Antigen-Specific B-Cell Responses to Porcine Reproductive and Respiratory Syndrome Virus Infection[▽]

Prasad Mulupuri,¹† Jeffrey J. Zimmerman,² Joseph Hermann,² Craig R. Johnson,¹ Jean Paul Cano,³ Wanqin Yu,¹ Scott A. Dee,³ and Michael P. Murtaugh^{1*}

Departments of Veterinary and Biomedical Sciences¹ and Veterinary Population Medicine,³ University of Minnesota, St. Paul, Minnesota, and Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, Iowa²

Received 10 May 2007/Accepted 4 October 2007

Porcine reproductive and respiratory syndrome virus (PRRSV) causes an acute, viremic infection of 4 to 6 weeks, followed by a persistent infection lasting for several months. We characterized antibody and B-cell responses to viral proteins in acute and persistent infection to better understand the immunological basis of the prolonged infection. The humoral immune response to PRRSV was robust overall and varied among individual viral proteins, with the important exception of a delayed and relatively weak response to envelope glycoprotein 5 (GP5). Memory B cells were in secondary lymphoid organs, not in bone marrow or Peyer's patches, in contrast to the case for many mammalian species. Potent anti-PRRSV memory responses were elicited to recall antigen *in vitro*, even though a second infection did not increase the B-cell response *in vivo*, suggesting that productive reinfection does not occur *in vivo*. Antibody titers to several viral proteins decline over time, even though abundant antigen is known to be present in lymphoid tissues, possibly indicating ineffective antigen presentation. The appearance of antibodies to GP5 is delayed relative to the resolution of viremia, suggesting that anti-GP5 antibodies are not crucial for resolving viremia. Lastly, viral infection had no immunosuppressive effect on the humoral response to a second, unrelated antigen. Taking these data together, the active effector and memory B-cell responses to PRRSV are robust, and over time the humoral immune response to PRRSV is effective. However, the delayed response against GP5 early in infection may contribute to the prolonged acute infection and the establishment of persistence.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-sense single-stranded RNA virus in the family *Arteriviridae*. It is a recent pathogen of swine that emerged in the late 1980s as the causative agent of a severe reproductive disease of pregnant sows and a respiratory disease of young and growing pigs (8, 18). Swine are the only known host of PRRSV, and myeloid cells, particularly macrophages and dendritic cells, are the primary permissive cells.

Various features of PRRSV infection and the ensuing immune response suggest that humoral immunity is aberrant or dysregulated. The acute, viremic infection lasts for 4 to 6 weeks and is followed by a persistent period in lymphoid tissues that lasts for several months before complete resolution of infection (2, 5, 7, 11, 32, 46, 47, 64). The T-cell response is weak and variable and appears transiently at 4 to 8 weeks after infection (35, 67). Anti-PRRSV antibody responses are detected at 7 to 10 days after infection (21, 33, 42), but the appearance of neutralizing antibodies is delayed to 21 to 28 days after infection (43), at which point viremia may no longer be detectable (48). There often is no anamnestic antibody response after rechallenge of immune animals (29). It also has been reported that B-cell responses in PRRSV infection are aberrant and nonspecific in neonatal pigs (41). These observations are con-

sistent with the failure of vaccine-based approaches to effectively control the disease in swine-growing regions throughout the world, even though efficacy is readily demonstrated in controlled trials (29, 36–38, 45, 50).

Humoral immunity to PRRSV typically has been based on serological assessments of raised antibody levels using an enzyme-linked immunosorbent assay (ELISA) that is licensed as a positive/negative herd test (6, 16, 63). However, to better understand the antibody response of swine to PRRSV and whether prolonged viremia and persistent infection are associated with its dysregulation, we sought to obtain more specific information about local and systemic immune responses than traditional methods allow. Here, we examined the antibody response to specific viral polypeptides throughout the course of primary and persistent infection. We also examined both plasma cell responses in lymphoid tissues, since serum antibody levels are determined by actively secreting plasma cells, and memory B-cell responses, because of their role in protective immunity. In acute infection, we found a robust humoral immune response that is strongest in spleen and draining lymph node, and in persistent infection a robust antiviral B-cell response was most pronounced in tonsil tissue. In contrast to their responses in mice, long-lived plasma cell and memory cell responses in pigs were not localized to bone marrow but were present in secondary lymphoid tissues. Potent memory responses were routinely elicited *in vitro*, even though a second infection did not increase the B-cell response *in vivo*. In addition, there was no evidence that PRRSV infection suppressed the antibody response to an unrelated antigen.

* Corresponding author. Mailing address: Department of Veterinary & Biomedical Sciences, University of Minnesota, 1971 Commonwealth Avenue, St. Paul, MN 55108. Phone: (612) 625-6735. Fax: (612) 625-5203. E-mail: murta001@umn.edu.

† Present address: Immunology and Molecular Pathogenesis Graduate Program, Emory University, Atlanta, GA 30322.

[▽] Published ahead of print on 17 October 2007.

MATERIALS AND METHODS

Animal procedures and housing. Conventional cross-bred commercial pigs of various ages that were negative for PRRSV by IDEXX ELISA were used as follows.

Twenty-two weaned pigs, 2 to 3 weeks of age, were maintained at the Livestock Infectious Disease Isolation Facility, Iowa State University, Ames, IA. Animals were assigned to four treatment groups at random and observed daily. Rectal temperatures and clinical scores were recorded every other day. After 1 week of acclimatization, four pigs were inoculated intramuscularly with 2 ml of 10^5 50% tissue culture infectious doses (TCID₅₀) of the VR2332 isolate of PRRSV, four pigs were inoculated intramuscularly with keyhole limpet hemocyanin (KLH) emulsion, 12 pigs were inoculated with both PRRSV and KLH, and two pigs were mock inoculated with 2 ml of saline diluent (PRRSV control) and incomplete Freund's adjuvant (KLH control). KLH emulsions were prepared by mixing equal volumes of KLH (2 mg in 1 ml saline; Sigma Chemical Co., St. Louis, MO) and Freund's incomplete adjuvant (Sigma Chemical Co.) in Micromate interchangeable syringes (Popper and Sons Inc., New Hyde Park, NY). Animals were immunized intramuscularly in the ham and boosted on day 14 with 50 µg of KLH emulsion.

The design was replicated one time for a total of 44 animals. Whole blood was collected in EDTA tubes and serum samples in clot tubes at weekly intervals. At 27 and 120 days after infection, animals were sacrificed and samples of spleen, tonsil, inguinal lymph node (ILN), sternal lymph node (SLN), and femur were collected in RPMI 1640 medium containing 10 µg/ml of gentamicin (GIBCO BRL, Grand Island, NY).

B-cell responses in persistent infection were characterized by inoculating 3- to 4-week-old conventional pigs, maintained at the Livestock Infectious Disease Isolation Facility, Iowa State University, Ames, IA. Animals were assigned to treatment groups at random and, after 1 week, were inoculated intramuscularly with 2 ml of 10^4 TCID₅₀ per ml of PRRSV strain VR2332. Both PRRSV-infected and uninfected animals were sacrificed at postinoculation days 98, 112, 147, 175, and 203 for the isolation of mononuclear cells (MNCs) from various lymphoid tissues.

To further analyze B-cell responses in lymphoid tissues, 6- to 8-week-old PRRSV naïve pigs were housed at the Swine Disease Eradication Center (University of Minnesota) research farm. Animals were assigned to treatment and control groups at random. Animals were inoculated intranasally with 2 ml of cell culture fluid containing 3×10^4 TCID₅₀ of PRRSV (MN-30100 field isolate) alone, with strain MN-30100 at day 0 and a boost with 2 ml of Ingelvac PRRS MLV vaccine (Boehringer Ingelheim Vetmedica) on day 7, or with 2 ml of saline diluent only. Animals were sacrificed to isolate MNCs from various lymphoid tissues at 37 days after the first infection.

Pigs at 4 weeks and 2 years of age were maintained at Vet Resources, Ames, IA. After acclimatization, animals were infected with the virulent PRRSV strain JA142 or were given diluent alone. Pigs were sacrificed 63 days after infection, and bone marrow as well as ILN and SLN tissues were harvested for fluorescence-activated cell sorter analysis.

All of the experiments were performed under IACUC-approved animal protocols at the University of Minnesota or Iowa State University.

Viruses and viral quantitation. Strains of PRRSV used, doses, and routes of inoculation used in each experiment are described separately in Results. Virulent PRRSV isolates VR2332, MN-30100, and JA142 and the vaccine Ingelvac MLV were used in this study. A titrated stock of PRRSV strain MN-30100 (5×10^5 TCID₅₀) was used to generate a quantitative one-step real-time reverse transcriptase PCR (qRT-PCR) standard curve for use as a positive control. RNA was extracted from 200 µl of serum using a Nucleospin II RNA isolation kit (BD Biosciences, San Jose, CA), eluted in 50 µl of RNase-free water, and stored at -80°C . For qRT-PCR assays, samples were dried in a Savant SpeedVac (GMI, Inc., Ramsey, MN) and resuspended in 5 µl of RNase-free water. Primers and a dual-labeled 6-carboxyfluorescein-6-carboxytetramethylrhodamine probe were designed from the *orf7* 3'-untranslated region of PRRSV (58). PRRSV RNA reverse transcription and PCR were performed on infected samples, a water-only negative control, and positive standards in duplicate in a single one-step TaqMan reaction mix (Perkin-Elmer Applied Biosystems, Foster City, CA). Each 20-µl reaction mixture contained 10.0 µl TaqMan One-Step Mastermix, 0.2 µl enzyme mix (catalog no. 4309169; Perkin-Elmer Applied Biosystems), 0.3 µM forward primer (5'-TGATGGGCTGGCATTCTT-3'), 0.3 µM reverse primer (5'-ACACGGTGGCCTAATTG-3'), 0.2 µM dual-labeled probe (6-carboxyfluorescein-TGTGGTGAATGGCACTGATTGACA-6-carboxytetramethylrhodamine), 2 µl of extracted RNA, and 5.6 µl RNase-free water. The qRT-PCR was carried out with an ABI 7500 sequence detection system (Perkin-Elmer Applied Biosystems). Thermocycler conditions were 50°C for 30 min followed by 95°C for 10

min, then 45 cycles of 95°C for 15 s and 60°C for 1 min. The detection limit was approximately 10 TCID₅₀/ml of stock virus.

Isolation of MNCs from blood and lymphoid tissues. Animals were sacrificed by intravenous administration of Beuthanesia-D solution (Schering Plough Animal Health, Union, NJ). All of the tissue and cell culture reagents were obtained from Mediatech except as noted. Blood samples were collected in EDTA tubes (Vacutainer; Sherwood Medical, St. Louis, MO). Spleen, tonsil, ILN, SLN, and femur samples were collected in RPMI 1640 containing 10 µg/ml of gentamicin (GIBCO BRL, Grand Island, NY). MNCs from spleen, tonsil, ILN, and SLN samples were isolated by mechanical disruption using 80-mesh screens in a cell strainer (Collector, St. Petersburg, FL). Bone marrow cells were isolated by being flushed with sterile phosphate-buffered saline (PBS). Peripheral blood MNCs (PBMC) were isolated by density gradient centrifugation of blood diluted 1:2 with sterile PBS on lymphocyte separation medium (ICN Biomedicals, Aurora, OH). PBMC and isolated cells from other lymphoid tissues were filtered through 70-µm cell strainers (BD Falcon, Bedford, MA), washed with PBS, and freed of red blood cells by hypotonic water lysis. Cells were resuspended in RPMI complete medium containing 5% fetal bovine serum (Sigma Chemical Co.), 2 mM L-glutamine, 0.02 mM nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 U), streptomycin (100 µg/ml) (GIBCO BRL), 50 mM HEPES, pH 7.2, and gentamicin (20 µg/ml) (GIBCO BRL). Cell viability was confirmed by trypan blue exclusion, and cells were counted using a hemocytometer.

Antigens used for ELISA and enzyme-linked immunospot (ELISPOT) assays. Recombinant PRRSV nucleocapsid (N), envelope glycoprotein 5 ectodomain (GP5 5' total), envelope glycoprotein 5 endodomain (GP5 3'), and nonstructural protein 2 (nsp2) proteins and polypeptides were prepared by PCR amplification and cloning from cDNA of PRRSV strain VR2332 (4, 31). The GP5 ectodomain contains 52 amino acids external to the viral envelope, is highly polymorphic, and includes the putative primary neutralization epitope (4, 21, 53). The GP5 endodomain contains the 72 carboxyl-terminal amino acids within the viral envelope, is highly conserved, and is recognized by serum from pigs infected with a wide variety of PRRSV isolates (unpublished data). DNA sequences were subcloned into expression plasmid pET24b as fusion proteins containing an amino-terminal myc tag and a carboxyl-terminal 6× histidine tag and were expressed in BL21(DE3)-RP cells (Stratagene, La Jolla, CA) (4, 31). Proteins were expressed and purified using a modification of the Qiagen Ni-nitrilotriacetic acid agarose affinity column purification method for denatured protein isolation.

Denatured proteins were dialyzed overnight in 0.1 M Tris-HCl, pH 8.0, 6 M guanidine HCl, 2 mM EDTA at 4°C and were adjusted to a concentration of 3 mg/ml. Dithiothreitol was added to 300 mM, and the solution was filtered through a 0.45-µm membrane. Reduced protein was added into refolding buffer, filtered (0.22 µm), and stirred overnight at 4°C . The purified protein was concentrated by tangential flow filtration (Pellicon XL Ultracel PLC 5 KD; Millipore) and dialyzed against 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. Protein concentrations were analyzed by Bradford assays (Bio-Rad, Hercules, CA). Purified protein solutions were stored at -80°C . For studying memory B-cell responses, a cocktail containing equal amounts of nsp2, N, and GP5 3' was prepared, dialyzed overnight in RPMI medium, and stored at -80°C .

ELISPOT assay for antigen-specific ASC. ELISPOT assays were performed on the day of MNC isolation to enumerate antigen-specific plasma cells actively secreting antibodies *in vivo*. Briefly, nitrocellulose-based 96-well microtiter plates (Millipore, Bedford, MA) were pretreated with 70% ethanol for 60 s and were washed three times with sterile water. The wells were coated with 500 ng/well of recombinant KLH, nsp2, N, or GP5 3' polypeptide separately to measure antigen-specific B-cell responses. After overnight incubation at 4°C , wells were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) for 1 h at 37°C . Plates were washed three times with PBS, and MNCs were plated at three fivefold dilutions in duplicate wells, starting with 5×10^5 cells/well. After incubation for 12 to 16 h at 37°C in a 5% CO₂ incubator, ELISPOT plates were washed three times with PBS and four times with PBS containing 0.05% Tween 20 (Fisher Scientific, Pittsburgh, PA). To each well we added 100 µl of horseradish peroxidase-labeled affinity-purified goat anti-pig antibody specific for immunoglobulin G (IgG) (KPL, Gaithersburg, MD), and Bethyl Laboratories, Montgomery, TX), IgM, or IgA (both from Bethyl Laboratories) diluted 1:2,000 in PBS-Tween and containing 1% BSA for 2 h at 37°C . Plates were washed three times in PBS and developed with 3-amino-9-ethylcarbazole (Sigma Chemical Co). Spot patterns were analyzed using the immunospot image analyzer (Cellular Technology, Cleveland, OH) in the laboratory of Bernhard Hering, Department of Surgery, University of Minnesota. Data are presented as the mean numbers of antigen-specific antibody-secreting cells (ASC) per 10^6 MNCs from duplicate wells of each sample. The assay was performed as described here for all samples, including analysis at three fivefold dilutions of the cell number so that at least one dilution was within the counting range of the image analyzer.

Memory B-cell response assay. Memory B-cell responses were assessed by *in vitro* stimulation of MNCs with recombinant PRRSV antigens and mitogens for 4 days in cell culture, followed by measuring antigen-specific, actively secreting B cells. The duration of antigen stimulation and the dose of antigen were optimized and standardized as previously described and were based on the culture conditions that yielded the greatest number of ASC in the ELISPOT assay (19). MNCs were plated in 24-well cell culture plates at 2×10^6 cells/well in 2 ml of RPMI complete medium supplemented with phytohemagglutinin (2.5 μ g/ml; Sigma Chemical Co.), pokeweed mitogen (2.5 μ g/ml; Sigma Chemical Co.), and 1.67 μ g/ml each of nsp2, N, and GP5 3'. Cells from each lymphoid tissue from PRRSV-infected and uninfected pigs were cultured in 12- or 24-well plates at 37°C for 4 days in a 5% CO₂ incubator. On the second, third, and fourth days of incubation, 0.5 ml of RPMI complete medium was added to each well. On the fifth day, cells were collected, washed three times with PBS, resuspended in RPMI complete medium, counted, and plated in antigen-coated ELISPOT plates using three fivefold dilutions in duplicate wells, starting with 5×10^5 cells/well as described above. Memory B-cell responses were quantified based on the number of spots obtained in response to restimulation.

Protein-specific ELISA. Microtiter plates were coated overnight with 100 ng/well of KLH, nsp2, or N or 200 ng/well of either GP5 5' total or GP5 3' in carbonate buffer (pH 9.6). Plates were washed and blocked with 5% nonfat dry milk (pH 9.6) in PBS containing 0.05% Tween 20 for 1 h. Serum samples were diluted 1:1,000, and 100 μ l of each sample was applied to duplicate wells on ELISA plates (Corning, Acton, MA) for 1.5 h. Plates were washed and incubated for 1 h with horseradish peroxidase-conjugated goat-anti-pig antibody, either anti-IgM at 1:1,500 dilution (Bethyl Laboratories), anti-IgG at 1:5,000 (KPL), or anti-IgA at 1:5,000 (Bethyl Laboratories). Plates were washed, and color development was performed with 100 μ l of tetramethylbenzidine substrate (KPL). Reactions were stopped with 100 μ l of 1 M phosphoric acid, and plates were read at 450 nm on a Molecular Devices ThermoMax plate reader (Sunnyvale, CA).

Statistical analysis. Statistical analysis was performed using nonparametric one-way analysis of variance in GraphPad Prism software (San Diego, CA). Multiple pair-wise comparisons were performed using MacAnova (www.stat.umn.edu/macanova/).

RESULTS

Characterization of the humoral antibody response to PRRSV infection. We characterized the humoral response to PRRSV by determining specific IgM and IgG antibody responses in serum to individual structural and nonstructural proteins of PRRSV. We compared these results to the antibody response to KLH, a common protein antigen, administered in the presence or absence of viral infection. The IgM response to KLH was present at day 3 (the earliest time point examined), peaked at day 7, and declined thereafter (Fig. 1A). The pattern of response to KLH was the same irrespective of concurrent PRRSV infection. A similar IgM response was observed to PRRSV nsp2 and N; both showed a peak at day 7 and a decline to low levels by day 21 (Fig. 1B and C). In striking contrast, the IgM response to GP5 endodomain epitopes (GP5 3') was inapparent; IgM antibodies were not significantly increased at any time point up to 56 days (Fig. 1D). To determine if an IgM response to another region of GP5 was present, an ELISA was performed with GP5 ectodomains (GP5 5' total), which contain the putative neutralization epitopes. IgM antibody levels were substantially delayed by 2 weeks, peaked at 21 to 28 days of infection, and even persisted to about 50 days (Fig. 1E).

IgG antibody titers to KLH were robust at day 14 and increased steadily to a peak at day 91 (Fig. 1F). IgG specific to PRRSV nsp2 was present at day 7, increased to a peak at about day 35, and remained high thereafter (Fig. 1G). The peak of anti-N IgG response was similar to that of anti-nsp2 IgG response, but the level decreased markedly at later time points (Fig. 1H). The appearance of IgG responses to GP5 3' and

GP5 5' total epitopes was delayed by 2 weeks, first being detected at 21 days after infection and peaking at day 35 (Fig. 1I and J). Anti-GP5 levels were maintained over time on average, but in the case of the ectodomain epitopes (GP5 5' total) there was marked variation among animals, as indicated by the large error bars.

The delayed IgG response to PRRSV proteins was not due to low-level or delayed production of viral antigens. A high level of viremia was present 3 days after infection and remained high for about 2 weeks (Fig. 2). Individual animals resolved the acute, viremic infection by 21 to 28 days of infection. Thus, viral antigens were actively produced for an extended period of 3 to 4 weeks, during which the onset of antiviral IgM responses varied from 3 days (nsp2) to no apparent response (GP5 3').

Overall, PRRSV infection had no substantial effect on the antibody response to an unrelated antigen, KLH, and the presence of KLH antigen had no effect on anti-PRRSV responses. In all cases, only infected or immunized animals responded, demonstrating that all of the humoral responses were antigen specific. Although PRRSV is reported to induce polyclonal B-cell activation and hypergammaglobulinemia (39, 41, 54), we observed no significant difference in anti-KLH antibody levels in the presence or absence of PRRSV infection (Fig. 1F).

Distribution of PRRSV- and KLH-specific plasma cells in acute and persistent infection. The persistent infection of lymphoid tissues by PRRSV suggests that antigenic stimulation of B-cell differentiation to actively secreting plasma cells is occurring for an extended period of time. However, anti-PRRSV antibody levels consistently declined over time, whereas anti-KLH antibody levels continued to increase for more than 60 days after the boost (Fig. 1G to J). Since serum antibody levels are determined by actively secreting plasma cells, we analyzed the distribution of plasma cells in various lymphoid tissues near the end of acute infection (27 days) and in persistent infection (120 days) (Fig. 3). In uninfected or unimmunized animals, antigen-specific plasma cells were not observed at any time (data not shown). Because the frequencies of antigen-specific IgM and IgA isotypes were low or undetectable following infection, further studies examined only IgG-secreting cells.

At the end of acute infection (27 days), the frequency of IgG plasma cell responses to all PRRSV antigens was highest in SLN, in the range of 100 to 200 ASC per million MNCs, for nsp2 and N (Fig. 3A). Slightly lower frequencies were observed in spleen, followed by tonsil and superficial ILN. ASC for the GP5 3' endodomain were present at substantially lower levels in all tissues, consistent with the lower levels of anti-GP5 3' endodomain antibody titers in serum at 27 days after infection. The presence of KLH did not affect the anti-PRRSV antigen response. IgG ASC specific for KLH were predominantly localized to spleen (~ 200 IgG ASC/ 10^6 cells) and tonsil (~ 150 to 200 IgG ASC/ 10^6 cells), followed by superficial levels in ILN and SLN. The presence of PRRSV infection had no effect on the plasma cell response to KLH. Interestingly, few plasma cells specific for either PRRSV proteins or KLH were detected in bone marrow under any treatment condition.

At 120 days, far into the persistent phase of infection, the frequencies of plasma cells specific for PRRSV antigens were low, in the range of 10 to 40 ASC/ 10^6 cells, and without differences in tissue distribution (Fig. 3B). At this time, the num-

FIG. 1. Serum antibody response kinetics after PRRSV and KLH treatment. Pigs either were mock inoculated or were inoculated intramuscularly with PRRSV VR2332, KLH, or both, as indicated in the treatment legend. Antigen-specific serum IgM (panels A to E) and IgG (panels F to J) antibody responses to KLH (A and F), nsp2 (B and G), N (C and H), GP5 3' (endodomain) (D and I), and GP5 5' total (ectodomain) (E and J) were characterized by ELISA at a 1:1,000 serum dilution. One hundred nanograms of coating antigen per well was used for KLH, nsp2, and N, and 200 ng/well was used for GP5 3' and GP5 5' total. Data are from 12 KLH/PRRSV-treated animals, 4 KLH-treated animals, 4 PRRSV-treated animals, and 2 untreated animals. Assays in panels D, E, I, and J were repeated a second time with the same results. A KLH boost was administered at 14 days.

bers of plasma cells secreting anti-GP5 3' endodomain were equal to the numbers of cells secreting anti-nsp2 and anti-N. The numbers of anti-KLH plasma cells also were low, though they still were more frequently observed in spleen and tonsil

than in other lymphoid tissues. Bone marrow showed no plasma cells specific for either PRRSV proteins or KLH at 120 days. Altogether, antigen-specific plasma cells exhibited different patterns of tissue distribution in KLH immunization and

FIG. 2. Viremia levels in pigs infected with VR2332 with ($n = 12$) or without ($n = 4$) KLH immunization and boosting at 14 days. Viral levels were determined by qRT-PCR as described in Materials and Methods. Data from the two groups were pooled after no significant differences were found and are presented as box-and-whisker plots, with the horizontal bar representing the mean, boxes at the 75th percentile, and the whiskers extended to the minimum and maximum values.

PRRSV infection. PRRSV-specific cells were predominantly localized to SLN draining the lungs, which is the primary site of acute infection. KLH-secreting cells were predominantly localized to spleen, which filters systemic circulation, and tonsil, a B-cell-rich tissue that samples oral antigens.

To further characterize antibody and B-cell responses over time, results from several experiments involving the challenge of pigs with various PRRSV strains were pooled. Pooling was justified because the immune response of pigs to individual viral proteins is equivalent and not substantially affected by the strain of virus, even between attenuated and virulent strains (31, 32). We were able to determine the frequencies of PRRSV-specific plasma cells in lymphoid tissues from near the end of acute infection (27 days) and at days 37, 67, 98, 112, 120,

147, 175, and 203 after infection. Three animals infected with the VR2332 isolate of PRRSV were sacrificed at 27 days, six animals infected with MN-30100 were sacrificed at each of 37 and 67 days, and three animals infected with VR2332 were sacrificed at each time point from 98 days and thereafter. A peak abundance of plasma cells specific for all three viral proteins was observed at 37 days after infection and was predominantly localized in SLN and spleen (Fig. 4A). The response in tonsil consistently peaked at the 67-day time point at a level equivalent to the response in SLN at the same time. The strongest response was to nsp2, followed by that to N and then the GP5 3' endodomain. At the time of peak response, about 0.1% of total mononuclear leukocytes were actively secreting antibodies to these three viral proteins. These data also con-

FIG. 3. Effect of PRRSV infection and KLH immunization on antigen-specific plasma cell responses. Two-week-old pigs were mock inoculated or were inoculated intramuscularly with 10^5 TCID₅₀ of VR2332, 2 mg KLH, or both, as indicated in the treatment legend. Antigen-specific plasma cell responses to KLH, nsp2, N, and GP5 3' at (A) 27 days postinfection and (B) 120 days postinfection were determined by ELISPOT assay. BM, bone marrow.

FIG. 4. Kinetics of antigen-specific B-cell responses in various lymphoid tissues in PRRSV infection. An ELISPOT assay was used to determine the kinetics of (A) antigen-specific plasma cell responses and (B) memory B-cell responses to nsp2, N, and GP5 3'. For plasma cell assays, three animals infected with the VR2332 isolate of PRRSV were sacrificed at day 27, six animals infected with MN-30100 were sacrificed at each of days 37 and 67, and three animals infected with VR2332 were sacrificed at each time point from day 98 on. Memory cell responses were performed on days 37, 98, and at each time point thereafter. BM, bone marrow.

firm that bone marrow is not a site of plasma cell residence ($<20/10^6$ MNCs).

Characterization of memory B-cell responses. In vitro recall responses were measured in MNCs obtained from different lymphoid tissues at different times after PRRSV infection to assess antigen-specific memory B-cell responses. Anti-PRRSV memory B-cell responses already were high when first measured at 37 days after infection, remained high in the case of anti-nsp2- and anti-N-secreting cells for about 100 days, and declined but remained readily detectable at 200 days (Fig. 4B). Anti-GP5 3' endodomain memory cells were present at about one-quarter to one-third of the peak level of anti-nsp2- and anti-N-specific memory cells but did not decline; they were maintained at the same frequencies for 200 days. All of the lymphoid tissues contained anti-PRRSV memory B cells at substantially higher (threefold or greater) levels than plasma cells at all time points up to 200 days.

At 37 days of PRRSV infection, spleen and tonsil had equivalent frequencies of anti-PRRSV memory B cells. At later time points the frequencies were higher in tonsil and lower in spleen, particularly for anti-nsp2 and anti-N memory B cells (Fig. 4B). As we noted previously, bone marrow was not a site of residence for memory B-cell differentiation. Multiple pairwise comparisons of kinetics of tissue distribution of memory B-cell responses revealed no difference between SLN and tonsil at the peak of antibody response at 37 days, whereas memory B cells were predominantly localized to tonsil in persistent PRRSV infection (Table 1). Except as indicated, tonsil samples had a significantly higher number of memory B cells than other lymphoid tissues in persistent PRRSV infection ($P < 0.05$). This indicates that, unlike actively secreting plasma cells, memory B cells were predominantly localized to tonsil in persistent PRRSV infection, perhaps because of the higher viral loads in tonsil tissue in persistent PRRSV infection (9, 17, 40, 56, 64, 67).

A second exposure to PRRSV does not alter plasma cell or memory B-cell responses. Reexposure of an animal to an antigen, i.e., boosting, typically induces a large increase in antibody response. To better understand B-cell and anti-PRRSV-specific plasma cell responses of pigs upon reexposure to PRRSV, we determined the effect of inoculation with PRRSV MN-30100 alone or MN-30100 plus, 7 days later, Ingelvac MLV vaccine. Responses were assessed at 37 days after the first exposure. In animals that received MN-30100 alone, the strongest antigen-specific IgG-secreting plasma cell response was to nsp2, with approximately twice as many cells than responded to N and three to four times more cells than responded to GP5 3' (Fig. 5A). A second exposure to PRRSV Ingelvac MLV had no effect on antigen-specific plasma cell numbers in any of the tissues examined. Plasma cells specific for each of the three viral proteins were significantly more abundant in SLN than in other cells and tissues, including spleen ($P < 0.05$). The frequencies in spleen, in turn, were significantly higher than those in PBMC, tonsil, ILN, and bone marrow ($P < 0.05$). Thus, spleen and lymph nodes draining the lung appear to be the major sites of anti-PRRSV antibody production, with circulating lymphocytes, tonsil, and ILN playing minor roles and bone marrow playing no role at all. These results were antigen specific, since uninfected controls showed no antigen-specific actively secreting plasma cell response (data not shown).

Memory B-cell responses to recall antigen were two to three times higher than plasma cell responses, with the nsp2 response being the strongest, followed by N and GP5 3' endodomain responses (Fig. 5B). A second exposure to PRRSV had no effect in any of the tissues examined (Fig. 5B). Tonsil was equivalent to SLN and spleen in anti-PRRSV memory B-cell responses, in contrast to its minor role in actively secreting plasma cell production. Memory B cells were uncommon in blood and were at extremely low levels in bone marrow. Recall

TABLE 1. Analysis of tissue distribution of memory B-cell responses in PRRSV infection

Protein and day	Mean no. of memory B cells in ^a :					
	PBMC	Spleen	Tonsil	Superficial ILN	SLN	Bone marrow
nsp2						
37	382.5 ^D	1,605.8 ^B	1,979.2 ^A	1,057.5 ^C	2140 ^A	105.3 ^E
98	170.0 ^D	1,688.3 ^B	2,630.0 ^A	478.3 ^C	1,615.0 ^B	235.0 ^{CD}
112	262.5 ^B	848.3 ^B	2,052.5 ^A	700.0 ^B	1,525.0^A	285.0 ^B
147	240.3 ^C	630.0 ^B	1,670.0 ^A	368.3 ^C	1,365.0^A	191.7 ^C
175	72.7 ^C	633.3 ^B	1,098.3 ^A	241.7 ^C	666.7 ^B	105.7 ^C
203	19.3 ^D	560.0 ^B	866.7 ^A	286.7 ^C	395.0 ^{BC}	54.7 ^D
Nucleocapsid						
37	242.5 ^C	1,221.7 ^A	1,215.8 ^A	746.7 ^B	1,273.3 ^A	66.7 ^C
98	103.7 ^D	710.0 ^C	1,717.5 ^A	978.3 ^{BC}	1,145.0 ^B	275.0 ^D
112	258.0 ^{BC}	763.3 ^B	1,937.5 ^A	423.3 ^{BC}	1,513.3^A	226.7 ^C
147	88.0 ^D	603.3 ^C	1,645.0 ^A	476.7 ^C	1,168.3 ^B	183.3 ^D
175	72.7 ^C	633.3 ^B	1,098.3 ^A	241.7 ^C	666.7 ^B	105.7 ^C
203	25.0 ^D	688.3 ^B	1,120.0 ^A	236.7 ^C	576.7 ^B	86.0 ^{CD}
GP5 3'						
37	123.3 ^C	629.7 ^A	319.3 ^B	383.2 ^B	556.7 ^A	8.2 ^C
98	209.8 ^C	445.0 ^{AB}	627.5 ^A	181.7 ^C	460.0^{AB}	301.7 ^{BC}
112	165.0 ^C	600.0 ^{AB}	732.5 ^A	248.3 ^C	495.0 ^B	131.7 ^C
147	162.0 ^B	590.0 ^A	698.3 ^A	260.0 ^B	441.7^A	122.0 ^B
175	128.3 ^C	440.0 ^B	578.3 ^A	160.7 ^C	371.7 ^B	131.7 ^C
203	92.0 ^C	405.0 ^B	583.3 ^A	171.7 ^C	440.0^{AB}	146.0 ^C

^a Means in the same horizontal row and not sharing the same superscript are significantly different from each other. Multiple pairwise comparisons were performed using MacAnova. The mean numbers of memory B cells/10⁶ MNC from three animals at each time point in each tissue were computed. Superscripts indicating significance ($P < 0.05$) were ranked in order (A > B > C > D). Mean values that are not significantly different from those for tonsil samples in persistent infection are in boldface.

responses to nsp2, N, and GP5 3' were not detected in PBMC of uninfected animals (data not shown).

Antigen-specific and nonspecific B-cell responses in Peyer's patches. Since B-cell responses in bone marrow, the site of long-term plasma and memory B-cell production in some mammalian species (34, 59), were nil, and Peyer's patches are reported to be the principal site of B-cell differentiation in the pig (3, 14, 15), we examined anti-PRRSV and total primary and memory B-cell responses in both the jejunal and ileal Peyer's patches. Five-week-old conventional pigs were inoculated with PRRSV MN-30100 on day 0 and were reinoculated with Ingelvac MLV on days 7, 37, and 67. Animals were sacrificed 97 days after the initial infection, and Peyer's patch MNC preparations were analyzed. IgG- and IgA-specific ASC were present in both jejunal and ileal Peyer's patches at approximately 0 to 25 ASC per 10⁶ MNCs (Fig. 6A). Memory B-cell responses were about fivefold greater than plasma cell responses, and there was no IgM memory response (Fig. 6B). The antiviral response was highest to nsp2, followed by those to N and GP5 3', as was consistently observed previously. The antiviral memory response was about 1% of the total ASC population, which ranged from about 5,000 to 15,000 cells per million MNCs (Fig. 6C). Under all conditions tested, the frequency of IgA-secreting cells was slightly higher than the frequency of IgG-secreting cells.

Effect of PRRSV infection on B-cell frequency in lymphoid tissues. The decline in the number of antiviral plasma cells was unexpected, since the viral antigen is present for extended periods in lymphoid tissues (67). To determine whether the decline was secondary to a generalized reduction in B-cell frequencies in lymphoid tissues as pigs age, total IgG-secreting

cells were enumerated in PBMC, spleen, SLN, superficial ILN, tonsil, and bone marrow of pigs at 7, 12, and 20 weeks of age. As shown in Fig. 7, the proportion of in vivo ASC increased steadily over time in all tissues examined, with the highest numbers of responding cells in spleen and tonsil and the lowest numbers in bone marrow and blood (PBMC).

Since tissue differences in PRRSV-specific B-cell frequencies might be due to a global effect of viral infection on B-cell differentiation or survival, we enumerated the frequencies of IgM⁺ cells in spleen, tonsil, SLN, superficial ILN, and bone marrow of pigs that were infected at 4 weeks or 2 years of age with PRRSV strain JA142. As shown in Fig. 8, the frequencies of IgM⁺ cells was not affected by PRRSV infection in tissue of young or adult pigs. B-cell frequencies in spleen showed substantial individual variation among animals regardless of age; there was a tendency for lower frequencies in young pigs infected with PRRSV, but there was no such tendency in adult pigs. Young pigs also demonstrated more variation in B-cell frequencies in other tissues compared to those in tissues of adult pigs, with the variation being skewed to higher frequencies in all tissues of young pigs. IgM⁺ cells were absent or present at very low frequencies in bone marrow of young pigs. Cells could not be isolated from bone marrow of adult pigs.

DISCUSSION

In an effective mammalian immune response, the B-cell response to antigen involves the specific activation and proliferation of naïve B cells in the draining lymph nodes and spleen, resulting in the generation of plasma cells and memory B cells in a matter of days (20). Plasma cells and memory B cells

FIG. 5. Effect of a second dose of PRRSV in vivo on (A) antigen-specific plasma cell responses and (B) memory B cell responses in various lymphoid tissues at 37 days after initial infection. Six- to 8-week-old PRRSV-naïve pigs were inoculated intranasally with 2 ml of 3×10^4 TCID₅₀/ml of PRRSV MN-30100 (white circles). Another group of pigs was infected with MN-30100 on day 0 and boosted with 2 ml of Ingelvac PRRS MLV on day 7 (black circles). An ELISPOT assay was used to measure antigen-specific responses to nsp2, N, and GP5 3'. Each circle represents the response of an individual animal. The horizontal bar is the mean value of all data points within a group. BM, bone marrow.

migrate to the sites of infection and to bone marrow, which is the major site of sustained antibody production and memory B-cell residence (44, 60). Antigen-specific plasma cells secrete IgM or IgG into the circulation or release dimeric IgA adjacent to the mucosal epithelium to generate antiviral responses (13,

49). The porcine B-cell response conforms to this model with regard to viral infections caused by influenza and foot-and-mouth disease viruses (1, 12). Specifically, active infection with peak viremia occurring within 24 to 72 h is followed by rapid diminution of viral load in the circulation and clearance of

FIG. 6. Anti-PRRSV and total B-cell responses in Peyer's patches. Pigs were inoculated with PRRSV strain MN-30100 and reinoculated with Ingelvac MLV on days 7, 37, and 67 after primary infection. Animals were sacrificed 97 days after primary infection. Jejunal (circles) and ileal (triangles) Peyer's patches from three animals were dissected. MNCs were isolated and cultured to determine IgG-ASC, IgM-ASC, and IgA-ASC for PRRSV nsp2, N, and GP5 3', respectively, as well as total IgG-ASC and IgA-ASC. Each symbol is the mean response from one animal. Cultures of ileal Peyer's patch cells from one animal became contaminated and were discarded.

FIG. 7. Effect of age on IgG-secreting plasma cell numbers in blood and lymphoid tissues. MNCs from different lymphoid tissues from conventional healthy pigs were isolated and assayed with an ELISPOT assay to measure total IgG B-cell responses. BM, bone marrow.

virus by 4 to 7 days. The reduction and resolution of viral infection is associated with the appearance of antiviral antibodies (1, 12, 22). These previous studies demonstrated that swine possess the full repertoire of innate and adaptive immune responses to rapidly respond to and resist infections by naturally occurring viral pathogens. However, the porcine response to PRRSV deviates from this model in its dynamics, lymphoid tissue distribution, dissociation from viral clearance in blood, and absence of a boosting response.

Delayed response to PRRSV protein GP5. The IgM response to the viral proteins nsp2 and N following infection with PRRSV occurs rapidly, transiently, and before any appearance of IgG. However, no IgM response to the endodomain fragment of GP5 (GP5 3') was detected, and the response to the GP5 ectodomain (GP5 5' total) peaked at 28 to 35 days, when viremia essentially was resolved. The IgG response to both domains also was delayed relative to that of other viral proteins. Since serum samples were tested three times under a variety of conditions for anti-GP5 3' endodomain IgM, it appears that either transcription or translation is inefficient, secretion of the antibodies is at a very low level, or that anti-GP5 3' endodomain IgM is unusually sensitive to degradation or elimination from the blood. GP5 3' also might be poorly im-

FIG. 8. Effect of PRRSV infection on IgM-positive B-cell frequencies in lymphoid tissues. Pigs at 4 weeks of age (young) and 2 years of age (adult) were infected with PRRSV strain JA142. Lymphoid tissue samples were obtained at sacrifice on day 63 of infection. Isolated cells were analyzed by a fluorescence-activated cell sorter for IgM-positive B-cell frequencies from three to five tissues from five pigs per group. MNCs could not be isolated from adult bone marrow. The gray line is the isotype-matched control. BM, bone marrow.

munogenic, though antigenicity and hydrophilicity plots suggest the region is antigenic (data not shown). Masking epitopes in the GP5 5' total ectodomain, which have been hypothesized to account for a delayed appearance of neutralizing antibodies to PRRSV, do not explain our results, since a neutralizing function was not required for the detection of anti-GP5 antibody responses (51). Neutralizing antibodies are believed to be directed to conserved sequences in the GP5 ectodomain (24, 25, 27, 28, 43, 52, 53, 55, 61, 62, 65, 66). Our results indicate that anti-GP5 antibodies do not play a significant role in the resolution of viremia and suggest that their delayed appearance contributes to the establishment of persistent infection.

The atypical response to GP5 is particularly interesting, since viral infection did not alter the response to an unrelated antigen, KLH, or (apparently) to other viral proteins, as evidenced by N and nsp2. The more rapid kinetics of the IgG response to KLH may reflect the immediate presence of a high antigen load rather than a gradual accumulation of viral antigen through the spread of acute infection. However, viral load as measured by viremia was maximal at 2 to 7 days after infection, so other factors must be involved in the lag to peak antibody production, which occurs at about 4 weeks after infection with PRRSV and even longer after infection in the case of the anti-GP5 response.

Declining humoral response despite antigen persistence. A hallmark of PRRSV infection is the persistence of infectious virus and viral antigens in lymphoid tissues, particularly the tonsil, for 150 or more days (5, 7, 11, 17, 30, 56, 63). Nevertheless, the cell-mediated immune response to PRRSV declines over time (67). In this study, the plasma cell response to nsp2, N, and GP5 also decreases, and at 120 days it was lower than the plasma cell response to KLH. PRRSV cytolytic infection destroys macrophage and dendritic cells, but Xiao et al. (67) and Samsom et al. (57) showed that macrophage cell numbers as a whole in lung and lymphoid tissues are not affected by PRRSV infection. Infection might suppress macrophage or dendritic cell presentation of antigen. While little evidence supports this hypothesis, more expression studies of the molecules involved in antigen presentation processes may address the question.

The decline of anti-PRRSV antibody levels and plasma cell frequencies indicates a nonsignificant level of stimulation rather than a lack of responsive cells, as demonstrated by the presence of a large reservoir of B cells able to respond to recall antigen in lymphoid tissues. Figure 9, redrawn from data depicted in Fig. 5, shows that memory cells are abundantly distributed in lymphoid tissues at 37 days after infection (the MN-30100 plus in vitro restimulation treatment group), whereas plasma cell frequencies are 3- to >10-fold lower and are not increased by a booster inoculation with a second viral strain 7 days after primary infection (MN-30100 group + MLV in vivo). Thus, in sum, neither PRRSV persistence nor a second infection at 7 days, prior to the establishment of specific immunity, facilitates a robust humoral antibody response. The decline in plasma cell numbers after the peak at 27 days also suggests that viral replication resulting in cytolytic infection is low during this period. Lysis of infected cells would release nonstructural and structural proteins into extracellular space for recognition by PRRSV-antigen-receptor-bearing B cells

FIG. 9. Absence of anamnestic response in vivo to PRRSV despite the presence of recall memory in vitro. Data from Fig. 5 are redrawn to show the robust memory response in vitro to nsp2 but no increase in the B-cell response to nsp2 in vivo after a booster infection. BM, bone marrow.

and presumably activate renewed antibody production, as was demonstrated in vitro.

In vitro recall response without enhanced response to second infection in vivo. An interesting feature of PRRSV humoral immunity is the absence of an anamnestic response (29), even though recall responses in vitro to PRRSV antigens are present from early times of infection and are more than two-fold higher than plasma cell responses. The lack of an in vivo anamnestic response may be due to the depletion of permissive macrophages that renders the host resistant to reinfection (although evidence of macrophage resistance to infection is lacking [67]) or to an absence of antigen that occurs when re-exposure fails to result in reinfection (29). In this study, re-exposure to virus on day 7, before antigen-specific immunity is observed, did not enhance ASC numbers in vivo, whereas the memory response to recall antigen in vitro was quite strong in tonsil, spleen, SLN, and ILN (Fig. 9). Thus, other mechanisms of immune resistance appear to prevent antigen production following reinfection so that more PRRSV-specific plasma cells are not elicited.

Previous attempts to assess an immunosuppressive effect of PRRSV infection on porcine disease resistance have not demonstrated convincing proof that PRRSV exacerbates other disease conditions, nor have they provided a mechanistic model of immunosuppression (reviewed in references 26 and 47). Here, we show that PRRSV infection had no effect on the humoral antibody and B-cell response to a second, specific, independent antigen. In addition, viral infection did not reduce B-cell frequencies in lymphoid tissues.

Regionalization of plasma and memory cell responses. In acute PRRSV infection, the viral load is high in a variety of lymphoid tissues (56, 67), but antigen-specific plasma cells are predominantly localized to SLN and spleen at the peak of the antibody response (Fig. 5). A prominent role for the SLN is

expected because it drains the lungs, the primary site of acute infection. The minor role of ILN was surprising, since gross enlargement of the superficial ILN, often discernible to the naked eye, is a hallmark pathological sign of PRRS. PRRSV-specific B cells also were abundant in spleen, which may be the most important source of plasma and memory cells due to its large size and intimate contact with the circulation. Tonsil was not significant as a PRRSV-specific plasma cell reservoir but was equivalent to SLN and spleen for memory B cells specific for all PRRSV proteins. Overall, these findings indicate that spleen and SLN are the major effector and surveillance sites containing actively secreting plasma cells and memory cells. In contrast, tonsil is a site for surveillance but not effector function, since it contains memory cells but not plasma cells. The ILN shows B-cell characteristics similar to those of tonsil but is not a principal site of persistent infection.

In persistent PRRSV infection, both plasma cells and memory B cells exhibited a different pattern of anatomical localization than was present at the peak of antibody responses. Antigen-specific plasma cell responses declined dramatically in all of the lymphoid tissues, but memory B cells showed no significant change. The decline in plasma cells might be attributed to the migration from SLN and spleen to sites of infection or to disappearance through apoptosis. Unlike plasma cell responses, memory B cells remained at high levels up to 200 days after infection. Interestingly, memory B cells were predominantly localized to tonsil in persistent PRRSV infection, consistent with higher viral loads (67).

Because bone marrow is regarded as the major site of long-lived memory B cell production and residence (44, 60), the paucity of PRRSV-specific plasma cells and memory B cells in bone marrow was particularly interesting. In rotavirus infection, bone marrow also was not the primary site of antibody production or the site of residence of memory B cells until 83 days after infection (68). The failure of ASC and memory B cells to migrate to the bone marrow could be due to a species-specific lack of expression of homing molecules on the responding B cells. Alternatively, the ileal Peyer's patches, not bone marrow, may be the primary site of B-cell differentiation in swine (10, 15). We observed that ileal and jejunal Peyer's patches have high frequencies of ASC, as shown in Fig. 8, but the frequencies of PRRSV-specific B cells was unremarkable. Therefore, it appears that PRRSV-specific long-lived plasma cell and memory B-cell maturation occurs not in generalized sites of B-cell differentiation but in secondary lymphoid tissues that drain sites of PRRSV infection, especially SLN draining the lung (23, 67), and tonsil, the primary site of persistent PRRSV infection (17, 40, 63, 67). Consistent with this interpretation is the shift we observed in the tissue distribution of PRRSV-specific ASC from SLN and spleen in acute infection to tonsil and spleen in persistent infection.

In conclusion, PRRSV infection induces a significant and specific antibody and B-cell response to a variety of PRRSV proteins without affecting humoral antibody or B-cell responses to an irrelevant protein antigen, KLH. Thus, viral infection did not suppress the immune response to a second immunological challenge. The predominant localization of IgG plasma cells to SLN and spleen suggests that they are the primary sites of effector antibody production in acute infection. In persistent infection, memory B cells were predominantly

localized to tonsil, the primary site of viral persistence. Since viral antigen is widespread in lymphoid tissues (67), the striking localization of anti-PRRSV memory B cells in tonsils indicates that mechanisms of specific B-cell differentiation and growth are different here than in other lymphoid tissues. The IgM response to GP5 5' total, the putative neutralization determinant, was notably deficient, which might contribute to viral persistence. Memory B-cell responses generated *in vitro* were more than twofold higher than plasma cell responses and were maintained for 200 days. Durable recall responses are consistent with prolonged periods of protective immunity beyond 600 days (36). A lack of anamnestic responses in the presence of resistance to reinfection suggests that viral antigens do not encounter memory B cells in sufficient amounts to trigger their activation and proliferation.

ACKNOWLEDGMENTS

We appreciate the expert technical assistance of LaRae Peterson, Geoffrey N. Hirsch, and Colleen Finnegan. Editorial expertise was provided by Karin Matchett and Cheryl Dvorak.

The work was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2003-35204-13809, and by awards from the USDA-CSREES coordinated agricultural project number 2004-35605-14197 to M.P.M., J.J.Z., and Robert R. Rowland, Kansas State University. Additional support was provided by Boehringer Ingelheim Vetmedica and the Minnesota Agricultural Experiment Station.

Genetiporc, Inc., generously provided animals for experiments performed at the Swine Disease Eradication Center Research Farm.

REFERENCES

- Alexandersen, S., M. Quan, C. Murphy, J. Knight, and Z. Zhang. 2003. Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *J. Comp. Pathol.* **129**:268–282.
- Allende, R., W. W. Laegreid, G. F. Kutish, J. A. Galeota, R. W. Wills, and F. A. Osorio. 2000. Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. *J. Virol.* **74**:10834–10837.
- Andersen, J. K., H. Takamatsu, C. A. Oura, S. M. Brookes, L. Pullen, and R. E. Parkhouse. 1999. Systematic characterization of porcine ileal Peyer's patch. I. Apoptosis-sensitive immature B cells are the predominant cell type. *Immunology* **98**:612–621.
- Baker, R. B., W. Yu, M. Fuentes, C. R. Johnson, L. Peterson, K. Rossow, C. S. Daniels, A. M. Daniels, D. Polson, and M. P. Murtaugh. 2007. Prairie dog (*Cynomys ludovicianus*) is not a host for porcine reproductive and respiratory syndrome virus. *J. Swine Health Prod.* **15**:22–29.
- Batista, L., S. A. Dee, K. D. Rossow, J. Deen, and C. Pijoan. 2002. Assessing the duration of persistence and shedding of porcine reproductive and respiratory syndrome virus in a large population of breeding-age gilts. *Can. J. Vet. Res.* **66**:196–200.
- Batista, L., S. A. Dee, K. D. Rossow, D. D. Polson, Z. Xiao, M. Olin, M. P. Murtaugh, T. W. Molitor, H. S. Joo, and C. Pijoan. 2004. Detection of porcine reproductive and respiratory syndrome virus in pigs with low positive or negative ELISA s/p ratios. *Vet. Rec.* **154**:25–26.
- Batista, L., C. Pijoan, S. Dee, M. Olin, T. Molitor, H. S. Joo, Z. Xiao, and M. Murtaugh. 2004. Virological and immunological responses to porcine reproductive and respiratory syndrome virus in a large population of gilts. *Can. J. Vet. Res.* **68**:267–273.
- Benfield, D. A., E. Nelson, J. E. Collins, L. Harris, S. M. Goyal, D. Robison, W. T. Christianson, R. B. Morrison, D. Gorceyca, and D. Chladek. 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J. Vet. Diagn. Invest.* **4**:127–133.
- Beyer, J., D. Fichtner, H. Schirrmeyer, U. Polster, E. Weiland, and H. Wege. 2000. Porcine reproductive and respiratory syndrome virus (PRRSV): kinetics of infection in lymphatic organs and lung. *J. Vet. Med. B. Infect. Dis. Vet. Public Health* **47**:9–25.
- Bianchi, A. T., R. J. Zwart, S. H. Jeurissen, and H. W. Moonen-Leusen. 1992. Development of the B- and T-cell compartments in porcine lymphoid organs from birth to adult life: an immunohistological approach. *Vet. Immunol. Immunopathol.* **33**:201–221.
- Bierk, M. D., S. A. Dee, K. D. Rossow, S. Otake, J. E. Collins, and T. W. Molitor. 2001. Transmission of porcine reproductive and respiratory syn-

- drome virus from persistently infected sows to contact controls. *Can. J. Vet. Res.* **65**:261–266.
12. Brown, I. H., S. H. Done, Y. I. Spencer, W. A. Cooley, P. A. Harris, and D. J. Alexander. 1993. Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. *Vet. Rec.* **132**:598–602.
  13. Butcher, E. C., M. Williams, K. Youngman, L. Rott, and M. Briskin. 1999. Lymphocyte trafficking and regional immunity. *Adv. Immunol.* **72**:209–253.
  14. Butler, J. E. 1998. Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals. *Rev. Sci. Tech.* **17**:43–70.
  15. Butler, J. E., M. Sinkora, N. Wertz, W. Holtmeier, and C. D. Lemke. 2006. Development of the neonatal B and T cell repertoire in swine: implications for comparative and veterinary immunology. *Vet. Res.* **37**:417–441.
  16. Christopher-Hennings, J., K. S. Faaberg, M. P. Murtaugh, E. A. Nelson, M. B. Roof, E. M. Vaughn, K. J. Yoon, and J. J. Zimmerman. 2002. Porcine reproductive and respiratory syndrome (PRRS) diagnostics: interpretations and limitations. *J. Swine Health Prod.* **10**:213–218.
  17. Christopher-Hennings, J., L. D. Holler, D. A. Benfield, and E. A. Nelson. 2001. Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. *J. Vet. Diagn. Invest.* **13**:133–142.
  18. Collins, J. E., D. A. Benfield, W. T. Christianson, L. Harris, J. C. Hennings, D. P. Shaw, S. M. Goyal, S. McCullough, R. B. Morrison, H. S. Joo, D. E. Goreyca, and D. W. Chladek. 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J. Vet. Diagn. Invest.* **4**:117–126.
  19. Crotty, S., R. D. Aubert, J. Glidewell, and R. Ahmed. 2004. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. *J. Immunol. Methods* **286**:111–122.
  20. Cyster, J. G. 2003. Homing of antibody secreting cells. *Immunol. Rev.* **194**:48–60.
  21. Dea, S., C. A. Gagnon, H. Mardassi, B. Pirzadeh, and D. Rogan. 2000. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. *Arch. Virol.* **145**:659–688.
  22. de Avila Botton, S., M. C. Brum, E. Bautista, M. Koster, R. Weiblen, W. T. Golde, and M. J. Grubman. 2006. Immunopotential of a foot-and-mouth disease virus subunit vaccine by interferon alpha. *Vaccine* **24**:3446–3456.
  23. Dee, S. A., T. W. Molitor, and K. D. Rossow. 2000. Epidemiological and diagnostic observations following the elimination of porcine reproductive and respiratory syndrome virus from a breeding herd of pigs by the test and removal protocol. *Vet. Rec.* **146**:211–213.
  24. Delputte, P. L., N. Vanderheijden, H. J. Nauwynck, and M. B. Pensaert. 2002. Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *J. Virol.* **76**:4312–4320.
  25. Dobbe, J. C., Y. van der Meer, W. J. Spaan, and E. J. Snijder. 2001. Construction of chimeric arteriviruses reveals that the ectodomain of the major glycoprotein is not the main determinant of equine arteritis virus tropism in cell culture. *Virology* **288**:283–294.
  26. Drew, T. W. 2000. A review of evidence for immunosuppression due to porcine reproductive and respiratory syndrome virus. *Vet. Res.* **31**:27–39.
  27. Duan, X., H. J. Nauwynck, H. Favoreel, and M. B. Pensaert. 1998. Porcine reproductive and respiratory syndrome virus infection of alveolar macrophages can be blocked by monoclonal antibodies against cell surface antigens. *Adv. Exp. Med. Biol.* **440**:81–88.
  28. Duan, X., H. J. Nauwynck, H. W. Favoreel, and M. B. Pensaert. 1998. Identification of a putative receptor for porcine reproductive and respiratory syndrome virus on porcine alveolar macrophages. *J. Virol.* **72**:4520–4523.
  29. Foss, D. L., M. J. Zilliox, W. Meier, F. Zuckermann, and M. P. Murtaugh. 2002. Adjuvant danger signals increase the immune response to porcine reproductive and respiratory syndrome virus. *Viral Immunol.* **15**:557–566.
  30. Horter, D. C., R. M. Pogranichniy, C. C. Chang, R. B. Evans, K. J. Yoon, and J. J. Zimmerman. 2002. Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. *Vet. Microbiol.* **86**:213–228.
  31. Johnson, C. R., W. Yu, and M. P. Murtaugh. 2007. Cross-reactive antibody responses to nsp1 and nsp2 of porcine reproductive and respiratory syndrome virus. *J. Gen. Virol.* **88**:1184–1195.
  32. Johnson, W., M. Roof, E. Vaughn, J. Christopher-Hennings, C. R. Johnson, and M. P. Murtaugh. 2004. Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. *Vet. Immunol. Immunopathol.* **102**:233–247.
  33. Joo, H. S., B. K. Park, S. A. Dee, and C. Pijoan. 1997. Indirect fluorescent IgM antibody response of pigs infected with porcine reproductive and respiratory syndrome virus. *Vet. Microbiol.* **55**:303–307.
  34. Karrer, U., C. Lopez-Macias, A. Osenius, B. Odermatt, M. F. Bachmann, U. Kalinke, H. Bluethmann, H. Hengartner, and R. M. Zinkernagel. 2000. Antiviral B cell memory in the absence of mature follicular dendritic cell networks and classical germinal centers in TNFR1^{-/-} mice. *J. Immunol.* **164**:768–778.
  35. Labarque, G. G., H. J. Nauwynck, K. Van Reeth, and M. B. Pensaert. 2000. Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. *J. Gen. Virol.* **81**:1327–1334.
  36. Lager, K. M., W. L. Mengeling, and S. L. Brockmeier. 1997. Duration of homologous porcine reproductive and respiratory syndrome virus immunity in pregnant swine. *Vet. Microbiol.* **58**:127–133.
  37. Lager, K. M., W. L. Mengeling, and S. L. Brockmeier. 1999. Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. *Am. J. Vet. Res.* **60**:1022–1027.
  38. Lager, K. M., W. L. Mengeling, and S. L. Brockmeier. 1997. Homologous challenge of porcine reproductive and respiratory syndrome virus immunity in pregnant swine. *Vet. Microbiol.* **58**:113–125.
  39. Lamontagne, L., C. Page, R. Larochelle, D. Longtin, and R. Magar. 2001. Polyclonal activation of B cells occurs in lymphoid organs from porcine reproductive and respiratory syndrome virus (PRRSV)-infected pigs. *Vet. Immunol. Immunopathol.* **82**:165–182.
  40. Lamontagne, L., C. Page, R. Larochelle, and R. Magar. 2003. Porcine reproductive and respiratory syndrome virus persistence in blood, spleen, lymph nodes, and tonsils of experimentally infected pigs depends on the level of CD8^{high} T cells. *Viral Immunol.* **16**:395–406.
  41. Lemke, C. D., J. S. Haynes, R. Spaete, D. Adolphson, A. Vorwald, K. Lager, and J. E. Butler. 2004. Lymphoid hyperplasia resulting in immune dysregulation is caused by porcine reproductive and respiratory syndrome virus infection in neonatal pigs. *J. Immunol.* **172**:1916–1925.
  42. Loemba, H. D., S. Mounir, H. Mardassi, D. Archambault, and S. Dea. 1996. Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. *Arch. Virol.* **141**:751–761.
  43. Lopez, O. J., and F. A. Osorio. 2004. Role of neutralizing antibodies in PRRSV protective immunity. *Vet. Immunol. Immunopathol.* **102**:155–163.
  44. McHeyzer-Williams, M. G., and R. Ahmed. 1999. B cell memory and the long-lived plasma cell. *Curr. Opin. Immunol.* **11**:172–179.
  45. Mengeling, W. L., K. M. Lager, and A. C. Vorwald. 1999. Safety and efficacy of vaccination of pregnant gilts against porcine reproductive and respiratory syndrome. *Am. J. Vet. Res.* **60**:796–801.
  46. Molitor, T. W., E. M. Bautista, and C. S. Choi. 1997. Immunity to PRRSV: double-edged sword. *Vet. Microbiol.* **55**:265–276.
  47. Murtaugh, M. P., Z. Xiao, and F. Zuckermann. 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. *Viral Immunol.* **15**:533–547.
  48. Nilubol, D., K. B. Platt, P. G. Halbur, M. Torremorell, and D. L. Harris. 2004. The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. *Vet. Microbiol.* **102**:11–18.
  49. Odendahl, M., H. Mei, B. F. Hoyer, A. M. Jacobi, A. Hansen, G. Muehlinghaus, C. Berek, F. Hiepe, R. Manz, A. Radbruch, and T. Dörner. 2005. Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. *Blood* **105**:1614–1621.
  50. Opriessnig, T., F. J. Pallares, D. Nilubol, A. L. Vincent, E. L. Thacker, E. M. Vaughn, M. Roof, and P. G. Halbur. 2005. Genomic homology of ORF 5 gene sequence between modified live vaccine virus and porcine reproductive and respiratory syndrome virus challenge isolates is not predictive of vaccine efficacy. *J. Swine Health Prod.* **13**:246–253.
  51. Ostrowski, M., J. A. Galeota, A. M. Jar, K. B. Platt, F. A. Osorio, and O. J. Lopez. 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J. Virol.* **76**:4241–4250.
  52. Plagemann, P. G. 2004. GP5 ectodomain epitope of porcine reproductive and respiratory syndrome virus, strain Lelystad virus. *Virus Res.* **102**:225–230.
  53. Plagemann, P. G. 2004. The primary GP5 neutralization epitope of North American isolates of porcine reproductive and respiratory syndrome virus. *Vet. Immunol. Immunopathol.* **102**:263–275.
  54. Plagemann, P. G., R. R. Rowland, and W. A. Cafrunty. 2005. Polyclonal hypergammaglobulinemia and formation of hydrophobic immune complexes in porcine reproductive and respiratory syndrome virus-infected and uninfected pigs. *Viral Immunol.* **18**:138–147.
  55. Plagemann, P. G., R. R. Rowland, and K. S. Faaberg. 2002. The primary neutralization epitope of porcine reproductive and respiratory syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. *Arch. Virol.* **147**:2327–2347.
  56. Rowland, R. R., S. Lawson, K. Rossow, and D. A. Benfield. 2003. Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. *Vet. Microbiol.* **96**:219–235.
  57. Samson, J. N., T. G. de Bruin, J. J. Voermans, J. J. Meulenbergh, J. M. Pol, and A. T. Bianchi. 2000. Changes of leukocyte phenotype and function in the broncho-alveolar lavage fluid of pigs infected with porcine reproductive and respiratory syndrome virus: a role for CD8⁺ cells. *J. Gen. Virol.* **81**:497–505.

58. Schurrer, J. A., S. A. Dee, R. D. Moon, M. P. Murtaugh, C. P. Finnegan, J. Deen, S. B. Kleiboeker, and C. B. Pijoan. 2005. Retention of ingested porcine reproductive and respiratory syndrome virus in houseflies. *Am. J. Vet. Res.* **66**:1517–1525.
59. Slifka, M. K., R. Antia, J. K. Whitmire, and R. Ahmed. 1998. Humoral immunity due to long-lived plasma cells. *Immunity* **8**:363–372.
60. Slifka, M. K., M. Matloubian, and R. Ahmed. 1995. Bone marrow is a major site of long-term antibody production after acute viral infection. *J. Virol.* **69**:1895–1902.
61. Vanderheijden, N., P. Delputte, H. Nauwynck, and M. Pensaert. 2001. Effects of heparin on the entry of porcine reproductive and respiratory syndrome virus into alveolar macrophages. *Adv. Exp. Med. Biol.* **494**:683–689.
62. Vanderheijden, N., P. L. Delputte, H. W. Favoreel, J. Vandekerckhove, J. Van Damme, P. A. Van Woensel, and H. J. Nauwynck. 2003. Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. *J. Virol.* **77**:8207–8215.
63. Wills, R. W., A. R. Doster, J. A. Galeota, J. H. Sur, and F. A. Osorio. 2003. Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. *J. Clin. Microbiol.* **41**:58–62.
64. Wills, R. W., J. J. Zimmerman, K. J. Yoon, S. L. Swenson, M. J. McGinley, H. T. Hill, K. B. Platt, J. Christopher-Hennings, and E. A. Nelson. 1997. Porcine reproductive and respiratory syndrome virus: a persistent infection. *Vet. Microbiol.* **55**:231–240.
65. Wissink, E. H., H. A. van Wijk, M. V. Kroese, E. Weiland, J. J. Meulenbergh, P. J. Rottier, and P. A. van Rijn. 2003. The major envelope protein, GP5, of a European porcine reproductive and respiratory syndrome virus contains a neutralization epitope in its N-terminal ectodomain. *J. Gen. Virol.* **84**:1535–1543.
66. Wissink, E. H., H. A. van Wijk, J. M. Pol, G. J. Godeke, P. A. van Rijn, P. J. Rottier, and J. J. Meulenbergh. 2003. Identification of porcine alveolar macrophage glycoproteins involved in infection of porcine respiratory and reproductive syndrome virus. *Arch. Virol.* **148**:177–187.
67. Xiao, Z., L. Batista, S. Dee, P. Halbur, and M. P. Murtaugh. 2004. The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. *J. Virol.* **78**:5923–5933.
68. Yuan, L., A. Geyer, and L. J. Saif. 2001. Short-term immunoglobulin A B-cell memory resides in intestinal lymphoid tissues but not in bone marrow of gnotobiotic pigs inoculated with Wa human rotavirus. *Immunology* **103**:188–198.