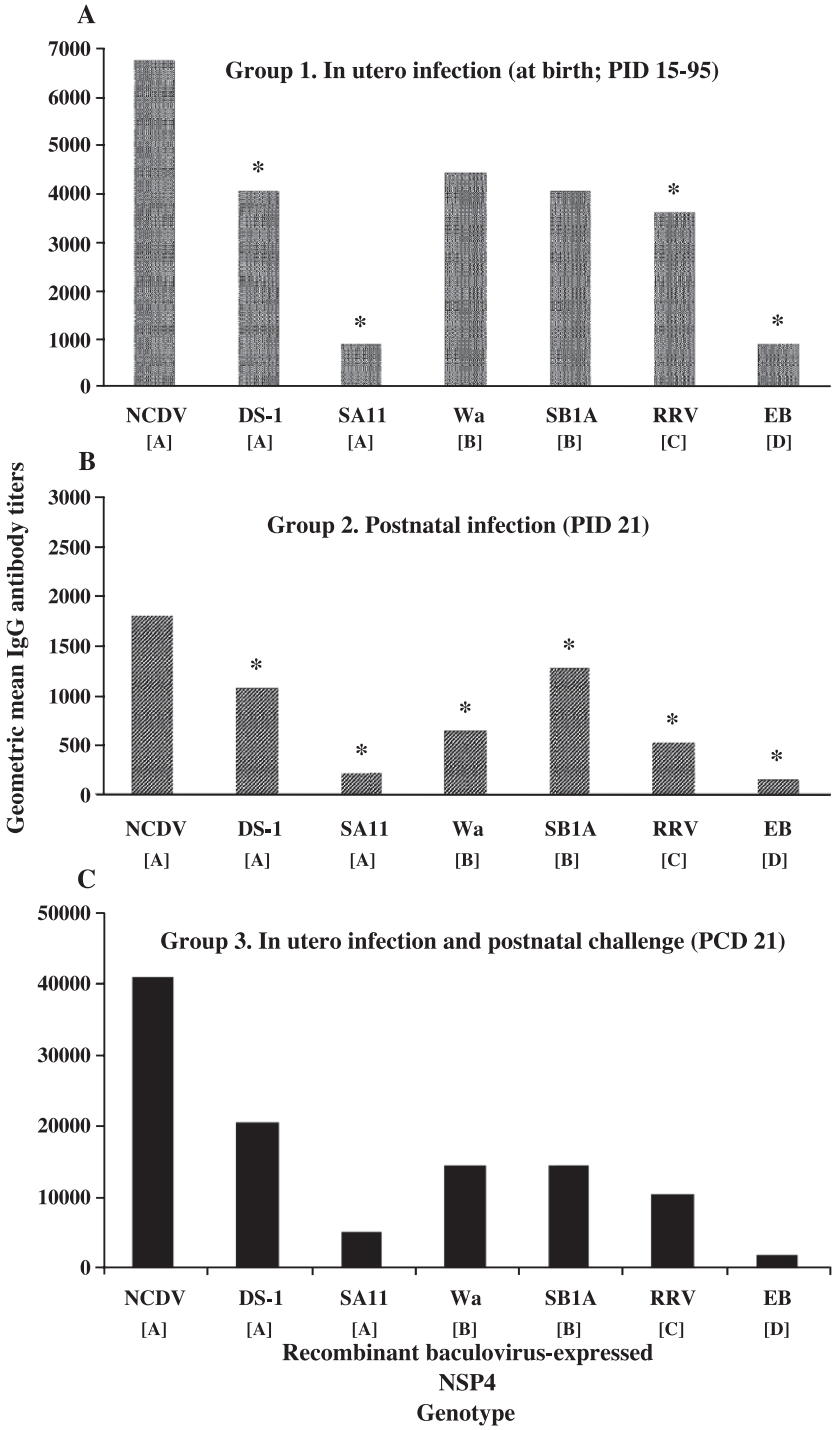

Fig. 1. Serum IgG antibody responses to NSP4s in gnotobiotic calves infected with NCDV bovine rotavirus (NSP4[A]). Serum IgG antibody titers to genotype [A], [B], [C], and [D] NSP4s were determined using recombinant baculoviruses expressing genotype [A] (bovine NCDV, human DS-1, and simian SA11), [B] (porcine SB1A and human Wa), [C] (simian RRV), or [D] (murine EB) rotavirus NSP4 in immunocytochemical staining assays. Light hatched bar, IgG antibody titers in cord blood of in utero-infected calves at birth (A); dark hatched bar, IgG antibody titers in serum of calves infected at 1 day old at PID 21 (B); solid bar, IgG antibody titers in serum of calves infected in utero initially and challenged at 1 day old at PCD21 (C). Asterisks denote significant differences in antibody titers compared to NCDV NSP4 genotype (Repeated Measures Analysis of Variance, $P < 0.05$).

from this uneven set of samples are also presented. No IgG or IgA NSP4 antibodies were detected in any piglets before rotavirus infection (PID 0) or in mock-inoculated pigs at PID 21. Substantially higher IgG antibody GMTs to homotypic and heterotypic NSP4s were detected in comparison to the level of IgA antibodies following primary infection (PID 21) of piglets with NSP4[B] OSU, Gottfried, or OSU × Gottfried reassortant porcine rotavirus (Figs. 2A and 2C, note the *y*-axis scale difference between IgG and IgA).

Table 1
Cross-challenge studies of porcine rotavirus strains OSU, Gottfried, and OSU × Gottfried reassortant in gnotobiotic piglets[a]

Group (no. of piglets)	Primary infection (4–5 days old)								Challenge (3 weeks after primary infection)							
	Rotavirus administered						Median days of		Rotavirus administered						Median days of	
	Strain	Origin of the virus	Genotype/serotype			Titer	Diarrhea	Virus shedding	Strain	Origin of the virus	Genotype/serotype			Titer	Diarrhea	Virus shedding
			NSP4	VP4	VP7						NSP4	VP4	VP7			
1 (4)	Gottfried	pig intestinal contents	[B]	P2B[6]	G4	4×10^6 ffu[b]	12	7.5	OSU	pig intestinal contents	[B]	P9[7]	G5	5×10^6 ffu	4.25	4
2 (4)	OSU	pig intestinal contents	[B]	P9[7]	G5	5×10^6 ffu	9	10	Gottfried	pig intestinal contents	[B]	P2B[6]	G4	4×10^6 ffu	4.5	3.5
3 (4)	OSU × Gottfried	cell culture lysates	[B]	P9[7]	G4	5×10^6 pfu[c]	7.25	7.75	OSU	pig intestinal contents	[B]	P9[7]	G5	5×10^6 ffu	none	1.5
4 (4)	OSU × Gottfried	cell culture lysates	[B]	P9[7]	G4	5×10^6 pfu	7.25	7.75	Gottfried	pig intestinal contents	[B]	P2B[6]	G4	4×10^6 ffu	none	none
5 (2)	none	NA[d]	[NA]	NA	NA	NA	NA	NA	OSU	pig intestinal contents	[B]	P9[7]	G5	5×10^6 ffu	5.5	10
6 (2)	none	NA	[NA]	NA	NA	NA	NA	NA	Gottfried	pig intestinal contents	[B]	P2B[6]	G4	4×10^6 ffu	7	7

[a] Adapted from Hoshino et al., 1988 (11).
[b] ffu = fluorescent-focus unit.
[c] pfu = plaque-forming unit.
[d] NA = not applicable.

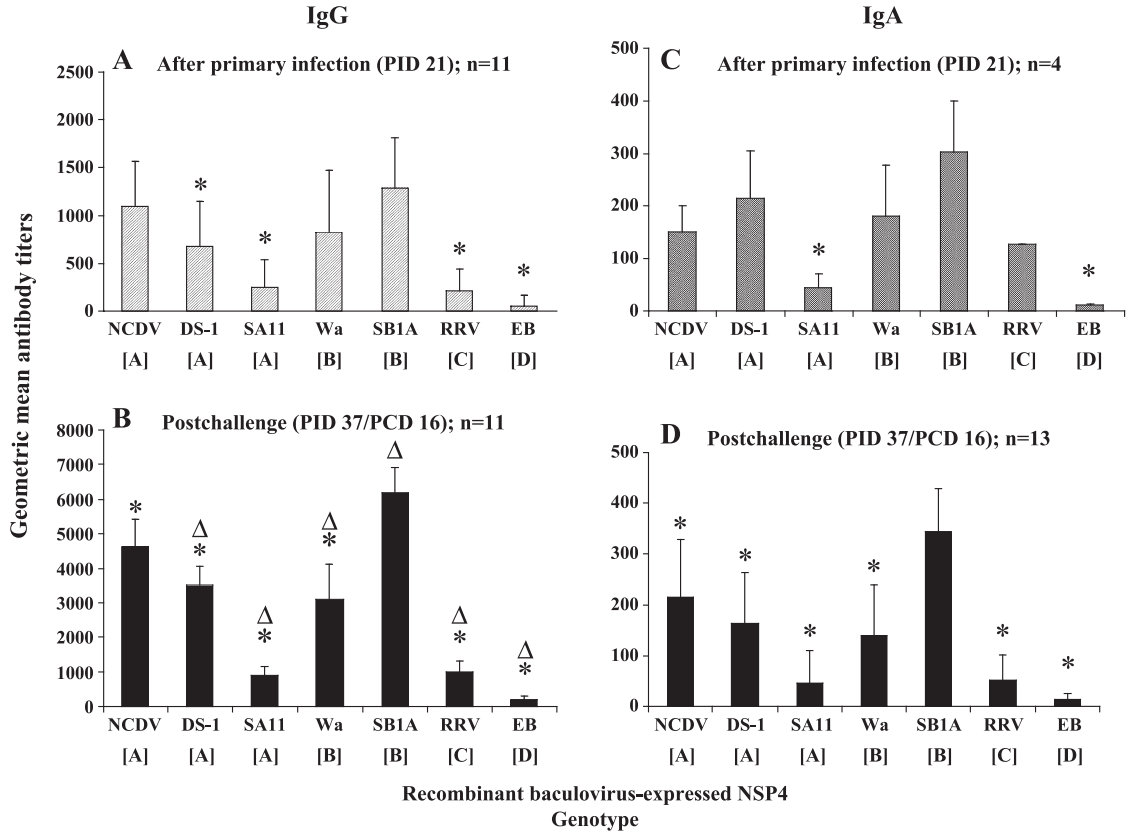

Fig. 2. Serum IgA and IgG antibody response to NSP4s in gnotobiotic piglets infected with OSU/Gottfried porcine rotavirus (NSP4 [B]) at PID 21 and PID 37/PCD 16. Serum IgG (left panel) and IgA (right panel) antibody titers to genotype [A], [B], [C], and [D] NSP4s were determined using recombinant baculoviruses expressing genotype [A] (bovine NCDV, human DS-1, simian SA11), [B] (porcine SB1A, human Wa), [C] (simian RRV), or [D] (murine EB) rotavirus NSP4 in immunocytochemical staining assays. Hatched bar, PID 21 (A, IgG; C, IgA); solid bar, PID 37/PCD 16 (B, IgG; D, IgA). Asterisks denote significant differences in antibody titers compared to SB1A NSP4 genotype (Repeated Measures Analysis of Variance, $P < 0.05$). Triangles denote significant increases in antibody titers postchallenge (Student's t test, $P < 0.05$). Eleven pairs of samples (from groups 1 to 4) were included for IgG antibody response at PID 21 and PID 37/PCD 16. Only four samples from piglets infected with OSU × Gottfried rotavirus (group 4) were available and included for IgA antibody response at PID 21 and 13 samples (from groups 1 to 4) were included at PID 37/PCD 16.

As expected, the highest IgG and IgA antibody responses were detected against the homologous host homotypic porcine (SB1A) NSP4[B]. The IgG antibody titers were significantly higher ($P < 0.05$) than those to heterologous host heterotypic human (DS-1) and simian (SA11) NSP4[A]s, simian (RRV) NSP4[C], and murine (EB) NSP4[D], and tended to be higher than those to heterologous host homotypic human (Wa) NSP4[B] and heterologous host heterotypic bovine (NCDV) NSP4[A] (Fig. 2A). The pattern of IgA antibody responses at PID 21 was similar to that of the IgG antibody responses, although with the small number of samples for the IgA analysis only two of these values achieved statistical significance (Fig. 2C). Unexpectedly, the IgG antibody titers to heterotypic NCDV NSP4[A] tended to be higher than those to homotypic Wa NSP4[B], consistent with the observation that antibody responses to NSP4 were not genotypic specific.

Following challenge with virulent NSP4[B] OSU or Gottfried rotavirus, IgG antibody titers to both homotypic (SB1A, Wa) and heterotypic (DS-1, SA11, RRV, EB) NSP4s increased significantly (3.5- to 5.1-fold of GMTs) (Fig. 2B). Interestingly, postchallenge IgA GMTs to both homotypic and heterotypic NSP4s remained similar to prechallenge GMTs. In addition, IgG and IgA antibody titers to homologous host homotypic porcine SB1A NSP4[B] were significantly higher ($P < 0.05$) than those to heterologous host homotypic Wa NSP4[B] and heterologous host heterotypic NSP4s (NCDV, DS-1, SA11, RRV, and EB) (Figs. 2B and 2D). In addition, the anamnestic IgG antibody responses to heterotypic NCDV NSP4[A] were higher than those to homotypic Wa NSP4[B]. These data suggested again that antibody responses were species specific rather than genotype specific and that there was a close antigenic relationship between bovine NCDV and the porcine rotavirus NSP4s.

Interspecies variable domain (aa 131–141) plays an important role in NSP4-specific antibody responses in calves and piglets

The pattern of antibody responses to NSP4s in calves and piglets infected with the homologous host rotavirus strains indicated that antibody responses to NSP4 were not

genotype specific (Figs. 1 and 2). To delineate the correspondence between antigenic relationship and phylogenetic relationship of NSP4s, we assessed the antigenic divergences between NSP4s using antibody GMT ratios of different NSP4s (Figs. 3 and 4, right *y*-axis). An alignment of aa sequences of full-length NSP4s of rotavirus strains employed in this study was made (Fig. 5), and the interspecies variable domain (aa 131–141), the potential epitope sites (ES I aa 150–169, II aa 135–149, III aa 112–133, and IV aa 1–25) (Borgan et al., 2003), and the enterotoxin domain (aa 114–135) are marked in boxes. The aa identities of full-length NSP4s and of the interspecies variable domain 131–141 were calculated and depicted in Figs. 3 and 4 (left *y*-axis). The aa 131–141 of NSP4s from selected human and animal rotavirus strains were compared in Table 2.

The aa sequence identities within 131–141 of NSP4s were lower overall than those of full-length NSP4s (Figs. 3 and 4). Species-specific sequences in this region of NSP4 were observed for all the NSP4 genotypes, except for NSP4[C] in which two human, two canine, one feline, and one simian rotaviruses shared 90–100% aa sequence identity (Table 2). Unexpectedly, the aa sequence identity of 131–141 of SA11 NSP4[A] with SB1A NSP4[B] or with NCDV NSP4[A] was substantially lower (approximately 2.5- to 3-fold) than the corresponding aa sequence identity of the full-length NSP4s (Figs. 3 and 4), which correlated closely with the characteristically high GMT ratios of SB1A/SA11 (Fig. 3) or NCDV/SA11 (Fig. 4) NSP4 antibodies, suggesting that the aa 131–141 region may be an immunodominant domain and played an important role in the antigenic divergence of NSP4s. This assumption correlated well with the recent report in which 11 of a total of 25 mAbs raised to an avian NSP4 were mapped to region aa 133–150 (Borgan et al., 2003).

Antibodies to NSP4 protein did not play a major role in resistance in gnotobiotic piglets to diarrhea induced by NSP4-homotypic virulent porcine rotavirus challenge

As shown in Table 1, which is a brief summary of our previously published data (Hoshino et al., 1988), group 1 piglets infected initially with Gottfried (NSP4[B], P2B[6], G4) and challenged with OSU (NSP4[B], P9[7], G5), and group 2 pigs infected initially with OSU and challenged with Gottfried rotavirus, developed diarrhea and shed viruses both after primary infection at 4–5 days of age and after challenge at PID 21. Thus, no significant protection was conferred by primary rotavirus infection against challenge with rotavirus strains bearing a homotypic NSP4 with nearly identical amino acid sequence, but

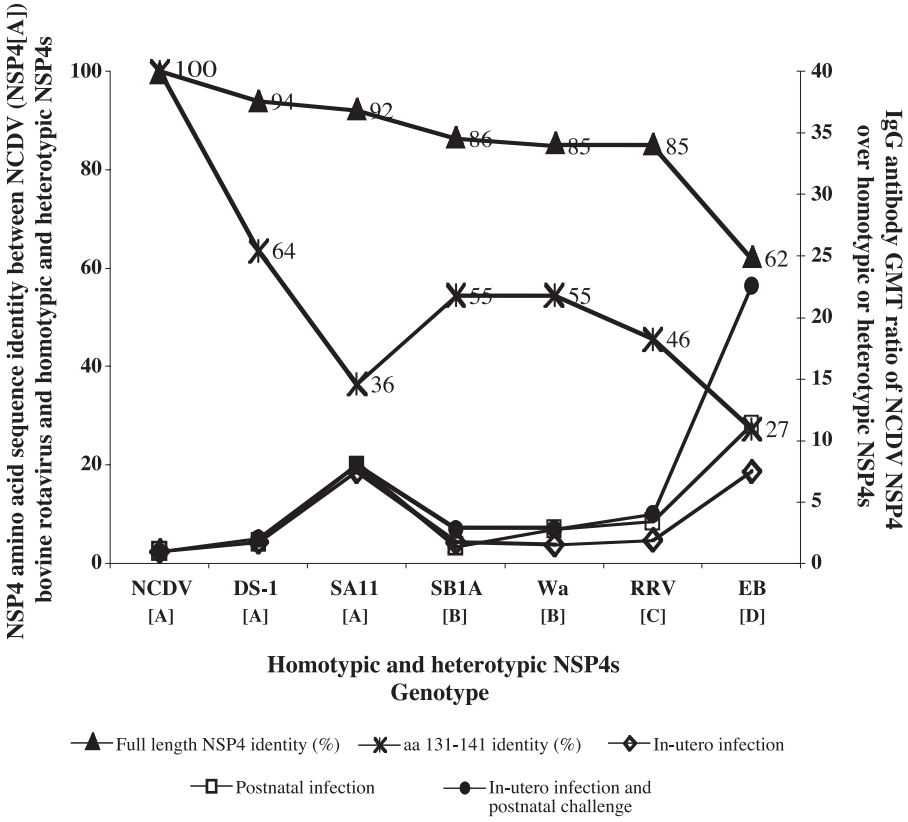

Fig. 3. Serological relationships versus phylogenetic relationships of NSP4s in calves infected with bovine rotavirus. Amino acid sequence identity between NCDV bovine rotavirus and the homotypic and heterotypic NSP4s is indicated by the left *y*-axis. IgG antibody GMT ratio of NCDV NSP4 over homotypic and heterotypic NSP4s was indicated by the right *y*-axis.

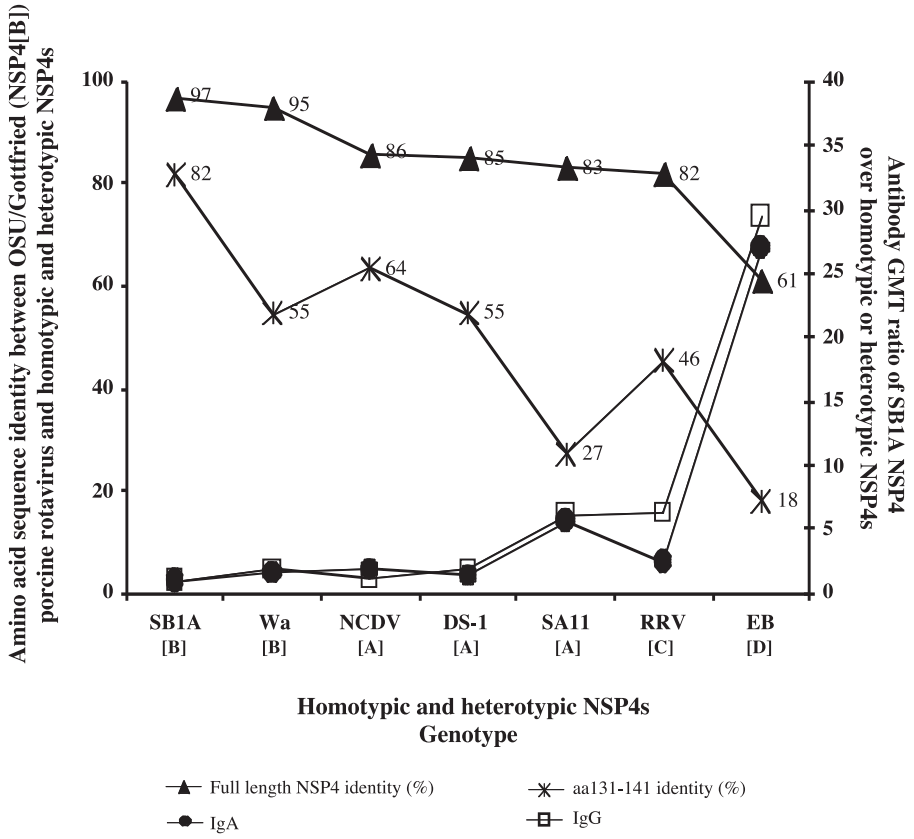

Fig. 4. Serological relationships versus phylogenetic relationships of NSP4s in piglets infected with porcine rotavirus. Amino acid sequence identity between OSU/Gottfried porcine rotavirus and the homotypic and heterotypic NSP4s is indicated by the left *y*-axis. Antibody GMT ratio of SB1A NSP4 over homotypic and heterotypic NSP4s was indicated by the right *y*-axis. The IgG and IgA antibody GMT ratios are presented as the average of GMT ratios at PID 21 and PID 37/PCD 16 for both IgG and IgA since there were no significant differences between those ratios at the two time points.

heterotypic VP4 and VP7 (although the duration of virus shedding and diarrhea was shorter in both groups 1 and 2 compared to mock-inoculated controls). In contrast, complete or nearly complete protection against rotavirus reinfection and diarrhea was observed in piglets inoculated initially with OSU × Gottfried single VP7 gene substitution reassortant (NSP4[B], P9[7], G4) and challenged with either VP4-homotypic OSU (group 3) or VP7-homotypic Gottfried rotavirus (group 4). Protection in these piglets was thus clearly associated with the homotypic VP4 (P9[7] of OSU) or VP7 (G4 of Gottfried) of rotavirus reassortant used in the primary infection.

Discussion

Using seven recombinant NSP4 proteins representative of four ([A], [B], [C], and [D]) genotypes derived from five mammalian species as detector antigens in an immunocytochemical staining assay, we analyzed serum samples obtained from gnotobiotic calves or piglets infected by the oral/alimentary or intraamniotic route with homologous host bovine or porcine rotaviruses, and evaluated the degree of antigenic divergence between various NSP4 genotypes and possible species-specific antibody responses to NSP4. Previously, we and others reported the cross-reactive nature of NSP4 proteins by analyzing serum samples derived from infants vaccinated with selected rotavirus vaccines (Yuan et al., 2004) or naturally infected with rotaviruses (Ray et al., 2003). However, the findings obtained from such studies may not reflect the true nature of NSP4-specific antibody responses, because, for example, in our previous study, almost all the infant vaccinees had rotavirus-specific IgG or IgA antibodies or both in their prevaccination sera (Yuan et al., 2004). The present study has demonstrated for the first time the true nature and extent of cross-reactivity and divergence of antibody responses to NSP4 in primary and secondary infections observed in a homologous virus–host system (i.e., gnotobiotic calves/bovine rotavirus and gnotobiotic piglets/porcine rotavirus) that mimics infants/human rotavirus infections in nature. This was possible because colostrum-deprived gnotobiotic calves and piglets are completely devoid of maternally derived antibodies and thus are true naive animals before virus inoculation.

Unexpectedly, the IgG and IgA antibody responses to NSP4 observed in gnotobiotic calves and piglets were not NSP4 genotype specific but rather NSP4 species specific. This finding was substantiated by our observation that antibody titers to homologous host homotypic NSP4 were

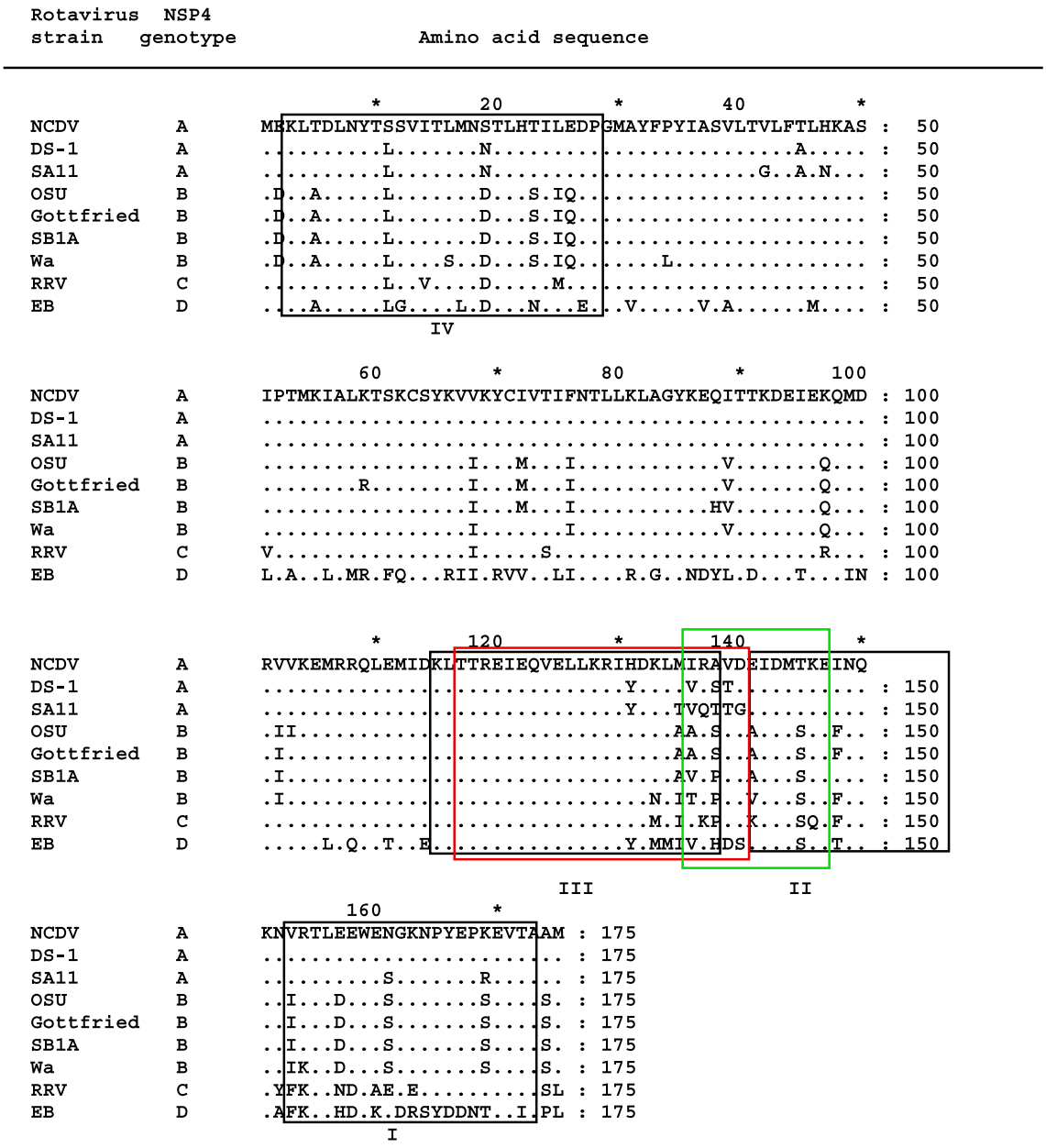

Fig. 5. Comparison of the deduced amino acid sequence of the NSP4s of genotype [A] (bovine NCDV, human DS-1, and simian SA11), genotype [B] (porcine OSU, Gottfried and SB-1A, and human Wa), genotype [C] (simian RRV), and genotype [D] (murine EB) rotavirus strains employed in this study. Potential epitope sites I (aa 151–169), II (aa 136–150), III (aa 112–133), and IV (aa 1–24) are marked in black boxes. Enterotoxin domain (aa 114–135) is marked in red box. Interspecies variable domain (aa 131–141) is marked in green box.

significantly higher or tended to be higher than those of heterologous host homotypic or heterologous host heterotypic NSP4s. For example, in gnotobiotic piglets infected with porcine NSP4[B] viruses (OSU or Gottfried or OSU × Gottfried), IgG and IgA antibody titers to the homologous host homotypic rotavirus NSP4[B] (porcine SB1A strain) tended to be higher after a primary infection and significantly higher after challenge than those of the heterologous host homotypic rotavirus NSP4[B] (human Wa strain), in spite of the similar full-length NSP4 aa sequence identity of OSU/Gottfried with SB1A (97%) or with Wa (95%) rotaviruses. Thus, the identities of full-length NSP4 aa sequence correlated poorly with the antigenic relationships of NSP4s. Analysis of the aa identity within the NSP4 aa sequence 131–141 region revealed unexpected and interesting figures. Within 131–141, the aa sequences of porcine strains OSU and Gottfried (NSP4[B]) were 82% identical to porcine SB1A (NSP4[B]), 63.6% identical to bovine NCDV (NSP4[A]), and only 54.5% identical to human Wa (NSP4[B]) rotavirus, respectively. Such phylogenetic relationships within the aa 131–141 region correlated closely with our observation of antigenic relationships that indicated that NSP4 of porcine rotavirus was more closely related antigenically to heterotypic NSP4 of bovine rotavirus than

Table 2
Alignment of deduced amino acid sequences 131–141 of NSP4 of selected human and animal rotavirus strains

NSP4 genotype	Species of origin	Rotavirus strain	Country of origin	Amino acid sequence
A	Bovine	NCDV	USA	131 : HDKLMI RAVDE : 141
		B223	USA	. . . . D . . . . .
		RV033	Japan	. . . . . V . . TD.
		CBNU-1	Korea	Y. . . . . TA.
		UK	UK	Y. . . . V . S TG.
	Equine	FI-14	USA	Y. . . . . T . .
		FI-23	USA	Y. . . . A . . T . .
		HI-23	Japan	Y. . . . . P T . .
		H-2	UK	Y. . . . . T . .
	Human	DS-1	USA	Y. . . . V . S T . .
		US1205	USA	Y. . . I V . S T . .
		RV5	Australia	Y. . . . V . S TG.
		MP409	India	. . . . . VQS TG.
		S2	Japan	Y. . . I V . S TG.
		MW47	Malawi	Y. . . I V . S T . .
		L26	Philippines	. . . . . VQS T . .
		1076	Sweden	. . . . . VQS TG.
		TD7	Taiwan	Y. . . I V . S TG.
	Lapine	ALA	USA	Y. R . T V . K T . .
		C-11	USA	Y. . . T V . K T . .
		R-2	Japan	Y. . . . . K T . .
	Simian	SA11	South Africa	Y. . . T VQT TG.
B	Human	Wa	USA	. . N . I T . P . . V
		RV3	Australia	Y. N . I T . P . NV
		BR1067	Brazil	. . N . I TKP . . V
		CH32	China	. . N . I TKPANV
		VA70	Italy	. . N . I T . P . . V
		AU32	Japan	. NN . I . KP . . V
		GR828/86	South Africa	. . N . I T . P . . V
		UG72	Uganda	. . N . I TRS . . V
		ST3	UK	. . N . I T . P . NV
		M37	Venezuela	. . N . I T . T . . V
	Porcine	OSU	USA	. . . . AA . S . . A
		Gottfried	USA	. . . . AA . S . . A
		SB1A	USA	. . . . AV . P . . A
		YM	Mexico	. . . . VT . P . . V
		A34	Venezuela	. . . . VT . P . . A
		A411	Venezuela	. . . . VA . S . . V
C	Canine	CU-1	USA	. . M. I AKP . . K
		RS15	Japan	. . M. I . KP . . K
	Feline	FRV1	Japan	. . M. I . KP . . K
	Human	AU1	Japan	. . M. I . KP . . K
		0291	Japan	. . M. I . KP . . K
	Simian	RRV	USA	. . M. I . KP . . K
D	Murine	EB	USA	Y. MMI V. HDS .
		EW	USA	Y. MMVV. QNR .
		EHP	Brazil	Y. MMVV. HDR .
		EC	UK	Y. MMVVCRDR.
E	Avian	PO-13	Japan	YEM . KFKKDE I
		Ty-1	UK	YEM . KFKKDEV
F	Avian	Ch-1	UK	YEL . KYKSEGN

to homotypic NSP4 of human rotavirus. The species-specific IgG antibody responses were more prominent in calves. Upon further sequence analysis of the aa 131–141 region of NSP4 derived from various animal species, antigenic similarity and diversity of the NSP4 protein were shown to reflect clearly the species specificity of aa sequence of the region. Our finding that the divergence of NSP4 residues 131–141 reflected more closely the overall divergence of antigenicity of NSP4s than that of the full-length NSP4 also implies that the aa sequence 131–141 is an immunodominant domain. This observation coincides with the report that monoclonal antibodies produced against NSP4 135–149 peptide of avian rotavirus PO-13 strain expressed in *Escherichia coli* reacted with not only avian

NSP4s but also with NSP4 of mammalian rotavirus strains of different NSP4 genotypes (Borgan et al., 2003). The 131–141 region has been shown to encompass portions of antigenic epitope sites II and III (Borgan et al., 2003). Previously, comparative sequence analyses of NSP4 sequences of rotavirus strains derived from various animal species have indicated that in general the NSP4s cluster according to species of origin (except for genotype [C] NSP4s) and suggested a constant pattern of evolution within species (Ciarlet et al., 2000), and this NSP4 species specificity is most prominent in the aa 131–141 region.

Strong serum IgG and IgA antibody responses to the homologous host homotypic NSP4[B] were observed in piglets 3 weeks after primary porcine rotavirus infection at the time of virulent porcine rotavirus challenge. Although VP4 and VP7 of OSU and Gottfried porcine rotaviruses belong to different P and G types (P9[7]G5 versus P2B[6]G4), NSP4s from virulent OSU and Gottfried rotavirus strains share nearly complete amino acid sequence identity (97% or 100% depending on the published sequences) (Chang et al., 1999; Zhang et al., 1998). Piglets infected with OSU, Gottfried, or OSU × Gottfried reassortant rotavirus shed viruses with similar titers and for similar time periods (Hoshino et al., 1988), indicating that the replication rates of these three rotaviruses in gnotobiotic piglets were comparable. Thus, the amount of viral proteins presented to the host and the magnitude of immune responses evoked in those animals by the infections should be comparable. This assumption was supported by our findings of similar magnitudes of IgG antibody responses to NSP4 among piglets infected with OSU (group 1), Gottfried (group 2), or OSU × Gottfried reassortant (groups 3 and 4) at PID 21 and after challenge (PID 37/PCD 16) (data not shown).

We found that protection against diarrhea or virus shedding after challenge was not associated with the existing homologous host homotypic NSP4 antibodies. Rather, protection was associated with high titers of homotypic VP4 or VP7 neutralizing antibodies (Hoshino et al., 1988). The IgA antibody responses (a critical known correlate of rotavirus immunity) to homotypic and heterotypic NSP4s at challenge in the four rotavirus inoculation piglet groups could not be compared due to the lack of samples at this time point. However, the serum IgA antibody responses to NSP4 detected at challenge in the samples from combined groups indicated that intestinal IgA antibody responses to NSP4s were also evoked at challenge. Further studies may be needed to analyze the role of antibodies to NSP4 in homotypic and heterotypic protective immunity in other species, including humans. Our findings in piglets demonstrated that antibodies to NSP4 did not play a significant role in protection against rotavirus infection or diarrhea.

In conclusion, species specificity within aa sequence of 131–141 correlated closely with the species specificity of antibody responses to NSP4 in gnotobiotic animals. The serological relationships of various NSP4 genotypes were dictated to a greater extent by the species-specific sequences of that region rather than the full-length NSP4 sequence identities (NSP4 genotypes) of homotypic and heterotypic rotavirus strains. In addition, in challenge studies in piglets, NSP4 antibodies failed to provide protection against challenge with a rotavirus that had a homologous host homotypic NSP4. Thus, antibodies to NSP4 do not seem to play a significant role in protection against rotavirus disease or infection in (i) experimental or natural rotavirus infections and (ii) an immunization strategy in which a live attenuated rotavirus vaccine is delivered orally.

Materials and methods

Construction and characterization of recombinant baculoviruses expressing rotavirus NSP4 proteins belonging to various genotypes

The construction and characterization of recombinant baculoviruses expressing selected recombinant rotavirus NSP4s were performed using pCR Bac vector (Baculovirus TA Cloning Kit) or pBlueBac4.5/V5-His-TOPO vector (pBlueBac4.5/V5-His TOPO TA Cloning Kit) according to the manufacturer's instructions (Yuan et al., 2004). Recombinant NSP4s employed in this study were derived from human rotavirus DS-1 (NSP4[A]) and Wa (NSP4[B]), simian rotavirus SA11 (NSP4[A]) and RRV (NSP4[C]), porcine rotavirus SB1A (NSP4[B]), bovine rotavirus NCDV (NSP4[A]), and murine rotavirus EB (NSP4[D]). The full-length or nearly full-length gene of each NSP4 was amplified by PCR using specific primers designed according to the gene sequence of the original rotavirus strain and inserted into baculovirus transfer vector and expressed in *Spodoptera frugiperda* 9 (Sf-9) insect cells. High-titer viral stocks of the recombinant baculoviruses were prepared in Sf-9 insect cells and their titers were determined using a plaque assay following the manufacturer's instructions (Bac-N-Blue Transfection Kit, Invitrogen Corporation, Carlsbad, CA). Working viral stocks of 1×10^7 PFU/ml was prepared with Grace's medium (GIBCO).

Western blot analysis was used to confirm specific protein expression of each recombinant NSP4 in Sf-9 cells. As shown in Fig. 6, a monoclonal antibody (mAb) B4-1/55 generated against Wa NSP4 (Petrie et al., 1984) reacted with all NSP4 proteins except for the EB protein (panel A), whereas serum from mouse hyperimmunized intranasally with recombinant EB protein reacted strongly with homologous EB NSP4 protein but very weakly with NSP4 of SA11, Wa, or SB1A (panel B).

Serum samples from gnotobiotic calves and piglets

Two sets of serum samples from previous rotavirus studies involving gnotobiotic calves (Wyatt et al., 1979) or piglets (Hoshino et al., 1988) were used in the present study. Six calf fetuses were infected with virulent bovine

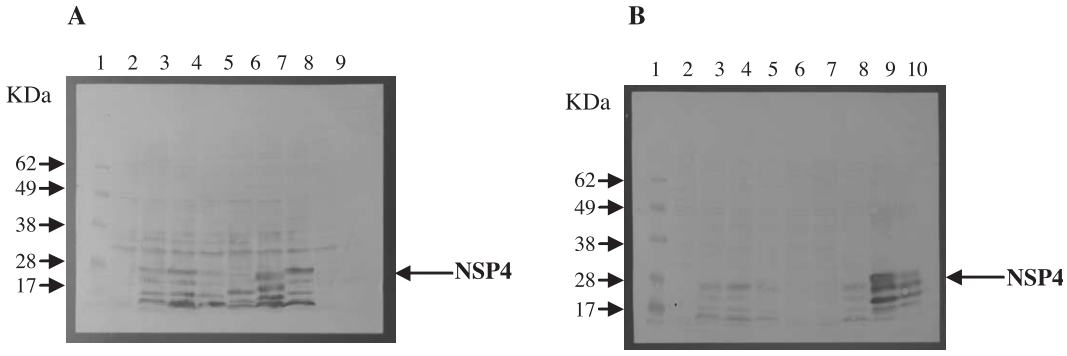

Fig. 6. Western blot analysis of various recombinant NSP4 proteins expressed in Sf-9 cells. Lane 1 = MW marker; lane 2 = uninfected Sf-9 cell lysates; lane 3 = DS-1 NSP4 cell lysates; lane 4 = NCDV NSP4 cell lysates; lane 5 = SA11 NSP4 cell lysates; lane 6 = Wa NSP4 cell lysates; lane 7 = SB1A NSP4 cell lysates; lane 8 = RRV NSP4 cell lysates; lane 9 = EB NSP4 cell lysates; and lane 10 = purified recombinant EB NSP4. Panel A shows that mAb B4-1/55 reacted with all the NSP4 proteins except for EB protein. Panel B shows that serum from mouse hyperimmunized intranasally with recombinant EB protein reacted strongly with homologous EB NSP4 protein but very weakly with NSP4 of SA11, Wa, or SB1A.

rotavirus NCDV (NSP4[A],P6[1],G6) (2% stool filtrate) intraamniotically 2–14 weeks (15–95 days) before delivery (group1: in utero infection, $n = 6$). All calves were delivered by cesarean section and maintained under gnotobiotic condition. One-day-old calves were injected with virulent NCDV directly in the duodenum at laparotomy (group 2: postnatal infection only, $n = 4$). Two additional calves inoculated in utero initially were later injected with virulent NCDV 1 day after birth via the same route as for group 2 calves (group 3: in utero infection and postnatal challenge, $n = 2$). Serum samples were collected at birth (cord blood) and 20–23 days after postnatal injection (designated as PCD 21) and stored at −20 °C.

As summarized in Table 1, colostrum-deprived 4- to 5-day-old gnotobiotic piglets were orally inoculated with OSU (NSP4[B],P9[7],G5) or Gottfried (NSP4[B],P2B[6],G4) porcine rotavirus, or a single VP7 gene substitution reassortant between OSU and Gottfried (NSP4[B],P9[7],G4), and then orally challenged 3 weeks later with virulent OSU (5×10^6 PFU) or Gottfried (4×10^6 PFU) virus. Serum samples were collected at postinoculation day (PID) 21 and postchallenge day (PCD) 16/PID 37 and stored at −20 °C.

Immunocytochemistry (Sf-9 cell staining) assay

Titers of serum IgA and IgG antibodies to homotypic and heterotypic recombinant rotavirus NSP4 proteins were determined using Sf-9 cell staining assay as previously described (Ishida et al., 1996). Briefly, recombinant baculovirus-infected, fixed Sf-9 cells on 96-well plates expressing various NSP4 proteins were used as detector antigens. The starting dilution for measuring IgA antibody titers was at 1:4, and for IgG antibody titers was at 1:40. Serial dilutions of the mAb to Wa NSP4 B4-1/55 were included on each NSP4 plate as a positive control to make sure that the amount of expressed NSP4 protein of NCDV, DS-1, SA11, Wa, or SB1A was equal and consistent. For EB NSP4 that did not react with the mAb, the protein expression level was monitored using a hyperimmune antiserum to recombinant EB NSP4. The test plates were used only when the titer variation of the mAb or hyperimmune antiserum was within 4-fold dilution. In addition, data were accepted for analysis only when the positive control titer was consistent on all plates of each NSP4 genotype. NSP4 antibodies bound to Sf-9 cells on the plates were detected with horseradish peroxidase-labeled goat anti-porcine or bovine IgA (α) or IgG (H + L) secondary antibodies (Bethyl Laboratory, Inc., Montgomery, TX). Horseradish peroxidase-labeled goat anti-mouse IgG (H + L) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used for the mAb B4-1/55. NSP4 antibody- and secondary antibody-bound Sf-9 cells were visualized with AEC substrate (3-amino-9ethyl-carbazole; Sigma). The NSP4 antibody titer was defined as the reciprocal of the highest dilution at which any positive cell staining could be detected under the microscope at 100× magnification as described previously (Ishida et al., 1996; Yuan et al., 2004).

Divergences of homotypic and heterotypic NSP4 antibody responses

Divergences between homotypic and heterotypic NSP4 antibody responses were calculated as ratios of antibody geometric mean titers (GMTs) of the homologous host homotypic NSP4 over (divided by) heterologous host homotypic or heterologous host heterotypic NSP4s. Greater values of GMT ratios indicate higher degree of divergence (or lower cross reactivity). In calves infected with NSP4[A] rotavirus (NCDV), ratios of IgG antibody GMTs were calculated as NCDV NSP4 over each of the six homotypic or heterotypic NSP4s. In pigs infected with NSP4[B] rotavirus (OSU or Gottfried), ratios of IgA or IgG antibody GMTs were calculated as homologous host homotypic SB1A NSP4 over each of the six homotypic or heterotypic NSP4s.

Statistical analysis

Student's t test was applied to logarithmically transformed (base 10) titers for comparisons of IgG or IgA

antibody responses in pigs pre- and postchallenge. One-way analysis of variance (ANOVA) (General linear model) followed by Duncan's multiple-range test was used to compare IgG antibody titers to each NSP4 among pigs in groups 1–4 at PID 21 or PID 37/PCD 16. Because there were no significant differences in IgG antibody titers among the four pig groups at PID 21 or PID 37/PCD 16, data from all pigs in these four groups were pooled for further statistical analysis of homotypic and heterotypic NSP4 antibody responses. For comparisons of IgG or IgA antibody responses between homotypic and heterotypic NSP4s in pig or calve samples, 'Repeated Measures Analysis of Variance' was used (antibody titers of each serum sample to various genotypes of NSP4 are not independent) followed by calculation of appropriate contrasts. No statistical analysis was performed on calves infected in utero initially and then challenged with NCDV (group 3) due to the small number of animals in the group. Statistical significance was assessed at $P < 0.05$ for all the analyses in the study (SAS Institute, Inc).

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