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Passive Transfer of Virus-Specific Antibodies Confers Protection against Reproductive Failure Induced by a Virulent Strain of Porcine Reproductive and Respiratory Syndrome Virus and Establishes Sterilizing Immunity

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Immune mechanisms mediating protective immunity against porcine reproductive and respiratory syndrome virus (PRRSV) are not well understood. The PRRSV-specific humoral immune response has been dismissed as being ineffective and perhaps deleterious for the host. The function of PRRSV antibodies in protective immunity against infection with a highly abortifacient strain of this virus was examined by passive transfer experiments in pregnant swine. All of a group of pregnant gilts ($n = 6$) that received PRRSV immunoglobulin (Ig) from PRRSV-convalescent, hyperimmune animals were fully protected from reproductive failure as judged by 95% viability of offspring at weaning (15 days of age). On the other hand, the totality of animals in a matched control group ($n = 6$) receiving anti-pseudorabies virus (PRV) Ig exhibited marked reproductive failure with 4% survival at weaning. Besides protecting the pregnant females from clinical reproductive disease, the passive transfer of PRRSV Ig prevented the challenge virus from infecting the dams and precluded its vertical transmission, as evidenced by the complete absence of infectious PRRSV from the tissues of the dams and lack of infection in their offspring. In summary, these results indicate that PRRSV-Igs are capable of conferring protective immunity against PRRSV and furthermore that these Igs can provide sterilizing immunity *in vivo*. © 2002 Elsevier Science (USA)

Key Words: PRRSV; swine arterivirus; antibodies; protective immunity.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the genus *Arterivirus*, together with equine arteritis virus (EAV), lactate-dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) (Meulenber *et al.*, 1993). The infection of domestic swine by PRRSV is characterized by respiratory dysfunction, late-term abortion, and a high incidence of stillborn, mummified, and debilitated newborn pigs. PRRSV is currently considered to cause the most economically significant infectious disease of swine (National Pork Producers, 2000). As is characteristic of arteriviruses, PRRSV is a small enveloped RNA virus which replicates primarily in macrophages. The PRRSV particle is 50 to 65 nm in diameter, with a central isometric nucleocapsid of approximately 30 to 35 nm in diameter (Meulenber *et al.*, 1993). The genome of PRRSV is a single-stranded polyadenylated RNA of 15 kb in length which contains eight open-reading frames

(ORFs). Of these, ORF-7 is known to code for the nucleocapsid protein, and ORFs 2, 3, 4, 5, and 6 are likely to code for envelope proteins (Meulenber and den Besten, 1996). The replication and gene expression of arteriviruses, involving a 3'-coterminal nested set of sub-genomic mRNAs, is similar to that of coronaviruses (Cavanagh, 1997; Plagemann, 1996).

Very little is known about the components of the immune response that are effective in the protective response of the pig to PRRSV infection. It is known that a certain degree of immune protection is conferred by some vaccines (Gorcyca *et al.*, 1995; Osorio *et al.*, 1998; Plana-Duran *et al.*, 1997b) or selected antigens of PRRSV (Pirzadeh and Dea, 1998; Plana-Duran *et al.*, 1997). Importantly, PRRS-convalescent animals show specific protective immunity (Gorcyca *et al.*, 1993; Labarque *et al.*, 2000; Lager *et al.*, 1997a,b). This protection seems to be preferentially strain specific, although a certain level of heterologous protection to other strains of PRRSV appears to exist (Lager *et al.*, 1999). Although the existence of this PRRSV-specific protective immunity is recognized, the molecules or cells that mediate this protection have not been clearly identified. It has been postulated that the main role in protection against PRRSV centers on the cell-mediated response. Infected pigs develop a transient T-cell-mediated PRRSV-specific lymphoproliferative

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response, starting at 4 weeks postinfection, lasting from 9 (Bautista and Molitor, 1997) to 14 weeks (Lopez Fuertes *et al.*, 1999) after this event. Virus-infected pigs also develop a delayed-type hypersensitivity response to the virus (Bautista and Molitor, 1997). The viral matrix protein and the envelope glycoprotein GP5 have been identified as targets of the lymphoproliferative response (Bautista *et al.*, 1999). The PRRSV-specific immune response appears to be characterized by an unusual delay in responsiveness observed in both the humoral and cellular components of the host response (Meier *et al.*, 2000). The response by PRRSV-specific γ -interferon-producing cells attains detectable levels not sooner than 4 weeks postinfection (Meier *et al.*, 2000). Likewise, the humoral immune response is characterized by the early appearance of a vigorous PRRSV-specific antibody that does not contain neutralizing activity until at least 3 or 4 weeks into the infection (Labarque *et al.*, 2000; Yoon *et al.*, 1994).

One important challenge for PRRSV research has been the identification of a model that would accurately represent protective immunity to PRRSV. Several different experimental challenge models have been used to evaluate protective immunity in PRRSV, including viremia (Pirzadeh and Dea, 1998) and quantitation of lesions postinfection: ranging from histopathological scoring in lungs (Halibur *et al.*, 1995) to the level of enlargement of lymph nodes (Mengeling *et al.*, 1996). Nonetheless, the evaluation of reproductive failure in pregnant females infected at the time at which the animals are most sensitive to PRRSV transplacental infection (i.e., 90 days of gestation) is an objective and sensitive indicator of PRRSV pathogenicity, thus ideal for evaluation of protective immunity against PRRSV (Gorcyca *et al.*, 1995; Lager *et al.*, 1999; Mengeling *et al.*, 1996; Mengeling *et al.*, 1998; Osorio *et al.*, 1998; Plana-Duran *et al.*, 1997a,b).

There are contrasting opinions about the protective significance of antibodies in PRRSV infections, specifically about the significance of the PRRSV-specific neutralizing antibody response that, as we noted, does not appear until at least 4 weeks PI. The absence of a detectable PRRSV-neutralizing activity during the first few weeks of infection plus the seemingly simultaneous detection of neutralizing antibodies and infectious PRRSV in the blood of infected animals lead several to postulate that neutralizing antibodies do not play a role in protection against this viral infection (Albina, 1997; Collins, 1998; Loemba *et al.*, 1996; Molitor, 1993; Molitor *et al.*, 1997; Rossow, 1998; Snijder and Meulenber, 2001). In addition, the observation that antibodies could enhance the PRRSV replication in macrophages (Yoon *et al.*, 1996) is considered to be an additional argument for the hypothesis that the PRRSV antibodies constitute a deleterious, nonprotective response. However, in direct contrast with these observations, some authors have reported on the protective effect of passive maternal

immunity that can be transferred to the piglets in the colostrum, which would result in protection of piglets against development of clinical symptoms and curtailment of the viremia (Gorcyca *et al.*, 1996). Importantly, the protective effect for piglets seems to disappear when colostrum antibodies become undetectable (Albina, 1997; Morrison *et al.*, 1996). Alternatively, it has been postulated that the protection conferred by the colostrum is not based on its antibodies but on its cell content instead (Bautista and Molitor, 1997; Molitor *et al.*, 1997). Nevertheless, there have been some reports in which DNA vaccination with the immunogenic PRRSV ORF-5 product (the gp 5 of 25 kDa MW) was accompanied by appearance of PRRSV-specific neutralizing antibodies and the concomitant establishment of protective immunity in young pigs (Pirzadeh and Dea, 1998). Likewise, there is a report that antibodies passively transferred to pigs at a sizable concentration (1:8 titer) cleared viremia of PRRSV effectively (Yoon *et al.*, 1996), while another report suggests that neutralizing antibodies would clear PRRSV from the lung during acute infection (Labarque *et al.*, 2000). Therefore, an assessment of the real role that antibodies may have in protective immunity against PRRS seems to be in order. To define the role of antibodies in conferring protective immunity and in modulating the infection of PRRSV in the natural host, we conducted passive transfers of PRRSV Igs obtained from hyperimmunized animals with high PRRSV-neutralizing titers. Our studies involving passive transfer of antibodies used the most definitive model of PRRSV pathogenesis: induction of reproductive failure by oronasal inoculation of PRRSV in 90-day-gestation pregnant females. Results of these experiments clearly indicate that passive transfer of PRRSV-Igs enriched in PRRSV-neutralizing activity fully prevented the transplacental infection of offspring and completely precluded the infection of PRRSV in the pregnant gilts.

RESULTS

Passive transfer of PRRSV-specific Igs fully prevents PRRSV-induced reproductive failure

As explained under Materials and Methods (Passive transfer experiments), three homogeneous groups of six pregnant gilts each received Igs: Group I received PRV Igs, Group II received PRRSV Igs, and Group III received normal (PRRSV and PRV antibodies free) Igs. Of these, only Groups I and II were challenged with PRRSV 3 days after passive transfer of Igs.

The clinical consequences of the challenge of pregnant females with virulent PRRSV at Day 90 of gestation are shown in Tables 1 and 2. The appearance of clinical signs followed one of two clearly distinct patterns among groups. There was no significant clinical alteration in Groups II and III. The animals in these groups maintained their normal appetite and alertness during the

TABLE 1
Offspring Viability Scores at Birth and at 15 Days
of Age (Weaning Time)

Group	Abs used for passive transfer	Gilt No.	Viability at birth		Viability at 15 days of age
			Dead	Live	
I	PRV	229	9	2	2
		230	11	1	1
		231	7	3	0
		232	10	4	0
		233	9	2	0
		241	13	1	0
II	PRRSV	234	0	14	14
		235	1	13	13
		236	1	10	10
		237	1	13	13
		238	1	11	11
		239	0	5	5
III	Normal Igs	244	1	10	10
		248	0	10	10
		249	2	11	9
		242	0	16	14
		243	0	12	12
		250*	N/A		

* Animal No. 250 (Group III) was withdrawn from experiment because of misdiagnosis of pregnancy status (open female detected at farrowing time).

remainder of gestation and did not show any abnormal clinical signs. On the other hand, the six animals of Group I (PRV Abs-treated group) presented significant clinical alterations starting at 24 h after PRRSV challenge. All of the animals in this group were clearly lethargic, lacked appetite, and presented mild fever for 7–10 days and a rough appearance for the rest of the gestation period. In this group three of the farrowings were advanced by 7, 6, and 5 days of the anticipated due date, respectively (see Table 1 for viability scores).

Upon challenge with PRRSV, the scores of offspring viability at birth and at weaning (Table 1) clearly show a statistically significant difference (Table 2) between Group I (18% of piglets born alive and 4% alive by Day 15

of age) on one side and Groups II (95% born alive and 95% alive by Day 15 of age) and III (95% born alive and 90% alive by Day 15 of age) on the other side. Group I, which had received the unrelated (PRV) antibody, exhibited unequivocal signs of reproductive failure which are the hallmark of infection with the highly virulent PRRSV strain that we used as viral challenge (Osorio *et al.*, 1998). The six gilts of Group I (specially those three that presented advanced farrowings at 5–7 days prior to estimated due date) delivered most of their litters dead (decomposed + stillborns) with just a few piglets surviving for a few (1–3) days. In addition to the significant mortality at birth, the overall survival of piglets born alive in this group was dramatically low (Table 1). The presence of PRRSV was confirmed in all of the pigs in the aborted litters by immunohistochemistry in thymus and other lymphoid tissue samples, as well as viral isolation or PCR on the thoracic fluid of the fetuses. The three piglets from this group that were still alive at weaning time (two piglets from gilt No. 229 and one from gilt No. 230, Table 1) were necropsied at the time of weaning. These piglets had pronounced pneumonitis, with their lymph nodes significantly enlarged and hemorrhagic, characteristic of PRRS in young pigs. PRRSV was isolated from several tissues of these animals and detected by immunohistochemistry in their lymphoid tissues (data not shown).

Piglets in Group II (PRRSV antibody-treated group) did not exhibit significant pre- or postnatal mortality, and the size and appearance of the litters exhibited no significant differences from the nonchallenged Group III (normal Ig-treated group, Tables 1 and 2). The few sporadic perinatal deaths observed in these two groups were due to routine factors that cause mortality in these animals (i.e., mechanical trauma caused by the dam, high number of pigs in the litter). No evidence of PRRSV infection was found in any of the four piglets born dead in Group II or in any of the three piglets born dead in Group III (Table 1). The survival of the piglets born alive was 100% in the PRRSV antibody-treated Group II and 95% in the Unchallenged Control Group III.

TABLE 2
Summary Statistics

	Group I		Group II		Group III	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Total piglets born	12.0 ^a	10.24–13.75	11.7 ^a	7.99–15.34	12.4 ^a	9.54–15.25
Proportion born alive	0.18 ^a	0.08–0.29	0.95 ^b	0.90–0.99	0.95 ^b	0.86–1.00
Proportion alive 15 days	0.04 ^a	0.00–0.12	0.95 ^b	0.90–0.99	0.90 ^b	0.74–1.00

Note. The average values and 95% confidence intervals for each group are presented. Values in a row with different superscripts are significantly different ($P \leq 0.01$) (Donner, 1991).

TABLE 3
Viremia in Gilts

Group	Gilt No.	Days PI							
		7		14		21		28	
		Infect ^a	RT/PCR	Infect	RT/PCR	Infect	RT/PCR	Infect	RT/PCR
I	229	4.7*	+	<1.2	+	<1.2	+	<1.2	-
	230	5.2	+	<1.2 ^a	+	<1.2	-	<1.2	-
	231	4.7	+	<1.2	-	<1.2	-	<1.2	-
	232	5.2	+	<1.2	+	<1.2	+	<1.2	-
	233	4.7	+	<1.2	-	<1.2	-	<1.2	-
	241	4.2	+	<1.2	-	<1.2	-	<1.2	-
II	234	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	235	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	236	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	237	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	238	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	239	<1.2	-	<1.2	-	<1.2	-	<1.2	-
III	244	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	248	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	249	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	242	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	243	<1.2	-	<1.2	-	<1.2	-	<1.2	-
	250	<1.2	-	<1.2	-	<1.2	-	<1.2	-

^a Infectivity is expressed as log₁₀ of PRRSV titer expressed in TCID₅₀/ml of serum.

The intraperitoneal instillation of swine immunoglobulins per se does not cause reproductive failure

Group III, which received, via ip route, a dose of normal swine Ig equivalent to that received by the other two challenged groups (70 mg/ml of Ig in a 1.5-L volume) without receiving infectious challenge, did not exhibit any clinical abnormality. Importantly, such normal percentage of viability and survival observed in Group III rules out any concern that might be raised in relation to the protocol used for ip transfer of Igs as a possible cause of flaws in the viability scores observed in the different experimental groups.

Passive transfer of PRRSV-specific Igs precludes PRRSV infection of the dams and transfer of infection to offspring

Table 3 shows the kinetics of appearance of viremia in serum samples obtained from the pregnant females of the three groups. To minimize stress to the pregnant females which could possibly distort the offspring viability results in all the groups, we collected serum samples only at weekly intervals, starting at 7 days pi until Day 28 pi. In the clinically affected Group I, viremia was evident in all of the dams, being detectable for the first week PI by infectivity assays and for a longer period (up to third week PI) by RT/PCR (Table 3). In contrast, the clinically normal Group II did not exhibit PRRSV viremia either by

infectivity assays or by RT/PCR at any point. Likewise, the unchallenged Group III did not exhibit PRRSV viremia at any point, which was expected as this group had not been challenged with PRRSV and had been maintained under appropriate isolation throughout the experiment.

At Day 15 postbirth, immediately after weaning of the offspring, the dams were killed and necropsied. The tissues from the dams were used to confirm whether PRRSV infection could be detected in those groups in which a typical viremia had not been detected (Groups II and III). RT/PCR and inoculation of MARC 145 cells were used to attempt PRRSV detection or isolation in individual, not-pooled, samples of tonsils, lung, lung lavage, and lymph nodes (including bronchial lymph nodes) in each of the gilts from Groups II and III. In all of these cases the viral isolation and RT/PCR assays were negative (data not shown). Negative results from Group III were expected as this group had not been challenged with PRRSV. It became important, however, to further verify, at the highest level of sensitivity, if PRRSV was absent from the tissues of the dams in the nonviremic, PRRSV-challenged Group II. Therefore pools of tissues of each of the 6 gilts from Group II were used to inoculate individual young PRRSV-free pigs to attempt isolation of infectious PRRSV by the highly sensitive bioassay procedure. No PRRSV sero-conversion or viremia could be detected in any of the six bioassay inoculates throughout a complete observation period of 28 days (data not shown).

The PRRSV serologic profiles observed in Groups I and II after the passive transfer of Igs and subsequent PRRSV challenges are shown in Fig. 1. These profiles are represented by the mean virus-neutralizing (VN) titers and Idexx ELISA S/P (signal to positive) ratios. The transferred PRRSV antibodies (Group II) followed a continuous decline starting from the initial titer of 1:16, which was reached immediately after transfer. The VN activity decayed to undetectable levels in the gilts at the time of farrowing, with a concomitant increase in colostrum (i.e., with VN titers reaching up to 1:128 in colostrum and milk and subsequent transfer to the piglets) (data not shown). The S/P of ELISAs, while decaying more slowly, reached near negligible levels at the end of the observation period in the dams (at weaning of the offspring at Day 15 of age) (Fig. 1). The decline of passively acquired pseudorabies virus (PRV)-neutralizing antibodies in the animals of Group I, which occurred at a kinetics equivalent to the decay of the PRRSV antibodies in the animals of Group II, is not shown in Fig. 1.

The PRRSV antibody response (VN and ELISA) in Group I, which received unrelated (PRV) antibodies and developed full clinical symptoms of PRRS, conforms to a typical primary response upon exposure to PRRSV (Fig. 1). This primary response is characterized by the precedence of the ELISA response over a more delayed VN response. The dams from Group III did not develop

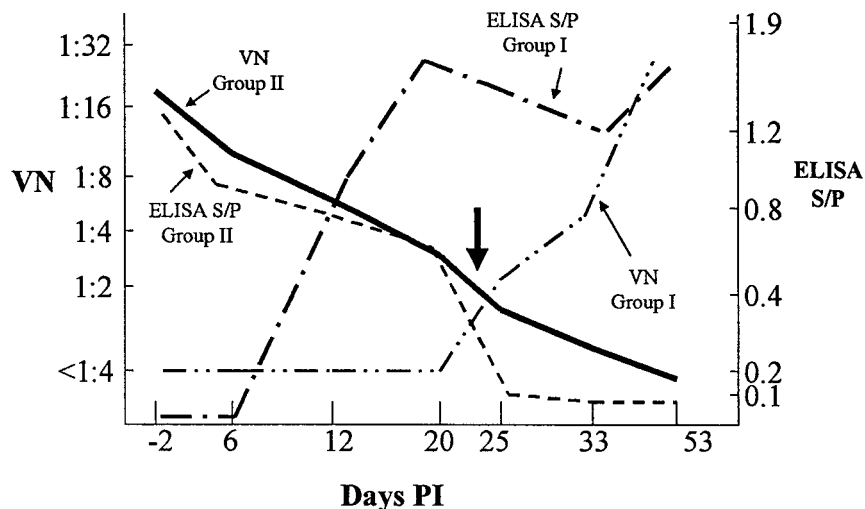


FIG. 1. PRRSV antibody profile in the dams of challenged Groups I and II: mean ($n = 6$) of ELISA (S/P) and SN titers. Solid arrow indicates average time for farrowing.

PRRSV serologic status at any moment throughout the experiment (data not shown).

As noted above, the PRRSV infection status of the offspring born from Group I was readily confirmed as positive by both RT/PCR and viral isolation when necropsied at weaning time (two piglets from gilt No. 229 and one from gilt No. 230, Table 1). Importantly, serologic and virologic assays indicated that the offspring born from the dams of Group II were free of PRRSV infection. Half of the offspring weaned by Group II's females ($N = 33$) were necropsied at 15 days of age and their tissues used for virologic detection of PRRSV. The remaining half of Group II's weaned offspring were maintained in isolation until Day 65 of age. During that time all the animals were

periodically sampled to ascertain their PRRSV serologic profile and possible viremia. At Day 65 of age the animals were killed and the tissues used for viral isolation and RT/PCR. As shown in Fig. 2, the antibody levels obtained by colostrum ingestion progressively declined until becoming negative (by either VN or ELISA assays) at approximately Days 28 and 42 postbirth. No subsequent rise in antibodies by either ELISA or VN (which would typically indicate active immunity due to continuous infection with PRRSV upon disappearance of maternal antibodies) was detectable at any point through necropsy of these animals at Day 65 of age (Fig. 2). Pools of tissues (lung, lymph nodes, tonsils, and spleen) of the totality of the offspring weaned from Group II ($N = 66$)

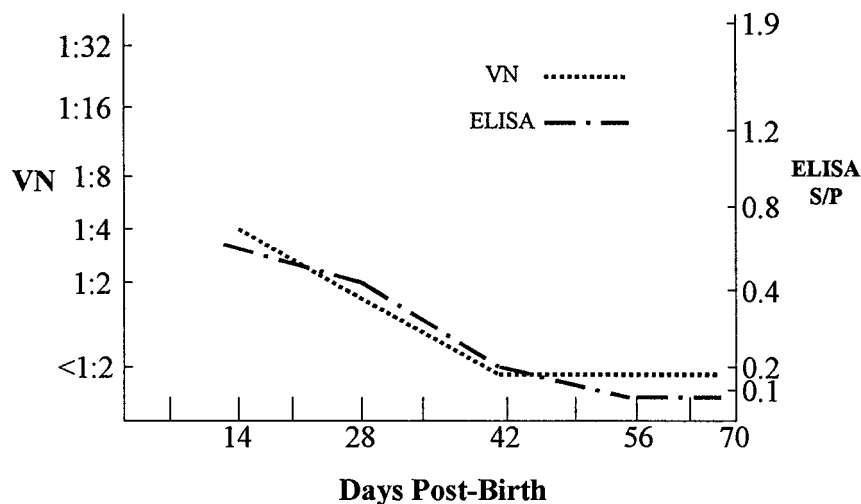


FIG. 2. Mean PRRSV antibody profile in offspring of Group II (group passively transferred with PRRSV antibodies). $N = 66$ for samples collected at Day 15 of age and $N = 33$ for samples collected on Days 28, 42, 56, and 65 of age. Ranges for VN individual values were Day 15, 1:8-1:2; Day 28, 1:4-<1:2; Days 42, 56, and 65, no range. Range for ELISA S/P individual values were Day 15, 1.235-0.69; Day 28, 0.978-0.301; Day 42, 0.234-0.000; Day 56, 0.090-0.000; and Day 65, 0.050-0.000. S/P: signal to positive ratio of the commercial ELISA.

were scored negative by both RT/PCR and viral isolation in MARC 145 cells (data not shown).

DISCUSSION

The results of these passive transfer experiments in pregnant gilts unambiguously indicate that the sole transfer of immunoglobulins specific for PRRSV conferred complete protection against reproductive failure induced by this virus. This total protection was first evidenced by complete absence of viremia in the challenged animals, which consequently suggests that the PRRSV-immunoglobulins were able to block the transplacental infection which is known to occur approximately 7 days after a pregnant female is infected with PRRSV at Day 90 of gestation (Mengeling *et al.*, 1996). Furthermore, and more importantly, the complete protection conferred by antibodies alone is confirmed by the normal viability at birth and survival of the piglets born from the gilts which received PRRSV Igs but not from those that received unrelated (anti-PRV) Igs. In addition, the passive administration of PRRSV Igs prior to challenge appears to have precluded the establishment or stopped the progress of the PRRSV infection in the gilts. This is evidenced by the absence of viremia in the PRRSV-Ig-protected gilts, the absence of infectious virus from these females' tissues at 45 days post challenge, and the lack of transmission of infection to the offspring, which remained PRRSV-free until their necropsy at Day 65 of age.

Regarding the basis for this humoral passive transfer of protection, it is important to note that our protective Ig stock solution had been prepared from individual hyperimmune sera with a high content of PRRSV-neutralizing antibodies. Therefore PRRSV-neutralizing antibodies were highly represented in the Ig stock solution that was passively transferred as well as in the circulation of the gilts shortly upon transfer and prior to challenge (Fig. 1). We believe that the PRRSV-neutralizing Igs may have a significant role in the protective effect of the passive transfer of Igs. Previous reports from several laboratories, including ours, support the notion that an association exists between *in vitro* neutralizing capacity and protection *in vivo* against PRRSV infection (Gorcyca *et al.*, 1995; Gorcyca *et al.*, 1996; Labarque *et al.*, 2000; Morrison *et al.*, 1996; Osorio *et al.*, 1998; Pirzadeh and Dea, 1998). Investigators that described the sequential appearance of non-PRRSV-neutralizing followed by PRRSV-neutralizing antibodies during the response of pigs to PRRSV infection have not described any protective effect conferred by the nonneutralizing fraction of PRRSV Igs (Labarque *et al.*, 2000; Yoon *et al.*, 1996). Instead, in those experiments the transfer or appearance of PRRSV-neutralizing antibodies protected pigs against viremia (Yoon *et al.*, 1996) and cleared the lung of infection (Labarque *et al.*, 2000). Likewise, immunization of pigs with PRRSV

ORF5 DNA conferred protection that seemed to correlate closely with the appearance of PRRSV-neutralizing antibodies (Pirzadeh and Dea, 1998). Moreover, we have previously reported (Osorio *et al.*, 1998) that in gilts which had received an attenuated PRRSV vaccine there was an anamnestic VN response shortly after challenge of the vaccinated animals with virulent PRRSV. This anamnestic VN response correlated closely with protection against PRRSV-induced reproductive failure (Osorio *et al.*, 1998). In summary, our current results on passive transfer of immunity and those of other researchers suggest that passively transferred anti-PRRSV Igs alone will protect swine against PRRSV infection and that such protection may be caused by the PRRSV-neutralizing activity of these antibodies. The PRRSV-neutralizing antibodies would then represent a significant component of the homologous protective immune response to PRRSV and the first bona fide correlate of protective immunity to be defined for PRRSV. The capacity to arise PRRSV-neutralizing antibodies against one or several reference strains could therefore help to estimate the efficacy of a PRRSV vaccine.

Despite the body of evidence suggesting that PRRV-neutralizing antibodies would confer protection *in vivo*, we cannot rule out a possible contribution of nonneutralizing (but protective) antibodies on this effect. We know, from other viral systems, that protective antibodies may function not only by virus neutralization, but also by other mechanisms such as antibody-dependent cell-mediated cytotoxicity, or complement-dependent antibody-mediated cell lysis (Lindsay and Oldstone, 1996). Interestingly, it has been demonstrated that nonneutralizing antibodies directed against the nonstructural glycoprotein NS1 of the flavivirus tick-borne encephalitis virus (TBEV) can mediate and passively transfer protective immunity (Kreil *et al.*, 1998).

One result of our experiments that is particularly remarkable is that passively acquired PRRSV antibodies conferred sterilizing immunity to the gilts when these animals were challenged with PRRSV. This phenomenon could be the consequence of the preexisting antibodies precluding the mucosal spread of the PRRSV challenge, thus stopping the progress of the infection. It is well documented that antibodies alone can confer protection against clinical disease in many other viral infections, including several of the most economically significant infections of swine (Onisk *et al.*, 1994; Terpstra and Wenswoort, 1988; Marchioli *et al.*, 1988). Antibodies mediate this function primarily by providing a major barrier for virus spread between cells and tissues and restricting virus spread by blood. However, it is commonly accepted that complete elimination of a virus infection cannot be achieved by antibodies alone, and that other effector mechanisms of the immune response, namely cell-mediated immunity, are needed for complete viral clearance. A typical example of this situation would be

the infections by pseudorabies virus, a member of the alphaherpesvirus subfamily. In this case neutralizing PRV-antibodies are known to be very effective in preventing initial infection, and in restricting the infection to the mucosal portal of entry. However, the ability of PRV to establish neurotropic latency allows this virus to bypass the protective circulating antibodies and perpetuate the infection in the form of neural latency (Mettenleiter, 1996). Despite the common perception that antibodies may prevent but not fully clear viral infections, there is now documented evidence for complete, sterilizing immunity being mediated exclusively by antibodies to HIV-1 (Gauduin *et al.*, 1997; Shibata *et al.*, 1999). Several mechanisms have been proposed to explain the absolute *in vivo* HIV-1 neutralization observed in these passive transfer experiments which involved either SCID mice (Gauduin *et al.*, 1997) or pig-tail macaques (Shibata *et al.*, 1999). Of these putative mechanisms, the strong binding of antibody to highly conformational epitopes in virions or in oligomeric gp120 expressed on the surface of infected cells remains the best explanation for this sterilizing capacity of anti-HIV antibodies (Cho *et al.*, 2000).

We can speculate on the possible mechanism for the occurrence of the sterilizing immunity produced by the passive transfer of PRRSV antibodies herein reported. The primary target of PRRSV infection is known to be macrophages, specifically the alveolar macrophages (Rossow *et al.*, 1995). It is well accepted that either by airborne route or by mucosal spread of the oro-nasal inoculum, the PRRSV reaches the macrophages of the alveolae where the primary replication of PRRSV takes place prior to the viremic and lymphoid dissemination of the infection to other tissues (Collins, 1998; Rossow *et al.*, 1995). In our experiments, the PRRSV-neutralizing antibodies obtained by hyperimmunization, precipitated by ammonium sulfate treatment, and transferred to the gilts were mostly of the IgG type, as demonstrated by the swine isotype-specific ELISA that we used to quantitate the precipitated immunoglobulins. The IgG is known to act as a major surface immunoglobulin in the lung of domestic species, including swine (Tizard, 2000). High titers of IgG perfuse into the alveolae by simple transudation (Nathanson, 1997). It is then conceivable that the high-titered anti-PRRSV IgG, translocating into the alveolar lumen or surface might be a major factor in precluding the macrophage-to-macrophage progress of the infection, leading then to conclusion of the infection.

It might be argued that the high content of PRRSV-specific antibodies of the solution of Igs used for these experiments may have created a condition of "antibody saturation" in the tissues of the passively transferred animals. According to this logic, the VN titers obtained by our passive transfer would therefore be artificially higher than the normal antibody levels that could be achieved by regular active immunization procedures. However, it must be born in mind that the maximum levels of VN

TABLE 4

In Vitro VN Endpoint of the Protective PRRSV Ig Stock Solution Used in the Passive Transfer Experiment Described in Table 1, against Several Isolates of PRRSV

Year of isolation	Strain used for VN assay	Endpoint VN titer
1996	IA 977895 ^a	256
1997	16244B ^b	256
1990	VR2332 ^b	256
1995	12068 ^c	128
1993?	MICH-1 ^c	256
1990	Lelystad ^c	64

^a Strain used several times for inoculation of the animals during preparation of hyperimmune serum and also as challenge for protective immunity assessment (homologous strain).

^b Strains used once for inoculation of the animals during preparation of hyperimmune serum.

^c Strains not used for inoculation of animals during preparation of hyperimmune serum. These strains are included to check cross-reactivity of the Ig solution.

antibodies that we observed in the females upon passive transfer, immediately prior to PRRSV challenge (serum titers at 1:16), are consistent with the VN levels obtained upon active immunizations as reported by different authors (Gorcyca *et al.*, 1995; Osorio *et al.*, 1998; Pirzadeh and Dea, 1998). Such VN titers, attained by active immunization (Gorcyca *et al.*, 1995) or as a result of an anamnestic increase after challenge of vaccinated animals with a heterologous PRRSV strain, showed close correlation with protective immunity (Osorio *et al.*, 1998; Pirzadeh and Dea, 1998).

Considering that PRRSV strains exhibit a high degree of genetic variability and that PRRSV-neutralizing activity has been reported to be strain specific, it would have been of interest to determine the degree of protective immunity achieved had we used a different challenge strain (i.e., one not used for hyperimmunization of the donor animals). If we accept that that *in vitro* VN can be used as a correlate of protective humoral immunity, Table 4 can then provide some interesting predictors of immunity. In that table are shown the endpoints obtained in the *in vitro* VN assay when different homologous and heterologous strains of PRRSV were used as challenge for the assay. It is interesting that strains which were not used in the hyperimmunization exhibit a sizable VN endpoint titer similar to that attained by the homologous challenge strain used for repeated immunizations of the donor animals. This suggests that broadly cross-reactive neutralizing epitopes exist in the PRRSV antigenic composition. Identifying these broadly cross-reactive neutralizing epitopes will be of great value for the design of "new generation," protective vaccines against PRRSV. In this respect, an important point that remains to be elucidated is the antigenic specificity of the protective antibodies that mediate the passive protection herein reported. It is

known that most of the neutralizing epitopes would reside in the envelope glycoprotein 5, the product of PRRSV ORF5 (Pirzadeh and Dea, 1997; Pirzadeh and Dea, 1998; Weiland *et al.*, 1999; Yang *et al.*, 2000). In addition, at least one other neutralizing epitope has been identified by a monoclonal antibody in the envelope glycoprotein 4 (Yang *et al.*, 2000) and another one in the M envelope protein coded for by ORF6 (Yang *et al.*, 2000). We should also bear in mind the recent reports demonstrating that antibodies against viral nonstructural proteins such as the cell-secreted glycoprotein NS1 of tick-borne encephalitis (Kreil *et al.*, 1998) and the NSP4 of rotavirus (Estes *et al.*, 2001) have great significance for *in vivo* protective immunity. In the case of PRRSV, one nonstructural glycoprotein in certain strains of PRRSV, the glycoprotein 3, which seems to be secreted from virus-infected cells, has been reported to be protective but not an inducer of neutralizing antibodies (Plana-Duran *et al.*, 1997b). The possible significance of nonstructural PRRSV proteins in protection is also highlighted by a recent publication by Oleksiewicz *et al.*, which locates the majority of a group of immunodominant B-cell epitopes, identified by phage display mapping of the PRRSV genome, in the NSP2 nonstructural gene of PRRSV (Oleksiewicz *et al.*, 2001).

MATERIALS AND METHODS

Animals

Adult female swine of mixed breed (Landrace × Duroc), which weighed about 300 lb each, were used for hyperimmunization with the goal of obtaining PRRSV-neutralizing or PRV-neutralizing Igs. The animals were purchased from a specific-pathogen-free herd that had certified records of absence of PRRSV and PRV infection. Normal swine Igs were obtained from serum collected from animals purchased from this same herd, killed, and bled out to harvest the maximum amount of serum possible.

For passive transfer experiments, mixed breed (Landrace × Duroc) gilts were obtained from the swine farm of the Animal Science Department of the University of Nebraska at Lincoln. These animals are free of PRV or PRRSV infection (as determined by clinical history and frequent serology) and show minimal incidence of clinical disease. The animals had been impregnated by artificial insemination and confirmed pregnant by ultrasound test at 56 days of gestation. The anticipated farrowing dates for all these pregnant gilts clustered within a time period of 48 h.

Virus strains and cells

We used in these experiments the following PRRSV isolates: (i) PRRSV IA 97-7895 (Allende *et al.*, 2000b; GenBank Accession No. AF325691) which had been iso-

lated by the Diagnostic Virology Unit of NVSL USDA/APHIS in a farm located in S.E. Iowa in December 1996. This strain corresponded to one of the cases of exacerbated reproductive failure (also called “atypical PRRSV”) that affected the region. This highly abortifacient strain had been previously used by our lab for the evaluation of PRRS vaccines by challenge in pregnant swine (Osorio *et al.*, 1998), (ii) PRRSV 16244B, which is a strain isolated in 1997 in Nebraska from a field investigation for “atypical PRRS” (Allende *et al.*, 2000a; GenBank Accession No. AF046869), (iii) PRRSV NVSL strain (provided by NVSL, USDA, APHIS/Ames, IA), (iv) The American prototype PRRSV strain VR-2332 (American Type Culture Collection), (v) The modified-live vaccine strain RespPRRS (NOBL Labs., Ames, IA), and (vi) the modified-live vaccine virus Prime PacPRRS (Schering Plough Animal Health, Elkorn, NE). All these strains were propagated and titrated in MARC-145 cells (Kim *et al.*, 1993). These cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). None of the strains were propagated more than twice in MARC 145 cells after the last isolation from infected tissues (wt strains) or after retrieving from the commercial vaccine vial (MLV strains).

Preparation of PRRSV antibodies from hyperimmunized animals

A total of 13 PRRSV-free female swine, of about 300 lb of body weight each, were initially infected by oro-nasal inoculation with 6×10^6 TCID₅₀ of the PRRSV IA strain 97-7895. At 5 weeks PI, the animals were super infected with a mixture of 5 additional PRRSV strains, which included 16244B, 2332 (ATTC), NVSL, RespPRRS, and Prime PacPRRS strains. Each strain was given at a dose of 5×10^6 TCID₅₀ through oro-nasal inoculation. After 4 weeks following this exposure to multiple PRRSV strains, each of the animals received a dose of 10^5 TCID₅₀ of strain PRRSV IA strain 97-7895, emulsified in 2 ml of Freund’s (complete) adjuvant via intramuscular route. At intervals of month and a half thereafter, the animals received a similar dose of PRRSV IA strain 97-7895 emulsified in 2 ml of Freund (incomplete) adjuvant for a range of 3 to 6 applications. The PRRS VN antibody titers in the peripheral blood of the immunized animals against the primary inoculation strain (PRRSV IA 97-7895) were monitored by a rapid neutralization assay of fluorescent foci on MARC 145 cells and was confirmed by a regular 4-day VN assay on Marc-145 and porcine alveolar macrophages as well. This VN endpoint titer gradually increased in each of the hyperimmunized animals. Within a period of time that ranged from 7 months (including 3 applications of virus + incomplete Freund’s) to 14 months (including 6 applications of virus + incomplete Freund’s), all of the 13 animals reached a final endpoint titer that ranged between 1:32 and 1:128, with most of the

animals exhibiting an endpoint VN titer of $\geq 1:64$. This point was considered to be the end of the hyperimmunization process and the animals were killed and exsanguinated, all of their sera individually collected, and serum Igs precipitated and concentrated by NH_2SO_4 treatment (Onisk *et al.*, 1994). The concentrated Igs from individual animals were consolidated in a single Ig stock solution to be used for passive transfer experiments.

Preparation of PRV-Igs from hyperimmunized animals

Hyperimmunization for preparation of PRV Igs was initiated by vaccinating each of 8 PRRSV-free female swine with a PRV-modified live virus vaccine (Syntrovect Marker Blue, Syntrovect, Lennexa, KS) followed 3 weeks later by intranasal and conjunctival inoculation with *wt* PRV (Becker) strain. Two months post-*wt* PRV infection all of the animals had reached a 1:32/1:128 range in their PRV VN endpoint titers. The animals were then killed and exsanguinated, and the PRV Ig NH_2SO_4 precipitated as previously described for the preparation of PRRSV-specific antibodies.

Preparation of normal Igs

Normal Igs collected from animals of the SPF herd (PRRSV and PRV-free) were prepared following the same procedures as described for anti-PRRSV and PRV Igs.

Standardization of the Igs stocks

The total content of swine Igs in each of the three concentrates of Igs (PRRSV-antibodies, PRV antibodies, and normal Igs) was determined by an indirect ELISA specific for swine IgG (Bethyl Laboratories Inc., Montgomery, TX). The level of endotoxin contamination in each of the three Ig solutions was determined in all of the three master Igs solutions using a commercially available detection kit based on *Limulus* amoebocyte lysate (Associates of Cape Cod Inc., Falmouth, MA). Likewise, the possible interferon activity contained in the three solutions of Igs was measured by a vesicular stomatitis virus/porcine kidney cells interferon assay (Mawie, 1996), using genetically expressed porcine α -interferon as positive control (source: Dr. F. A. Zuckermann, College of Veterinary Medicine, University of Illinois). In all of the cases, the level of viral interference activity was negligible and identical in the three stock solutions of Igs. The endpoint of PRRSV-neutralizing activity attained for the resulting master stock solution was 1:256, while the resulting stock with PRV-neutralizing activity reached 1:512 by regular PRV VN assay. The three stocks were confirmed by RT/PCR as free of contaminant PRRSV. No titer against PRRSV was evident in the PRV Ig stock solution. Likewise, neither titer against PRRSV or PRV was evident in the normal Ig stock solution.

Passive transfer experiments

The Ig solutions prepared and standardized as described above were used for passive intraperitoneal transfer into pregnant gilts. In a typical experiment, the gilts received the ip instillation in the left flank, at Day 87 of gestation, in standing position, with subcutaneous local anesthesia at the point of injection, using an atraumatic teat cannula. Typically, the time of instillation for an entire dose of Ig stock solution was 10–15 min/gilt. The dilution of the Igs in the body of recipient gilts was evaluated and compared using as a reference the endpoint dilution of VN antibodies. Prior to the transfer experiment, the PRRSV- and PRV-neutralizing stock solutions were tested using two pregnant gilts in each case. In these initial trials, intraperitoneal instillation of 1.5 liters of Ig stock solution containing 70 mg/ml of Igs and a PRRSV-neutralizing endpoint of 1:256 consistently lead to the establishment of a PRRSV-neutralizing titer in peripheral blood of 1:16 by Day 89 of gestation. Likewise, at Day 89 of gestation, upon instillation of 1.5 liters of PRV master stock Ig solution containing 60–70 mg/ml of Igs and a PRV-neutralizing endpoint of 1:512, the gilts exhibited a peripheral PRV-neutralizing titer of 1:32/1:64. Once the required transfer dose of Igs was determined in each case, the main passive transfer experiment was carried out as follows: Three homogeneous groups of 6 pregnant gilts each were intraperitoneally instilled with 1.5 liters of Ig stock solutions at Day 87 of gestation. Group I received PRV Igs (Control of Ab Specificity Group) and the resulting PRV-neutralizing titer in their peripheral circulation was, 48 h later, 1:64. Group II (Principal Group) received PRRSV Igs and the resulting PRRSV-neutralizing titer in the peripheral circulation of any of these animals was, 48 h later, 1:16. Finally Group III received normal (PRRSV and PRV antibody-free) Igs (Control of the Safety of the ip Instillation Procedure). At 3 days after ip instillation (Day 90 of gestation) Groups I and II (Ab Specificity Control and Principal Groups) were challenged oronasally with 2 ml containing $10^{5.4}\text{TCID}_{50}$ of PRRSV IA strain 97-7895 (second passage in MARC 145). Group III (Group for Control of Safety of the ip Instillation Procedure) was just mock-infected receiving 2 ml of DMEM through oronasal instillation. The three groups were maintained in isolation from each other during the entire experiment.

Evaluation of protection against PRRSV-induced reproductive failure

A clinical and virological evaluation of the level of protection attained in the gilts that had been passively transferred with different Igs was conducted by: (i) number and proportion of viable offspring at birth, (ii) number and proportion of viable offspring at Day 15 of age (weaning time), (iii) measurement of viremia in the dams at 7, 14, 21, and 28 days after challenge, (iv) isolation of PRRSV from the tissues of the gilts in the principal group upon

necropsy conducted at weaning time, and (v) evaluation of the infection status of the offspring by (va) PRRSV detection in tissues of offspring (half of which were killed at weaning time and the other half at Day 65 of age) by RT/PCR and by viral isolation on MARC 145 cells and (vb) analysis of the kinetics of the PRRSV ELISA antibody response (Idexx PRRSV ELISA, Portland, ME) present in the sera of the offspring maintained alive until Day 65 of age.

Detection of PRRSV in tissues and blood

The tissues collected from the offspring at necropsy were analyzed for the presence of PRRSV by infectivity assays on MARC 145 cells, by immunohistochemistry, and by RT/PCR. The protocols for viral isolation and immunohistochemistry were reported previously (Sur *et al.*, 1998). The samples assessed included tonsil, serum, lymph nodes (retropharyngeal and bronchial lymph nodes), serum, and lung. Likewise, samples of serum collected from all of the dams of the two PRRSV-challenged groups at 7, 14, 21, and 28 days postchallenge were used to titrate viremia in MARC 145 cells and detect PRRSV RNA by RT/PCR. For tissues and blood from gilts or piglets we used a protocol of nested RT-PCR using primers capable of directing the amplification of 403- and 150-bp fragments of ORF6 of the PRRSV IA 97-7895 strain (GenBank Accession No. AF325691), which was used for challenge of the passively transferred females. The sense and antisense primers for the outer PCR were 5'-AGGTGCTCTTGCGCTTCTCTATT-3' (nucleotides 14424 to 14447) and 5'-GCTTTTCTGCCACCCAACACG-3' (nucleotides 2848 to 2869), respectively. Primer sequences for the nested PCR were 5'-CCTCCAGATGCCGTTTGTGCT-3' (nucleotides 14661 to 14682) and 5'-TGCCGTTGACCGTAGTGGAGC-3' (nucleotides 14790 to 14811). Cycling parameters for both PCRs were 95C 1 min, 60C 1 min, 72C 1 min in standard PCR mix with 4 mM MgCl₂ for a total of 30 cycles. The RNA extraction from samples, RT, and PCR protocols have been described previously (Sur *et al.*, 1996).

The detection of PRRSV in the tissues of the gilts from the Principal Group was also approached by *in vivo* inoculation of PRRSV-susceptible pigs (swine bioassay) as previously described (Allende *et al.*, 2000b). Briefly, we inoculated 1- to 2-week-old piglets, obtained from an unvaccinated PRRSV-free herd, to assay for the presence of infectious PRRSV in tissue collected from the gilts of the principal group killed at weaning of offspring (at 45 days postchallenge with PRRSV IA 97-7895 strain). Each homogenate typically consisted of 50 to 80 mg of ground tissues including tonsil, lung, and pool of retropharyngeal and bronchial lymph nodes. Each homogenate was maintained at -80°C for parallel attempts of viral isolation assay in cell cultures. For bioassay each homogenate was diluted to a volume of 8 ml with MEM, supple-

mented with gentamicin (50 µg/ml) and used to inoculate the piglets. Tissue samples taken at necropsy from the infected animals were ground separately. Typically each homogenate consisted of 5 to 10 g each of ground lung, lung lymph node, and tonsil tissues and 4 ml of serum. MEM supplemented with gentamicin (100 µg/ml) was added in a volume of 15 ml. The suspension was then frozen and thawed and clarified by centrifugation at 2000 rpm for 10 min. Supernatants were then transferred to new sterile tubes and diluted 1 to 5 in MEM containing 100 µg/ml of gentamicin. A volume of 12 ml from each sample suspension was used to inoculate the piglets. Bioassay experimental piglets were inoculated with 1 ml of the supernatants from tissue suspension delivered in each nostril with the remaining 10 ml of inoculum being delivered intraperitoneally. Each inoculated animal remained individually isolated during the bioassay experiments for a period of 3 weeks. The progress of the experimental inoculation was monitored by daily clinical observation and weekly serum sampling which was used for the assessment of viremia and PRRSV antibody response by ELISA in the recipient young pigs.

Statistical analysis

All statistical analyses were performed using SYSTAT 9.0 (SPSS, Inc., Chicago, IL). Differences between groups were evaluated by Kruskal-Wallis analysis of variance and pairwise rank-sum test (Donner, 1991).

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