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## Protection against Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Infection through Passive Transfer of PRRSV-Neutralizing Antibodies Is Dose Dependent<sup>∇†</sup>

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**Previous work in our laboratory demonstrated that passive transfer of porcine reproductive and respiratory syndrome virus (PRRSV)-neutralizing antibodies (NA) protected pregnant sows against reproductive failure and conferred sterilizing immunity in sows and offspring. We report here on the dose requirement for protection by passive transfer with NA in young weaned pigs. The presence of a 1:8 titer of PRRSV-NA in serum consistently protected pigs against viremia. Nevertheless, their lungs, tonsils, buffy coat cells, and peripheral lymph nodes contained replicating PRRSV similar to the infected control group. Likewise, these animals excreted infectious virus to sentinels similar to the infectivity control animals. In an attempt to reach complete protective immunity equivalent to that previously observed in sows, the pigs were transferred with a higher titer of PRRSV-NA (1:32), and even then apparent sterilizing immunity was attained in only 50% of the animals. In conclusion, the presence of anti-PRRSV-NA in serum with a titer of 1:8 is enough to block viremia but not peripheral tissue seeding and transmission to contact animals. While a relatively low level of NA in blood is capable of conferring sterilizing immunity against PRRSV in sows, the amount of NA necessary to obtain full protection of a young weaned pig would be significantly higher, suggesting that differences exist in the PRRSV pathogenesis between both age groups. In addition, the titer of NA could be a helpful parameter of protection in the assessment of PRRSV vaccines.**

Porcine reproductive and respiratory syndrome causes devastating economic losses due to late-term reproductive failure and severe pneumonia in neonatal pigs. The etiological agent for porcine reproductive and respiratory syndrome is an RNA virus (PRRSV), classified in the family *Arteriviridae*, order *Nidovirales*. PRRSV consists of an enveloped, 50-nm virion, with a single-stranded RNA<sup>+</sup> genome of approximately 14.5 kb in length. Infection in late-gestation sows produces transplacental infection, leading to premature farrowing of still-born, partially autolyzed, and mummified fetuses.

Although the establishment of PRRSV-specific convalescent or postvaccination protective immunity is well known to occur (5–7), the molecules or cells that mediate this protection have not been fully identified. PRRSV-vaccinated or convalescent animals are protected against reinfection (in a preferentially homologous rather than heterologous PRRSV strain-specific manner). However, it is well accepted that there is a generalized failure of the protective immune effectors to clear the acutely replicating PRRSV *in vivo*, as illustrated by the late and meager response of both PRRSV-NA and PRRSV-specific gamma interferon-producing cells in naturally infected

animals (9). The consequence of this delayed protective immune response is the inability to clear off the PRRSV from the tissues during the acute periods of infection when most of tissue damage, abundant viral replication, excretion, and contact transmission take place. It has been recently proposed that the ensuing persistence of PRRSV in infected animals would represent the inability of adaptive immunity to overcome the ongoing infection (21). These authors assign instead a more pivotal role in such a process to the combined effect of innate immunity and the mere fluctuation or depletion in the number of subpopulations of macrophages permissive to PRRSV infection that are available *in vivo* (21), as had been previously proposed for the murine arterivirus LDV (15). The actual disappearance of the persistent PRRSV from tissues of individual animals does not take place until much later in the infection process, at postinfection times well beyond the actual appearance of NA or gamma interferon-producing cells to permit any correlation of these immune factors' appearance with viral clearance (2).

Although it is clear that the adaptive immune effectors are unable to mediate rapid viral clearance during natural PRRSV infection, strong evidence exists indicating that some of these same immune parameters may provide solid protective immunity when present in the body at the time of viral exposure (or evoked shortly thereafter). A previous experiment in our laboratory has shown that PRRSV-NA alone can fully prevent the transplacental infection with PRRSV and extinguish the infection of PRRSV in pregnant females (11). This observation closely correlates with previous reports of protective vaccines or immunization regimes inducing the appearance of PRRSV-NA which seemed to be associated with protection (12, 14). Based on these results, NA

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would then represent a true correlate of protective immunity against PRRSV (11) and thus be an important parameter by which to evaluate the efficacy of a vaccine against PRRSV. Since the ability to induce the appearance of NA may have strong implications for the development of efficient PRRSV vaccines, we focused on the study of this parameter in detail. In the experiments reported here, we used a model of PRRSV challenge in young pigs that have been passively transferred with antibodies, which would permit us to study the optimal conditions of neutralization *in vivo* and the efficiency of antibodies to provide protective immunity.

#### MATERIALS AND METHODS

**Animals.** Fifteen-day-old, weaned pigs were purchased from a specific-pathogen-free herd with a certified record of absence of PRRSV infection. All animals were tested for absence of anti-PRRSV antibodies by enzyme-linked immunosorbent assay (ELISA; Idexx Labs, Portland, ME).

**Virus strains.** PRRSV IA 97-7895 (1) (GenBank accession no. AF325691) was used as previously described (11). The virus was passaged and titrated in MARC-145 cells using Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Aliquots (1 ml) of the supernatant were stored at  $-80^{\circ}\text{C}$  until use.

**Preparation and standardization of PRRSV-NA and non-neutralizing antibodies.** The PRRSV-NA used in these experiments corresponded to the same stock of PRRSV-NA previously used in experiments with pregnant sows (11). These NA had been prepared by infection (PRRSV IA 97-7895), followed by hyperimmunization of adult pigs. Serum was obtained by complete exsanguination of the hyperimmunized animals and used for extraction of immunoglobulin G (IgG) by precipitation with ammonium sulfate as described previously (11). For standardization of the immunoglobulin stocks, the total content of swine IgG was determined by an antigen-capture ELISA specific for swine IgG1 and IgG2 (Bethyl Laboratories, Inc., Montgomery, TX) (11, 13). The level of potential endotoxin contamination in the immunoglobulin solutions was determined by using a commercially available detection kit based on *Limulus* amoebocyte lysate (Associates of Cape Cod, Inc., Falmouth, MA) as described previously (11). Likewise, the possible interferon activity contained in the solution of immunoglobulins was measured by a vesicular stomatitis virus-porcine kidney cell-interferon assay (8) using genetically expressed porcine alpha interferon as a positive control (F. A. Zuckermann, College of Veterinary Medicine, University of Illinois). In all cases, no viral interference activity was detectable. The endpoint of PRRSV-neutralizing activity attained for the neutralizing stock solution was 1:128, which was 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 cells and porcine alveolar macrophages as well. PRRSV-specific non-neutralizing antibodies were prepared according to the same protocol of infection of adult pigs used for the preparation of PRRSV-NA, but in this case 3 weeks after infection the pigs were bled out by exsanguination upon testing for active seroconversion by ELISA but the absence of detectable PRRSV-neutralizing activity. Their sera were inactivated for 30 min at  $56^{\circ}\text{C}$ , pooled, and precipitated as described for NA. No infectious virus was detected in these pools by addition to MARC-145 cells and also by inoculation into one PRRSV-free 15-day-old pig. The non-neutralizing antibodies showed no neutralizing activity as monitored by regular PRRSV VN assay. The IgG content of this non-neutralizing antibody fraction was standardized by indirect ELISA specific for swine IgG1 and IgG2 as described for PRRSV-NA.

**Passive transfer experiments, followed by viral challenge.** The immunoglobulin solutions prepared and standardized as described above were used for passive intraperitoneal transfer into 2-week-old piglets. A pilot experiment was performed to determine the amount of stock solution of NA (70 mg of immunoglobulin/ml) needed to reach different virus-neutralizing titers in 14-day-old pigs, respectively, and calculations were made based on body weight, considering a value for total blood volume as corresponding to 7% of body weight for the pig in order to calculate the dilution factor for the instilled immunoglobulins. At 24 h after intraperitoneal instillation and immediately before challenge, the animals were bled, and the titer of NA in the serum of recipient piglets was evaluated by a regular PRRSV serum virus neutralization (SVN) assay. Thus, the experiments were performed by intraperitoneal injection of different volumes of the immunoglobulin stock solution to attain virus-neutralization titers of 1:4, 1:8, 1:16, and 1:32 in the circulation of the recipients. Animals receiving non-neutralizing antibodies (60 to 70 mg/ml) were transferred with equivalent volumes and concentrations (determined by equivalent ELISA signal-to-positive ratios) of non-

neutralizing antibodies to the group that attained a virus-neutralizing titer of 1:32 in blood. In all cases, 1 day later, the animals were challenged with  $5 \times 10^5$  50% tissue culture infective dose(s) (TCID<sub>50</sub>) of PRRSV IA 97-7895 by the intranasal route. PRRSV-free sentinel animals obtained from the same source were used to determine excretion and transmission of infectious PRRSV by the principal animals.

**Viral isolation from blood and tissues.** Tissue samples (lungs, bronchial lymph nodes, tonsils, and a pool of the mesenteric, mediastinal, inguinal, and popliteal lymph nodes) were taken at necropsy from the infected animals by using individual surgical instruments. Each tissue was cut in small pieces and homogenized, and 1-g samples were taken to assess infectivity on MARC-145 cells as previously described (11).

**Virus neutralization assay.** The titer of NA (expressed as SVN) was determined by using the fluorescence focus neutralization assay as previously described by Wu et al. (20).

**ELISA.** Titers of pooled sera, as well as seroconversion of the contact sentinel animals, were determined by a commercial ELISA (Idexx PRRSV; Idexx, Portland, ME) according to the kit's instructions.

**PBMC.** Blood samples were collected in heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Accu-Paque lymphocytes (Accurate, Westbury, NY). Washed PBMC from each animal were resuspended in phosphate-buffered saline and kept at  $-80^{\circ}\text{C}$  until RNA extraction.

**PCR for PRRSV detection.** PRRSV RNA was extracted from samples as previously described (2). RNA was subjected to reverse transcription-PCR (RT-PCR) using primers capable of directing the amplification of 403- and 150-bp fragments of ORF6 of the PRRSV IA 97-7895 strain, which was used for challenge of the passively transferred animals. This RT-PCR assay has a sensitivity threshold for RNA detection of  $10^3$  RNA molecules (determined by an RNA mimic). The sense and antisense primers for the outer PCR were 5'-AG GTGCTCTTGCGGTTCTCTATT-3' (nucleotides 14424 to 14447) and 5'-GCT TTTCTGCCACCAACACG-3' (nucleotides 2848 to 2869), respectively. The primer sequences for the nested PCR were 5'-CCTCCAGATGCCGTTTGTG CT-3' (nucleotides 14661 to 14682) and 5'-TGCCGTTGACCGTAGTGGAG C-3' (nucleotides 14790 to 14811). The cycling parameters for both PCRs were  $95^{\circ}\text{C}$  for 1 min,  $60^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min in a standard PCR mix with 4 mM MgCl<sub>2</sub> for a total of 30 cycles.

**Experimental design. (i) Experiment 1.** To determine the minimum level of NA protecting against viremia, six randomly selected groups of six piglets each were intraperitoneally instilled with an immunoglobulin stock solution to attain titers of neutralizing antibodies in serum of 1:4, 1:8, 1:16, and 1:32. The attainment of the targeted SVN in the peripheral circulation in each group of pigs was confirmed 24 h after instillation. The SVN measured in each case was 1:4, 1:8, 1:16, and 1:32 for groups 1, 2, 3, and 4, respectively. The animals of group 5 were intraperitoneally instilled with non-neutralizing antibodies (PRRSV-specific antibodies obtained from serum of infected pigs at 21 days postinfection, when ELISA titers are high but no PRRSV-neutralizing activity is yet detectable). The level of anti-PRRSV antibodies, as measured by ELISA S/P, in the latter group was similar to the level (also measured in ELISA S/P units) attained in the group that received NA at the 1:32 level. Group 6 did not receive antibodies. At 24 h after immunoglobulin transfer, the six groups were challenged intranasally with PRRSV IA 97-7895 strain. The rectal temperature was obtained daily until euthanasia. Blood samples were collected immediately before challenge and at 4 and 7 days postinfection (p.i.). Viremia (detected on MARC-145 cells and by RT-PCR in serum) and SVN assays were performed on these samples. At 7 days p.i. the animals were euthanized, tissue samples were collected, and the presence of PRRSV in tissues was detected by infectivity assay on MARC-145 cells.

**(ii) Experiment 2.** For the second experiment two randomly selected groups (1 and 2) of five young piglets each were intraperitoneally instilled with NA to attain a 1:8 titer (as described previously for experiment 1) immediately prior to intranasal challenge with  $10^5$  TCID<sub>50</sub> PRRSV IA 97-7895 1 day later. Animals from group 1 were euthanized at day 7 p.i., and animals from group 2 were euthanized at day 14 p.i. A third group ( $n = 3$ ) was used as an untreated, infected control. Blood was collected before challenge and at days 4, 7, 10, and 14 p.i. and assayed for SVN and viremia. RT-PCR was performed at day 7 p.i. in serum and in buffy coat cells collected by Ficoll gradient centrifugation. Four sentinel animals were used in each group as previously described (19) to determine whether the NA-transferred animals were able to transmit PRRSV to uninfected animals. The first two sentinel pigs were put in contact at 2 days p.i. and kept in contact with the group for seven consecutive days. The sentinel animals were then removed at day 9 p.i. to a clean room for an additional 21 days to verify infection by the development of viremia or seroconversion detected by commercial ELISA. Two additional sentinel pigs were added to the principal group on

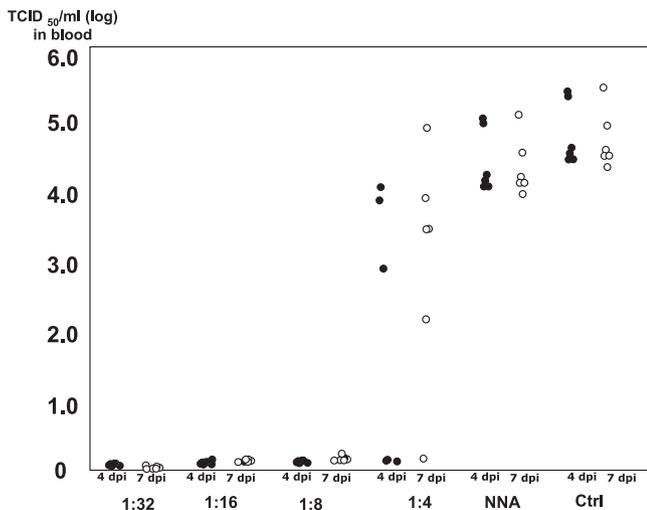


FIG. 1. Correlation between titer of NA and virus in serum detected by viral isolation. Two-week-old pigs were passively transferred with different amounts of a stock of immunoglobulin with NA that attained a range of SVN titers (1:32, 1:16, 1:8, and 1:4). One group received non-neutralizing antibodies (NNA), and another group did not receive antibodies at all (Ctrl). One day later, animals were intranasally challenged with  $5 \times 10^5$  TCID<sub>50</sub> of PRRSV, and the virus levels in serum were determined by viral isolation from MARC-145 cells and RT-PCR (the RT-PCR data is not shown in figure but is mentioned in the text) at days 4 and 7 p.i. as indicated in Materials and Methods.

day 9 p.i. and kept in contact with the infected group until 14 days p.i. These sentinel animals were removed to a clean room and maintained for an additional 21 days.

(iii) **Experiment 3.** In the third experiment a group of six animals received anti-PRRSV immunoglobulins with the goal of attaining an SVN titer in serum of at least 1:32 or more. One day later the principals plus a group of unprotected, infected animals ( $n = 3$ ) were infected with PRRSV IA 97-7895 as described for the previous two experiments. At 7 and 14 days p.i., blood samples were collected and viremia and SVN assays were performed. Viral isolation and RT-PCR from tissues were performed with tissue samples at day 14 p.i. Also in this experiment, sentinel animals were added as described above for experiment 2.

**Statistical analysis.** Statistical significance was determined by using the Student *t* test (see Fig. 2 and 3, showing the amounts of virus on tissues at 7 and 14 days p.i., respectively) and the protective least-significant-difference test (SAS statistical program; SAS Institute, Cary, NC).

**RESULTS**

**The minimum virus-neutralizing antibody titer in serum that protects against viremia is 1:8.** We had previously demonstrated that the passive transfer of an immunoglobulin solution enriched for NA against PRRSV protected pregnant sows against reproductive failure (11). Moreover, this immunoglobulin solution conferred sterilizing immunity in these sows and their offspring. The protection conferred by NA to these sows was characterized by the complete absence of viremia, which we failed to detect, either by isolation in MARC-145 cells or by RT-PCR in serum. Our first goal in this new series of experiments was to determine the minimum neutralizing antibody dose for passive protection using a young pig model. In experiment 1, 2-week-old piglets were injected with different concentrations of PRRSV-NA, which was followed 24 h later by intranasal challenge with PRRSV. Animals receiving an amount of hyperimmune immunoglobulin sufficient to reach a PRRSV-neutralizing titer equal to or higher than 1:8 in serum did not develop cell-free viremia after

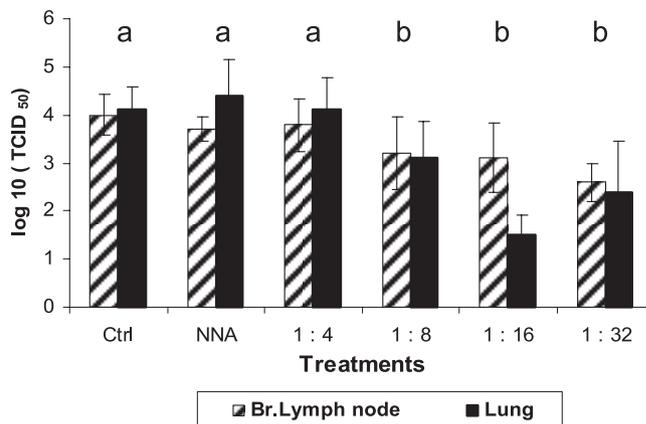


FIG. 2. Correlation between the titer of NA and the viral load in bronchial lymph nodes and lungs. Fifteen-day-old pigs were passively transferred with either neutralizing immunoglobulins to attain titers of 1:4, 1:8, 1:16, and 1:32, non-neutralizing antibodies (NNA), or no antibodies (Ctrl) and challenged with PRRSV 1 day later (experiment 1). The virus titers in the bronchial lymph nodes (▨) and lungs (■) at day 7 p.i. are shown. Columns with different letters are marginally significantly different in the lymph nodes ( $P = 0.06$ ) and significantly different in the lung ( $P < 0.05$  by Student *t* test, 11 df). Ctrl, animals were not transferred with antibodies; NNA, animals were transferred with non-NA. Groups 1:4, 1:8, 1:16, and 1:32 indicate the different groups of animals that, when passively transferred with different quantities of NA, attained peripheral SVN titers of 1:4, 1:8, 1:16, and 1:32, respectively.

viral challenge (Fig. 1). The absence of virus in serum was confirmed by RT-PCR (data not shown). On the other hand, animals with a titer of 1:4 at the time of challenge developed viremia. Virus titers in blood of the latter animals, however, were lower ( $P < 0.05$ , Student *t* test) than those in the infected controls. Moreover, three of three animals from this group did not show virus in blood 4 days p.i., indicating that the presence of some level of NA in blood can block viremia for at least a short time p.i. In addition, a group of animals receiving an equivalent dose of non-PRRSV-NA (i.e., immunoglobulins collected from PRRSV-infected animals within the first 3 weeks p.i.) showed titers of virus in blood similar to those seen in unprotected, infected controls, indicating that these immunoglobulin solutions containing the early appearing, non-PRRSV-NA were not protective against experimental challenge with PRRSV (Fig. 1).

The body temperature of the different groups in experiment 1, except for group 5, did not rise significantly during the 7 days of observation except for the last day of observation (day 7 p.i.), when a slight increase in the body temperature was observed in the groups 1, 2, and 6 (1:4, 1:8, and the “no antibodies” group). Group 5, receiving non-neutralizing antibody solutions, became hyperthermic between days 1 and 7 p.i. (data not shown).

**A titer of PRRSV-NA of 1:8 does not protect against replication of PRRSV in the primary site of viral replication and draining lymph nodes of challenged pigs.** Even though no virus was detected in the sera of animals exhibiting a titer equal to or higher than 1:8 (Fig. 1), PRRSV was detected in the lungs and draining bronchial lymph node of these animals when necropsied at day 7 p.i. (Fig. 2). It should be noted, however, that the amount of virus was significantly lower in lungs ( $P < 0.05$  by Student *t* test) and marginally significant in bronchial

TABLE 1. Association between titer of NA in blood and absence of viremia after challenge<sup>a</sup>

ID no.	SVN at <sup>b</sup> :		Viremia at day 7 p.i. <sup>c</sup>	SVN at day 14 p.i. <sup>b</sup>	Viremia at day 14 p.i. <sup>c</sup>	RT-PCR result <sup>d</sup>		Time necropsy was conducted (days p.i.)
	Day 0	Day 7 p.i.				Serum	Buffy coat	
61	1:8	1:4	Neg	N/A	N/A	Neg	Neg	7
63	1:8	1:4	Neg	N/A	N/A	Neg	Pos	7
64	1:8	1:8	Neg	N/A	N/A	Neg	Pos	7
66	1:8	1:4	Neg	N/A	N/A	Neg	Pos	7
73	1:8	1:2	10 <sup>2.6</sup>	N/A	N/A	Pos	Pos	7
44	1:8	1:8	Neg	1:4	10 <sup>3.0</sup>	Pos	ND	14
45	1:8	1:4	Neg	1:4	Neg	ND	ND	14
46	1:8	1:4	Neg	1:2	10 <sup>2.6</sup>	Pos	ND	14
47	1:8	1:8	Neg	1:4	Neg	ND	ND	14
48	1:8	1:8	Neg	1:4	Neg	ND	ND	14
67	Neg	Neg	10 <sup>5.2</sup>	Neg	ND	Pos	ND	14
68	Neg	Neg	10 <sup>4.0</sup>	Neg	ND	Pos	ND	14
70	Neg	Neg	10 <sup>3.5</sup>	Neg	ND	Pos	ND	14
71	Neg	Neg	10 <sup>3.6</sup>	Neg	ND	Pos	ND	14
74	Neg	Neg	10 <sup>4.6</sup>	Neg	ND	Pos	ND	14

<sup>a</sup> A group of 15-day-old pigs (*n* = 10) were transferred with NA to reach an SVN titer of 1:8 and subsequently challenged with PRRSV. A control group (*n* = 5) did not receive antibodies and was infected with PRRSV.

<sup>b</sup> Titer of PRRSV-NA transferred by passive transfer. The titer of NA (SVN) was determined at days 7 and 14 p.i. Neg, negative; N/A, not applicable.

<sup>c</sup> Viremia was determined at days 7 and 14 p.i. Values are TCID<sub>50</sub>/ml of serum. Neg, infectivity was below the threshold of detection (<10 TCID<sub>50</sub>/ml).

<sup>d</sup> RT-PCR was performed in PBMC in the animals necropsied at day 7 p.i. and in serum in the other necropsied animals.

lymph nodes (*P* = 0.06) in animals of groups with SVN titers higher than 1:8 at the time of challenge compared to the control group (Fig. 2).

**A titer of PRRSV-NA of 1:8 does not prevent local replication in lungs and dissemination of infection to other peripheral lymphoid tissues.** To further characterize the protective significance of the minimum level of PRRSV-NA necessary to protect against viremia, systemic dissemination and shedding to sentinels, we launched experiment 2. We used another two groups of pigs passively transferred to attain a serum PRRSV-neutralizing titer of 1:8 at the time of challenge, and the presence of virus in blood was monitored for 1 week in one group and for 2 weeks in the second group. Table 1 shows that these animals presented viremia when virus-neutralizing titers fall below 1:8, thus confirming our previous observation indicating a correlation between the titer of NA in blood and protection

against viremia after challenge. There is a clear correlation between the presence of NA in the circulation at a given threshold (1:8) and the blocking of the spread of PRRSV in plasma. Above the threshold of 1:8 of NA in serum, cell-free viremia (measured in this case in serum) is not detected.

In experiment 2, although no virus was detected in serum of animals with a titer of 1:8 (Table 1), PRRSV was detected in bronchial lymph node and lungs of all nonviremic animals necropsied at day 7 p.i. (data not shown), which is consistent with the prior results of experiment 1. Figure 3 shows the infectious PRRSV load in different tissues in all of the animals sacrificed at day 14 p.i. PRRSV has disseminated to the lymph nodes and tonsils of these nonviremic animals. However, the amount of virus was lower (*P* < 0.05) in animals with an SVN titer of 1:8 at the time of challenge. The presence of viral RNA in PBMC (buffy coat) measured at 7 days p.i. (Table 1) is

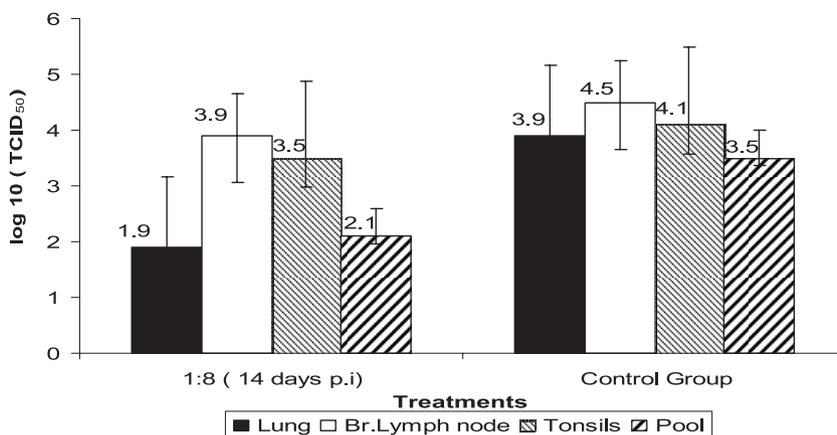


FIG. 3. Dissemination of PRRSV in PRRSV immunoglobulin-transferred pigs. Two-week-old pigs were transferred with serum to reach a titer of 1:8 and challenged 1 day later with a dose of 10<sup>5</sup> TCID<sub>50</sub>/ml (experiment 2). At 14 days p.i., five animals were sacrificed, and tissues were collected to determine the virus load in each tissue (10<sup>5</sup> TCID<sub>50</sub>/g of tissue). The amount of virus was lower (*P* < 0.05 [Student *t* test], 9 df) in pools from animals with a titer of 1:8.

TABLE 2. Dissemination of PRRSV in tissues in pigs with a titer of 1:32 of NA in serum<sup>a</sup>

Pig ID no.	Titer at day:					Viral load in tissues at day 14 <sup>b</sup>			
	0 (NA)	7 p.i. (NA)	7 p.i. (viremia) <sup>b</sup>	14 p.i. (NA)	14 p.i. (viremia) <sup>b</sup>	Lung	Lymph node	Tonsils	Pool
26	1:32	1:16	—	1:8	—	—	—	—	—
27	1:32	1:16	—	1:8	—	—	10 <sup>0.6</sup>	10 <sup>2.6</sup>	—
28	1:32	1:16	—	1:8	—	—	—	—	—
29	1:32	1:16	—	1:8	—	—	—	—	—
32	1:16	1:8	—	1:4	—	10 <sup>4.6</sup>	10 <sup>4.0</sup>	10 <sup>4.0</sup>	10 <sup>2.5</sup>
33	1:16	1:16	—	1:8	—	10 <sup>3.0</sup>	10 <sup>2.0</sup>	10 <sup>2.6</sup>	10 <sup>2.5</sup>
37	—	—	10 <sup>5.6</sup>	—	10 <sup>4.3</sup>	ND	ND	ND	ND
38	—	—	10 <sup>5.5</sup>	—	10 <sup>4.0</sup>	ND	ND	ND	ND
34	—	—	10 <sup>5.0</sup>	—	10 <sup>4.3</sup>	ND	ND	ND	ND

<sup>a</sup> A group (*n* = 6) of 15-day-old piglets received NA to attain a titer of 1:32 before challenge with PRRSV (experiment 3). Three animals were used as controls (non-serum transfer). At days 7 and 14 p.i., virus titers in serum were determined. At day 14 day p.i. the animals (except controls) were necropsied, and lung, lymph node, tonsils, and a pool of lymph nodes were collected and prepared as described in Materials and Methods. —, undetectable viremia.

<sup>b</sup> Values are TCID<sub>50</sub>/ml of serum. ND, not done.

consistent with a cell-associated pattern of viral spread by cells circulating in blood. The systemic dissemination of the infection in these nonviremic animals explains the readily demonstrated transmission to sentinel pigs in experiments 2 and 3 as described below.

**Passive transfer of PRRSV-NA at a higher concentration (SVN titer of 1:32) induces full protection, but only in some of the young pigs.** A new experiment (experiment 3) was aimed at determining the minimum amount of NA needed to protect against viral spread to organs, thus preventing transmission to contacts. Six 15-day-old pigs were then transferred with our immunoglobulin stock to attain a titer of 1:32 and challenged with PRRSV (PRRSV IA 97-7895, 10<sup>5</sup> TCID<sub>50</sub>), and the presence of virus in blood and several organs was studied. Three additional animals not treated with antibodies were used as unprotected, challenged controls. In addition, the principal and control animal groups were put in contact with two sets each of two sentinel pigs (one sentinel pig colodged with principals from day 2 p.i. to day 9 p.i. and was then rotated to be replaced by a second sentinel animal that was in contact with the principal animals from days 9 to 14 p.i.) as previously described for experiment 2. As shown in Table 2, no viremia was detected in any of the animals exhibiting an SVN titer of 1:16 to 1:32 since the start of the experiment up to 14 days p.i. At necropsy (14 days p.i.), three of the principals exhibited viral replication in peripheral tissues, as evidenced by sizable viral load in lungs, lymph nodes, and tonsils (Table 2). Interestingly, the remaining three nonviremic principals did not exhibit any detectable viral load in peripheral tissues, thus suggesting that sterilizing immunity may have been achieved in these cases. Thus, the presence of more elevated concentrations of NA in blood may protect against infection, as well as prevent the dissemination of the virus. As can be seen below, the full protective effect in three of the six principal animals determined that the transmission to contacts was significantly delayed, although not prevented (see below).

**Shedding and transmission of PRRSV occurs in the absence of viremia.** The definitive significance of the protective effect of PRRSV-NA is given by the ability of passive transfer of NA to block transmission of the infection to contact sentinel animals. To determine whether the amount of circulating NA correlates with blocking of shedding and transmission of virus, PRRSV-

free sentinel pigs were lodged during experiments 2 and 3 with animals that had received NA and subsequently been challenged with PRRSV. At 9 days p.i. both sentinel animals lodged with nonviremic animals having an SVN titer of 1:8 were scored as positively infected by viremia and seroconversion (Table 3); consequently, the second set of sentinel animals maintained in contact with the same “donor” group between days 9 and 14 p.i. were also scored as infected. Likewise, the control group consistently transmitted the infection throughout the entire period of 14 days (Table 3). In contrast, despite contact, the group that had received a higher dose of passive NA (to titers of 1:16 to 1:32) (Table 2) remained uninfected for the first 9 days p.i. (Table 3), and only during the last 5 days of contact did the transmission take place (Table 3). The overall results clearly indicate that the lack of viremia in the challenged (donor) animal does not correlate with the ability to shed and transmit PRRSV. Likewise, the results show a significant delay in the transmission of infection when higher titer of antibodies were transferred, which is in agreement with the fact that only half (three of six) of the “donor” animals had actually been infected (Table 2), thus reducing the overall load

TABLE 3. Shedding of virus from nonviremic pigs to contact animals<sup>a</sup>

SVN	Viremia in donors during contact period <sup>b</sup>	Transmission to sentinel pigs <sup>c</sup>	
		2 to 9 days p.i.	9 to 14 days p.i.
1:8 (expt 2)	—	+	+
1:16–1:32 (expt 3)	—	—	+
Not protected/infection controls	+	+	+

<sup>a</sup> Fifteen-day-old pigs were transferred with antibodies to attain a titer of 1:8 (experiment 2) or 1:16 to 1:32 (experiment 3). One day later the animals were challenged with PRRSV. Two sentinel animals per group were lodged with the challenged animals (“donors”) from days 2 to 9 after infection, removed for isolation for 14 days, replaced with another two new sentinel pigs that were lodged with the donor animals from days 9 to 14 p.i., and then kept in an isolation room for 14 more days.

<sup>b</sup> Virus in blood was determined at days 9 and 14 p.i.

<sup>c</sup> The presence of antibodies by PRRSV-ELISA was determined at days 16 and 21.

of virus shed and consequently delaying the transmission to uninfected contacts.

## DISCUSSION

Previously, we demonstrated that NA is a parameter of protection against PRRSV infection in pregnant sows (11). These results prompted us to determine the minimal dose of NA that will protect pigs from infection in an attempt to quantify the significance of this parameter of protection. With this aim, we performed a first experiment using a stock of NA administered to 2-week-old pigs in different quantities to attain different SVN titers in blood. The results of this experiment indicated that animals with titers of 1:8 or more did not develop viremia, whereas animals with a titer of 1:4 or less did develop viremia (Fig. 1). This experiment confirms the importance of NA as a parameter of protection against PRRSV and demonstrates that there is a threshold of neutralizing antibody titer (seemingly 1:8) above which animals are protected from viremia after challenge.

In addition, an important conclusion that results from experiment 1 is that immunoglobulin fractions containing NA are effective in clearing viremia and protecting pigs. No evidence of protection was exhibited by the group instilled with PRRSV-specific but non-neutralizing antibodies. This observation is important since it confirms that the protective effect of the passive transfer of immunoglobulins (i.e., the clearance of viremia) would be related to the neutralizing antibody fraction and that the early non-neutralizing antibodies that determine the seroconversion to PRRSV detectable by use of the commercial ELISA kits are not responsible for protection. On the contrary, the group of animals receiving non-neutralizing antibodies also showed significantly higher temperatures during the length of experiment 1 (7 days) and a tendency to develop interstitial pneumonia that was detected upon necropsy (data not shown). This would suggest a role for non-neutralizing antibodies in inducing inflammation and enhancement of infection as has been described previously (22). Alternatively, it is possible that the exacerbating effect on disease symptoms and pathology affecting the group treated with non-neutralizing PRRSV antibodies may have been caused by proinflammatory mediators that are systemically abundant in acutely PRRSV-infected pigs (17, 18). The PRRSV-specific non-neutralizing immunoglobulin solution, which was harvested from the sera of acutely infected pigs, may have carried these molecules as well.

From the results of experiments 1, 2, and 3, a major conclusion that can be drawn is that the transfer of PRRSV-NA at a moderate level may mitigate clinical symptoms but not necessarily clear infection in young weaned pigs. It was observed that a reduced viral load in tissues and a clearance of the viremia were observed in animals that had received NA at a titer of at least 1:8. However, the presence of anti-PRRSV-NA in serum at a titer of 1:8 interferes with the development of viremia but does not prevent peripheral tissue seeding and transmission to contact animals. Under the maximal concentration of NA attainable by passive transfer in our experimental system (1:32, Table 2), an apparent sterilizing immunity was achieved in at least three of six animals, which suggests that the full protective effect may be related to a threshold of replicating virus to be overcome by antibodies in young animals. The virus was de-

tected in the peripheral organs of the other animal exhibiting a titer of 1:32 (although at a relatively lower viral load level). Considering that the SVN assay provides a semiquantitative measurement of neutralizing antibodies, it is possible that this animal corresponds to a borderline level of protection. In contrast, the other two animals in which a titer of 1:32 was not reached were not protected from infection dissemination. Such a threshold could be higher in young pigs than in sows in which sterilizing immunity was attained in individuals at lower concentrations of neutralizing antibody transfer (11). A possible reason for the occurrence of this higher threshold of NA protection being required in young pigs may be based on the age dependence of the permissiveness of swine macrophages to PRRSV replication. It has been reported that the susceptibility of the macrophages of young pigs is higher than the macrophages of adult swine (10). It is conceivable that the overall infectious yield and load in young pigs infected with PRRSV is higher than in adults, thus requiring higher concentrations of NA to achieve protection in young pigs.

An apparent paradox was observed in our experiments: animals that had been treated with NA and had then achieved a nonviremic status (based on the lack of detection of either infectious PRRSV or RT-PCR signal in serum) not only prevented dissemination of infection to and replication of PRRSV in peripheral tissues but also were able to shed infectious PRRSV and infect sentinel animals (Table 3). These results indicate that pigs that are scored as "nonviremic" according to the routine diagnostic paradigms in use (i.e., animals that do not have free virus in blood, as detected in serum, which is the sample commonly submitted to a diagnostic lab) still can excrete PRRSV in sufficient doses to infect other animals. Although the sera of our nonviremic animals contained no PRRSV RNA, RT-PCR conducted in buffy coat cells of the same animals indicated the presence of PRRSV in PBMC (Table 1). This suggests that, in addition to cell-free virus in blood, the PRRSV pathogenesis and dissemination is based on the spread of infection and persistence through an intracellular route. Our observation is in agreement with previous reports indicating the presence of minute amounts of PRRSV in buffy coat cells of infected animals (3, 16) and gives support to previous work of other laboratory suggesting the possible occurrence of transmission to contact controls, from persistently infected sows, in the absence of detectable viremia in the index sows (4). Our experiments show a high viral load in the tonsils in nonviremic pigs transferred with serum to reach an SVN titer of 1:8 (Fig. 3). Tonsils are known to be as a site of persistent infection (2, 3); thus, this tissue could play a major role in the transmission to sentinels (Table 3). The cell-to-cell dissemination of PRRSV and spread through mononuclear cells might also help to explain the differences in pathogenesis and susceptibility to passive antibody protection that we observed between adult sows and young pigs. It is likely that in our previous report of passive protection in sows (11), the level of antibodies injected in the sows was sufficient to completely preclude the transplacental infection, just by completely clearing the circulation of cell-free PRRSV. Our results suggest that the blood mononuclear cells can also disseminate the infection. Therefore, a complete, more accurate *in vivo* assessment of the PRRSV infection status of a pig by RT-PCR can only be

obtained from an analysis of buffy coat cells in addition to serum.

In conclusion, these results identify certain threshold of SVN titer ( $\geq 1:8$ ) at which the dissemination of PRRSV in the serum of a young pig would be blocked, as well as a higher threshold ( $\geq 1:32$ ) that could imply complete protection of the animal from PRRSV infection. Finally, the results also indicate that the absence of PRRSV in the sera of young pigs does not necessarily imply complete freedom from PRRSV infection.

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