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Gradual development of the interferon-γ response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination

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Abstract

Infection of swine with virulent porcine reproductive and respiratory syndrome (PRRS) virus induced a rapid, robust antibody response that comprised predominantly nonneutralizing antibodies and waned after approximately 3 months. In contrast, the initial onset of virus-specific interferon (IFN)-γ-secreting cells (SC) in the pig lymphocyte population remained at a fairly low level during this period and then increased gradually in frequency, plateauing at 6 months postinfection. A similar polarization of the host humoral and cellular immune responses was also observed in pigs immunized with a PRRS-modified live virus (MLV) vaccine. Even coadministration of an adjuvant that enhanced the immune response to a pseudorabies (PR) MLV vaccine failed to alter the induction of PRRS virus-specific IFN-γ SC (comprising predominately CD4/CD8α double positive memory T cells with a minority being typical $CD4^{-}/CD8\alpha\beta^{+}$ T cells) and the generation of neutralizing antibodies. Moreover, unlike inactivated PR virus, nonviable PRRS virus did not elicit virus-neutralizing antibody production. Presumably, an intrinsic property of this pathogen delays the development of the host IFN-γ response and preferentially stimulates the synthesis of antibodies incapable of neutralization.

Keywords: PRRS virus; Interferon-γ; Swine T cell; Polarization of immunity; Neutralizing antibodies; Adjuvant; Cellular immunity; ELISPOT

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is currently considered to be the most significant and economically important infectious disease to afflict swine worldwide. The causative agent, PRRS virus, was first isolated in The Netherlands in 1991 (Wensvoort et al., 1991) and then a year later in the United States (Collins et al., 1992). This pathogen is a member of the Arteriviridae, a family of small, cytolytic, positive-stranded, enveloped RNA viruses (Benfield et al., 1992; Wensvoort, 1993) in the order Nidovirales (Cavanagh, 1997; Conzelmann et al., 1993; Meulenberg et al., 1993; Pringle, 1996). Other members of this family are equine arteritis virus, lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (Conzelmann et al., 1993; Meulenberg et al., 1993; Plagemann and Moennig, 1992). A common biological property of these viruses is their primary replication in host alveolar macrophages (Wensvoort et al., 1991) and some cells of the monocyte lineage (Pol and Wagenaar, 1992; Voicu et al., 1994), from where they may influence the host immune response. Additionally, PRRS virus can localize in various organ systems (Mengeling et al., 1995, 1996; Rossow et al., 1995; Shibata et al., 1997) and produce persistent infections in the absence of viremia (Allende et al., 2000; Van Reeth, 1997; Wills et al., 1997).

Although both clinical and basic research have increased our knowledge about the biology and pathogenesis of the PRRS virus, still little is known about its immunobiology or which components of the porcine immune response may be

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Table 1
Detection of PRRS viremia

Status	Time postinfection (weeks)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Infected[a]	4/3[b]	4/3	3/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−[c]	−	−
Uninfected[d]	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−	−	−

[a] Four pigs were intranasally infected with 10^5 $TCID_{50}$ of virulent PRRS virus strain 12068-96.
[b] Left designation indicates number of pigs considered to be viremic based on positive RT/PCR result, while the right designation represents the number of pigs considered to be viremic based on successful virus isolation.
[c] Only RT/PCR was performed.
[d] Two pigs were mock-infected with sterile saline.

important in establishing protective immunity against this pathogen. An important characteristic of the immunity elicited against PRRS virus is an abundant antibody response that exhibits minimal virus neutralizing (VN) activity (Albina et al., 1998b; Gonin et al., 1999; Nelson et al., 1994; Loemba et al., 1996; Vezina et al., 1996; Yoon et al., 1996). This inadequate production of VN antibody, in combination with the known ability of PRRS virus to produce a persistent infection, lasting up to 150 days (Allende et al., 2000), indicates a potential reliance by the host on cell-mediated immunity to protect itself against this microbe. Although limited studies have focused on this area, it has been demonstrated that infected pigs do develop a transient T-cell-mediated PRRS virus-specific lymphoproliferative response, starting at 4 weeks postinfection and lasting an additional 9 (Bautista and Molitor, 1997) to 14 weeks (Lopez-Fuertes et al., 1999). Targets of this response have been identified as the viral matrix protein and the envelope glycoprotein GP5 (Bautista et al., 1999). Moreover, immunization of pigs with a plasmid encoding GP5 has been shown to stimulate a lymphoproliferative response and provide some protection against challenge with virulent virus (Pirzadeh and Dea, 1998). Infected pigs also exhibit a delayed-type hypersensitivity response to PRRS virus (Bautista and Molitor, 1997), although the importance of this response in regard to protection is not known.

Since host interferon (IFN)-γ production is the major protective mechanism against infections by a variety of cytopathic viruses (Ramsay et al., 1993; Zinkernagel et al., 1996), it is likely that this cytokine plays a role in protection against PRRS virus in pigs. In this regard, IFN-γ mRNA has been detected in the lymph nodes, lungs (Rowland et al., 2001), and peripheral blood mononuclear cells (PBMCs) (Lopez-Fuertes et al., 1999) of PRRS virus-infected pigs. Moreover, through the use of cultured cells, porcine IFN-γ has been shown to block PRRS virus replication (Bautista and Molitor, 1999), apparently by the inhibition of viral RNA synthesis via a dsRNA inducible protein kinase (Rowland et al., 2001).

Despite this intriguing evidence supporting a primary role for a virus-specific IFN-γ response in porcine immunity against PRRS virus infection, a thorough analysis of this possibility has yet to be undertaken. We have now applied a sensitive and quantitative ELISPOT assay that enables enumeration of virus-specific IFN-γ secreting cells (SC) (Zuckermann et al., 1998) to examine the kinetics and magnitude of the IFN-γ response of pigs to exposure to virulent, attenuated, or inactivated PRRS virus. Simultaneously, we also conducted a temporal determination of the appearance and retention of both neutralizing and nonneutralizing anti-PRRS virus antibodies. For comparison, the effects of vaccination with a proven strong stimulator of both humoral (VN antibody) and cell-mediated (IFN-γ SC) porcine immunity, namely a pseudorabies (PR) modified live virus (MLV) vaccine (Zuckermann et al., 1999), were also monitored. Based on the results of these studies, we conclude that PRRS virus induces not only a poor VN antibody response but also a weak T-cell-mediated IFN-γ response that increases gradually in magnitude over a period of months. Moreover, the degree of PRRS virus virulence does not appear to influence the tardiness of this response.

Results

Duration of viremia in pigs infected with wild-type PRRS virus

To examine the kinetics of the development of the immune response to virulent PRRS virus, a group of 8-week-old piglets was intranasally inoculated with the field strain 12068-96. The success of the experimental infection was evidenced by the presence of viremia that could still be detected at 2 weeks postinfection (Table 1). Although at this point viable PRRS virus could be isolated from the blood of only three of the four infected pigs, the presence of virus in the serum of all four pigs was evident based on positive RT-PCR results. In contrast, neither of the two uninfected pigs exhibited viremia.

Humoral immune response to infection with wild-type PRRS virus

A specific antibody response to PRRS virus was detected in the serum of all four pigs within 2 weeks after being inoculated (Fig. 1A). The amount of these antibodies increased until peaking at approximately 9 weeks later, and then declined to borderline positive levels by 28 weeks

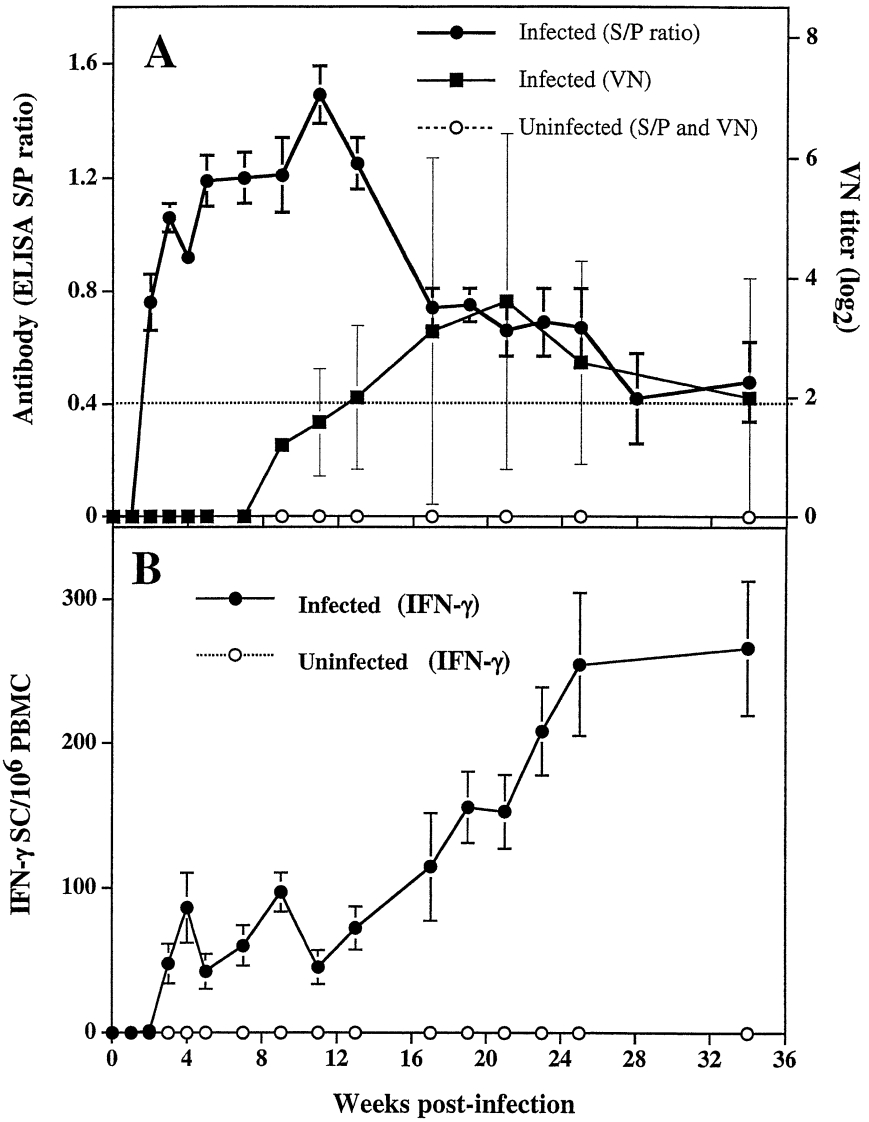

Fig. 1. Kinetics of the humoral and cellular immune responses of pigs to infection with virulent PRRS virus. Groups of pigs were inoculated intranasally with PRRS virus strain 12068-96 or mock-treated with sterile saline. At the indicated times after infection serum and PBMCs were obtained from their peripheral blood. For humoral immunity (A), results are expressed as *S/P* ratios measured by ELISA or as the $\log_2$ of the reciprocal of the largest dilution of serum that inhibited the development of a virus-induced cytopathic effect in cell culture (VN titer). *S/P* ratios <0.4 and VN titers <2 ($\log_2$) are considered to be negative. For cellular immunity (B), the frequency of PRRS virus-specific IFN-γ-SC/10^6 PBMCs in each sample was determined by using an ELISPOT assay. Each value represents the mean response of four (infected) or two (uninfected) pigs ± standard error of the mean (SEM).

postinfection. At this point one of the pigs was considered to be seronegative since the standard-to-positive (*S/P*) ratio of its serum in the ELISA test for PRRS virus was less than the established cut-off value of 0.4. As expected, uninfected animals maintained an *S/P* ratio below 0.4 throughout the study. Unlike the overall rapid production of anti-PRRS virus antibodies reactive in an ELISA assay, VN antibodies against this virus were not detected until 11 weeks postinfection, and then in only two of the four infected animals (Fig. 1A). Maximum titers of 1:16 and 1:4, respectively, were observed 9 weeks later and then declined such that at 34 weeks postinfection one of the two pigs was deemed to be seronegative.

Cell-mediated immune response to infection with wild-type virus

In contrast to anti-PRRS virus antibody formation, the development of cell-mediated immunity as measured by the frequency of PRRS virus-specific IFN-γ-SC in the pig PBMC populations was not detected until the third week postinfection (Fig. 1B). At this time and during the ensuing 10 weeks these values varied approximately twofold within a range of 50–100 IFN-γ-SC/10^6 PBMCs. Afterward, the frequency of PRRS virus-specific IFN-γ-SC cells began to increase and reached an average of 266 ± 35 cells/10^6 PBMC at 34 weeks postinfection. At termination of the

Table 2
Intensity and specificity of the host IFN-γ response to immunization with either porcine reproductive and respiratory syndrome (PRRS) or pseudorabies (PR) MLV vaccine

Immunization[b]	MLV vaccine	Challenge antigen (ELISPOT)[a]		
		Dose (MOI)	PRSS virus	PR virus
Primary	PRRS	1.0	82 ± 25[c]	2 ± 1
		0.1	33 ± 25[d]	1 ± 1
	PR	1.0	3 ± 1	289 ± 82[c]
		0.1	2 ± 1	164 ± 65[d]
Secondary	PRRS	1.0	164 ± 60[e]	20 ± 8
		0.1	45 ± 14[f]	4 ± 1
	PR	1.0	3 ± 1	587 ± 105[e]
		0.1	4 ± 2	362 ± 85[f]

[a] Results are presented as the number of IFN-γ-SC detected per 10^6 PBMC isolated from MLV-vaccinated pigs by an ELISPOT assay after a 20-h exposure of the lymphocytes to the indicated amount of virus.

[b] PBMCs were isolated from the pigs either at 3 weeks after primary vaccination or 2 weeks after secondary vaccination (5 weeks after primary immunization) with MLV.

[c] Difference between the two values is significant ($P < 0.05$).

[d] Difference between the two values is significant ($P < 0.05$).

[e] Difference between the two values is significant ($P < 0.03$).

[f] Difference between the two values is significant ($P < 0.05$).

experiment (48 weeks postinfection) the relative percentage of IFN-γ-SC was further enhanced to 394 ± 45 cells/10^6 PBMC (data not shown). A similar, relatively high frequency of PRRS virus-specific IFN-γ-SC (527 ± 49 cells/10^6 PBMCs) was observed in the blood from 11 boars at 45 weeks after infection with the same strain of PRRS virus. When 4 of these boars were tested 53 weeks later, the average frequency of PRRS virus-specific IFN-γ SC was still 450 ± 50 cells/10^6 PBMC. Thus, the host IFN-γ response to infection with virulent PRRS virus can be characterized as being prolonged in regard to its establishment and being relatively stable once established.

Comparison of the IFN-γ response to immunization with either PRRS or PR modified live virus vaccines

In comparison to that reported for PR virus (Zuckermann et al., 1999), the intensity of the porcine IFN-γ response to infection with virulent PRRS virus appeared to be weak during the 13 weeks immediately following inoculation. To determine if a similar phenomenon occurred with attenuated PRRS virus, the cell-mediated immune responses of age-matched pigs to immunization with either a PRRS or a PR MLV vaccine were monitored (Table 2). By 3 weeks after vaccination with PR virus, the respective animals had developed a group mean frequency of 289 ± 82 virus-specific IFN-γ-SC/10^6 PBMCs. In contrast, pigs receiving the PRRS virus vaccine generated a significantly ($P < 0.05$) lower number of virus-specific IFN-γ-SC (82 ± 25 cells/10^6 PBMCs). Even when the dosage of antigen used as stimulus in the ELISPOT assay was reduced 10-fold, the same relative difference in the frequencies of virus specific IFN-γ-SC was apparent. Since neither group of pigs had IFN-γ-SC that recognized the reciprocal virus, the specificity of both the immune response and the assay was verified. To further examine this difference in the intensity of the cellular immune response, both groups of animals were boosted with the respective original type of vaccine. Although at 2 weeks after this booster immunization the frequencies of both PR and PRRS virus-specific IFN-γ-SC doubled to 587 ± 105 and 164 ± 60 IFN-γ-SC/10^6 PBMCs, respectively, the IFN-γ response to PRRS virus remained significantly lower than the response to PR. Once again, a 10-fold reduction in the amount of viral stimulus used in the assay did not alter the relative difference in the extent of the IFN-γ response to the two viruses. Thus, the intensity of the short-term virus-specific IFN-γ response of swine to either a primary or a booster immunization with a PRRS MLV vaccine was lower than that to its PR virus counterpart.

Effect of coadministration of a conventional adjuvant on the porcine immune response to vaccination with either PRSS or PR modified live virus vaccine

Because of the observed relatively weak cellular immune response to exposure to the PRRS MLV vaccine, enhancement of this immunity was attempted by coadministration of an adjuvant. This approach was considered because inclusion of the commercial adjuvant Imugen was previously found to intensify the IFN-γ response to a PR MLV vaccine (Zuckermann and Martin, unpublished observations). Accordingly, pigs were injected intramuscularly with either a PRSS or a PR MLV vaccine alone or in combination with this adjuvant. Control animals received either adjuvant alone, a commercial inactivated PRRS virus (already formulated with an adjuvant) or a BEI-inactivated PR virus mixed with Imugen. All pigs were boosted with the same respective vaccine formulation 3 weeks later. Just prior to the second immunization, the number of PR virus-specific IFN-γ-SC in the blood of animals receiving the PR MLV vaccine mixed with adjuvant (366 ± 80 cells/10^6 PBMCs) was approximately 2.5-fold greater than that in the blood of pigs vaccinated with PR MLV alone (148 ± 21/10^6 PBMCs) (Fig. 2B). Likewise, enhancements of approximately 1.8-fold (730 ± 68 vs 400 ± 46 cells/10^6 PBMCs) and 1.4-fold (584 ± 57 vs 421 ± 46 cells/10^6 PBMCs) at 1 and 2 weeks after the booster vaccination, respectively, were also found to be associated with the inclusion of adjuvant during PR immunization. In contrast, the addition of the same adjuvant to the PRRS MLV failed to enhance the IFN-γ response to this virus even after a booster immunization (Fig. 2A). For instance, the virus-specific IFN-γ-SC frequency in the blood of pigs at 1 week after the second immunization with PRRS MLV given in the presence or absence of adjuvant was 113 ± 21/10^6 PBMCs and 120 ± 37 cells/10^6 PBMCs; respectively. Moreover, as observed in the first comparative study (Table 2), the cell-mediated immune response to the PRRS virus was consistently lower than that to the PR MLV. Apparently, both

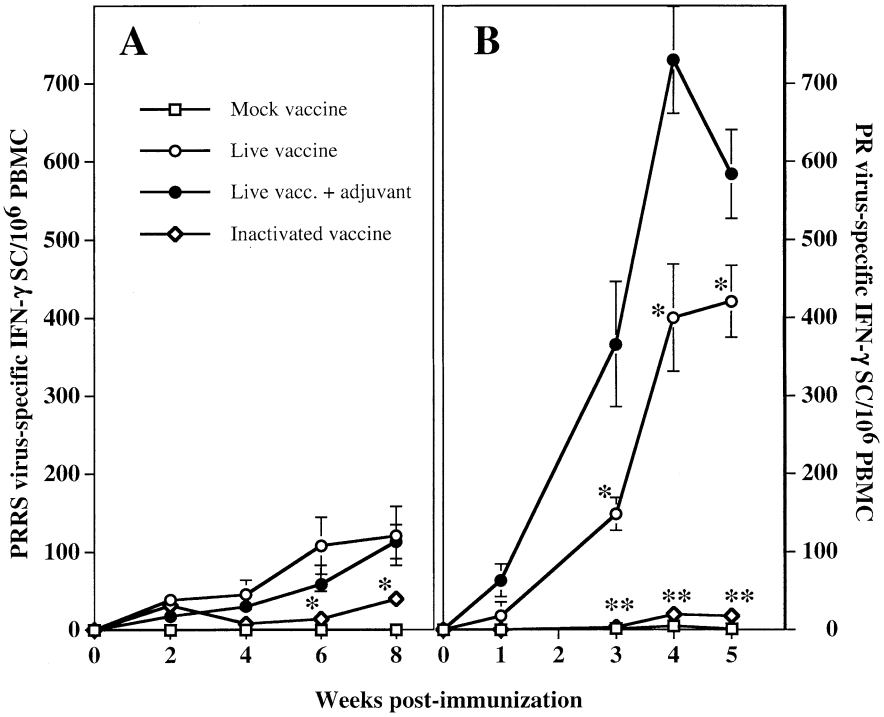

Fig. 2. Comparison of the intensities of the PRRS and PR virus-specific IFN-γ responses of pigs to vaccination either with live virus in the presence or absence of adjuvant or with inactivated virus. Groups of pigs were immunized either with an unmodified, commercially available PRRS (A) or PR (B) MLV vaccine or with the respective vaccines that had first been mixed with the oil-in-water adjuvant Imugen and received a booster of the same formulation 3 weeks later. Additional groups of pigs were immunized either with an inactivated PRRS virus vaccine (A) or BEI-treated PR virus (B) or mock-vaccinated with sterile saline (A, B) and were also boosted 3 weeks later. PBMCs were isolated from the pigs at the indicated times postimmunization and the frequency of PRRS (A) or PR (B) virus-specific IFN-γ-SC was determined by using an IFN-γ ELISPOT assay. Significant differences between the quantities of virus-specific IFN-γ-SC detected in the blood of animals immunized with PRRS MLV in the presence or absence of adjuvant versus those receiving the inactivated PRRS virus vaccine (A) are represented by an asterisk ($P < 0.05$). Likewise, significant differences between the quantities of virus-specific IFN-γ-SC detected in the blood of animals receiving PR MLV in the presence of adjuvant versus those receiving PR MLV in the absence of adjuvant or BEI-treated PR virus (B) are represented by an asterisk ($P < 0.01$) and a double asterisk ($P < 0.001$), respectively. These statistical determinations were obtained by using the FPLSD test. Each value represents the mean response of five pigs ± SEM.

viruses must be viable to elicit the generation of virus-specific IFN-γ-SC, since these entities were not detected in pigs receiving either a commercial inactivated PRRS virus vaccine or BEI-inactivated PR virus (Figs. 2A and B).

Despite the inability of inactivated PR virus to elicit a detectable cell-mediated immune response, it still readily induced a humoral immune response as evidenced by the production of VN antibodies at a level similar to that generated after PR MLV immunization (Fig. 3B). In contrast, a significant VN titer was not obtained in pigs receiving either the live or inactivated PRRS virus vaccine (Fig. 3A). Moreover, even the addition of adjuvant had no stimulatory effect on the ability of the PRRS MLV to elicit production of VN antibodies, although there was an approximately threefold enhancement ($P < 0.05$) of VN titer in the presence of this agent when the PR MLV vaccine was used (Fig. 3B).

Polarization of the humoral and cell-mediated immune responses of swine to vaccination with PRRS MLV vaccine

To ascertain if the host IFN-γ response to attenuated PRRS virus developed with kinetics similar to that observed in pigs infected with virulent PRRS virus (Fig. 1), the period for determining the frequency of virus-specific IFN-γ-SC in pigs vaccinated twice with PRRS MLV was extended past the 2-week postbooster time frame used in the initial study (Fig. 4A). In these animals the cell-mediated immune response varied approximately twofold within a range of 50–100 PRRS virus-specific IFN-γ-SC/10^6 PBMC during the 1 week preceding and the 6 weeks following the second immunization. Thus, no immediate effect of a boosting vaccination on the host INF-γ response was apparent. However, by 18 weeks after the second vaccination, the frequency of IFN-γ-SC had increased to 220 ± 10 cells/10^6 PBMCs, had reached a value of 443 ± 38 cells/10^6 PBMC 14 weeks later, and was even greater at 51 weeks postvaccination (641 ± 24 cells/10^6 PBMCs; data not shown). Remarkably, as seen in the pigs infected with wild-type PRRS virus, the kinetics of the host humoral immune response to the PRRS MLV (Fig. 4B) was the opposite of that of the IFN-γ response. There was a rapid production of anti-PRRS virus antibodies between 2 and 4 weeks post primary vaccination. Afterward, their quantity steadily declined and by 32 weeks after primary vaccination these

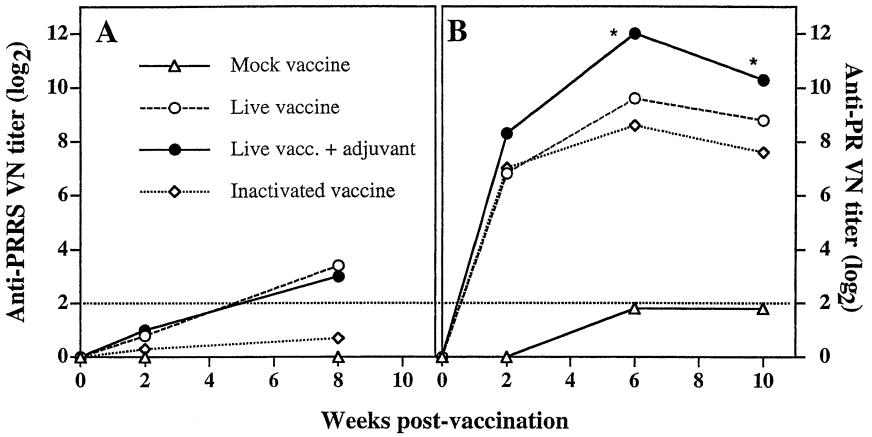

Fig. 3. Comparison of the intensities of the PRRS and PR virus-specific humoral immune responses of pigs to vaccination either with live virus in the presence or absence of adjuvant or with inactivated virus. Groups of pigs were immunized either with an unmodified, commercially available PRRS (A) or PR (B) MLV vaccine or with the respective vaccines that had first been mixed with the oil-in-water adjuvant Imugen and received a booster of the same formulation 3 weeks later. Additional groups of pigs were immunized either with an inactivated PRRS virus vaccine (A) or BEI-treated PR virus (B) or mock-vaccinated with sterile saline (A, B) and were boosted 3 weeks later. Sera were obtained from the pigs at the indicated times postimmunization and the titer of PRRS (A) and PR (B) VN antibody in the blood of these animals was determined by a VN test. Results are expressed as the reciprocal of the highest dilution of serum that inhibited the development of a virus-induced cytopathic effect in cell culture. VN titers >2 ($\log_2$) are considered to be positive. A significant difference between the quantity of VN antibodies detected in the blood of animals immunized with PR MLV in the presence or absence of adjuvant (B) is represented by an asterisk ($P < 0.05$). These statistical determinations were obtained by using the FPLSD test. Each value represents the mean response of five pigs ± SEM.

antibodies could hardly be detected by using an ELISA assay. Presumably, the initial, intense humoral immune response was due to the presence of nonneutralizing antibodies, since only half of the animals had a detectable level of VN antibodies. Moreover, their peak average titer of 2.6 $\log_2$ occurred at 14 weeks post primary vaccination—10 weeks after the maximum amount of anti-PRRS virus antibody was detected by ELISA in the serum of vaccinated pigs.

Phenotypic analysis of PRRS virus-specific IFN-γ-secreting cells

To establish the phenotype of the IFN-γ-SC, antibody-directed, complement-mediated depletion studies were performed with PBMCs isolated from pigs previously vaccinated twice against PRRS virus (Fig. 5). Ninety percent of the PRRS virus specific IFN-γ-SC were eliminated as a result of exposure to anti-porcine CD3 mAb. Likewise, incubation with mAbs reacting with either porcine CD4 or CD8α caused 95 and 96%, respectively, of these cells to be depleted. In contrast, treatment with anti-CD8β antibodies resulted in the elimination of less than 18% of the IFN-γ-SC population. Since exposure to antibody recognizing the porcine B-cell marker CD21 had a minimal reductive effect (1% depletion), PRRS virus-specific IFN-γ-SC appear to be predominately $CD3^+$ T cells that express both CD4 and CD8α, i.e., CD4/CD8 double positive (DP) T cells, with most of the remainder expressing CD8β. The later population represents the classical $CD4^-/CD8\alpha\beta^+$ cytotoxic T-cell population (Zuckermann et al., 1990). In general, these two T-cell subsets may be responsible for the host IFN-γ response to virus invaders, since similar results were obtained in depletion studies conducted with PBMCs originating from PR virus-immune pigs (data not shown).

Discussion

In the present study a remarkable temporal polarization of the host cellular and humoral immune responses to infection by either virulent or attenuated PRRS virus was observed. From its onset at 2–3 weeks postinfection and during the ensuing 10–12 weeks, the IFN-γ response was relatively weak and then its intensity began to increase gradually. In contrast, anti-PRRS virus antibodies incapable of neutralizing this virus were abundant until near the end of this period, when their numbers were dwindling. Coincident with their decline was the appearance of VN antibodies—a phenomenon previously observed during PRRS virus infection of swine (Albina et al., 1998b; Molitor et al., 1997; Nelson et al., 1994; Shibata et al., 2000). Interestingly, temporal segregation of the humoral response is actually directed against two epitopes on the same protein, as indicated by the appearance of neutralizing antibodies recognizing PRRS virus glycoprotein 5 as the level of antibodies reacting with an immunodominant nonneutralizing determinant in this protein declined (Ostrowski et al., 2002). Presumably, this two-step humoral immune response may actually contribute to the continuance of PRRS virus replication (Wills et al., 1997), possibly via antibody dependent enhancement (ADE) of infection (Yoon et al., 1996). If so,

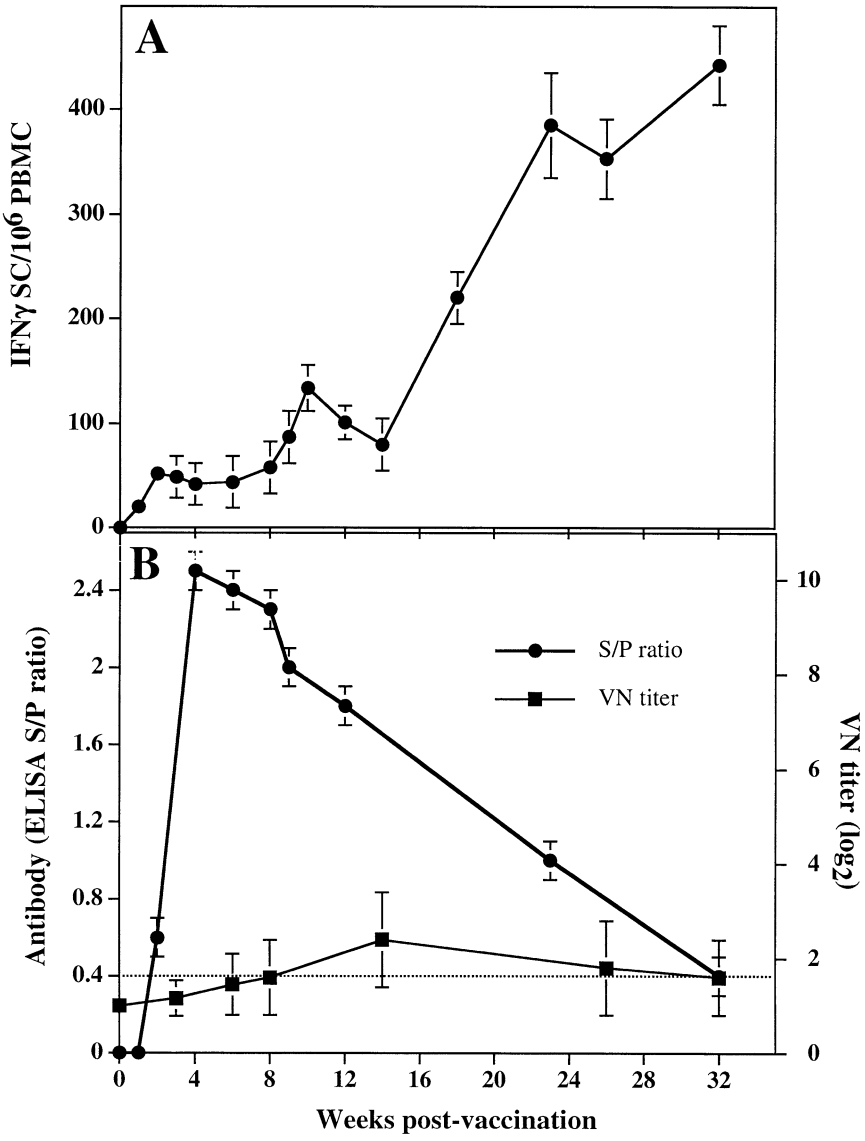

Fig. 4. Kinetics of the humoral and IFN- γ response of pigs to immunization with a PRRS MLV vaccine. A group of pigs were immunized with PRRS MLV vaccine and then were boosted with the same vaccine 3 weeks later. At the indicated times post primary immunization, serum and PBMC were obtained from their peripheral blood. For cellular immunity (A), the frequency of PRRS virus-specific IFN-γ-SC/10^6 PBMCs in each sample was determined by using an ELISPOT assay. For humoral immunity (B), results are expressed as *S*/*P* ratios measured by using ELISA or as the $\log_2$ of the reciprocal of the largest dilution of serum that inhibited the development of a virus-induced cytopathic effect in cell culture (VN titer). *S*/*P* ratios <0.4 and VN titers <2 ($\log_2$) are considered to be negative. Values up to and including 9 weeks represent the mean response of 35 pigs ± SEM. From 10 to 23 weeks, values represent the mean response of 12 pigs ± SEM and thereafter values represent the mean response of 6 pigs ± SEM.

then the initial onslaught of non-neutralizing anti-PRRS virus antibodies may represent a major impairment to the development of vaccine-induced protective immunity. A similar scenario has been found when examining the host response to human immunodeficiency virus (HIV). In this case within 2 months postinfection, most patients develop anti-HIV antibodies that can be detected by using ELISA or Western blot methodology. However, the titer of VN antibodies against the autologous isolate is fairly low or undetectable. Although neutralizing antibodies against T-cell line-adapted variants of HIV-1 are produced, at least >25–53 weeks pass before high titers against these strains are observed (Pilgrim et al., 1997). Because of this situation, the presence of enhancing antibodies against HIV and their potential harmful effects in volunteers immunized with HIV-1 candidate vaccines have been considered to be a major hindrance in the provision of immunity to this virus (Fust, 1997). Thus, both PRRS virus and HIV may possess a similar strategy for evading the potentially inhibitory activity of the host humoral immune response and present a similar challenge to effective vaccination.

That VN antibodies are protective against subsequent PRRS virus infections has been established (Osorio et al., 2002). Sows having acquired a VN antibody titer of 1:16 by

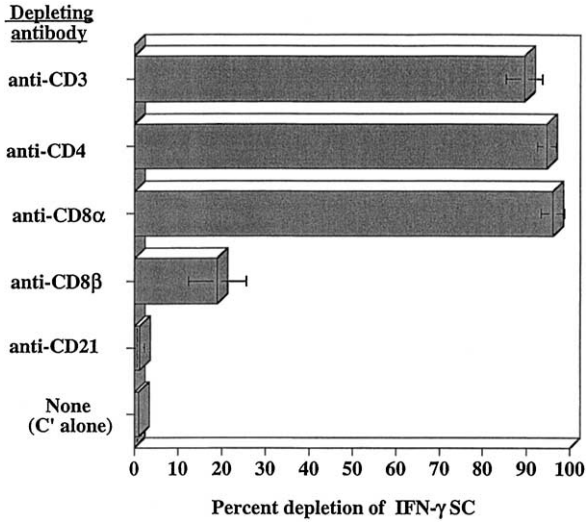

Fig. 5. Phenotypic characterization of PRRS virus-specific IFN-γ-SC. PBMCs from PRRS virus-immune pigs were incubated in the presence of one of the indicated anti-porcine CD mAbs or left untreated. After exposure to complement, the number of PRRS virus-specific IFN-γ-SC present in the unlysed cell populations was determined by using an ELISPOT assay. Results are shown as the percentage depletion, calculated using the formula 100 − [100 (number of spots in antibody + complement-treated well)/number of spots in complement only-treated well]. Data presented represent the average of six independent experiments performed with PBMC from four different pigs.

passive immunity could clear a PRRS virus infection when challenged at 90 days gestation and not experience reproductive failure (abortion). Unfortunately, as observed in the present study, the production of VN antibodies by pigs vaccinated (Mengeling et al., 1999) or infected (Gonin et al., 1999; Nelson et al., 1994; Shibata et al., 2000; Vezina et al., 1996) with PRRS virus is limited (titers are usually ≤1:8) and sporadic. Even our attempt to enhance the ability of a PRRS MLV vaccine to elicit VN activity by including an adjuvant in the vaccine formulation failed, despite the fact that the inclusion of the same adjuvant significantly increased the titer of VN antibodies generated in response to immunization with the PR MLV vaccine. Experimentally, a high titer of PRRS VN antibodies has been obtained in pigs but only after hyperimmunization consisting of sequential injections of wild-type virus mixed with Freund's adjuvant (Nelson et al., 1994; Osorio et al., 2002; Ostrowski et al., 2002). Clearly, the need to undergo such an intensive immunization regime indicates that a dominant and very effective mechanism normally prevents the creation of a strong VN antibody response against PRRS virus. The cause of this prevention is currently unknown, although the presence of decoy immunodominant epitopes is likely responsible (Ostrowski et al., 2002). A similar situation has been observed in the case of HIV, where antibodies against a neutralizing epitope in gp120 are not detected until several months after infection. Instead, antibodies against nonneutralizing epitopes in this protein are produced during the first 6 months following infection (Ho et al., 1991). Thus, it appears that PRRS virus, like HIV, delays the induction of neutralizing antibody formation by focusing the immune response to nonneutralizing epitopes on an immunodominant viral protein (Ostrowski et al., 2002).

Despite the limited humoral immune response to PRRS virus vaccination, pigs do efficiently clear subsequent homologous virus infections (van Woneseel et al., 1998). In fact, PRRS virus-infected pigs lacking detectable amounts of VN antibodies can apparently still eliminate this pathogen (Labarque et al., 2000). Presumably this ability is at least partially dependent on induced cell-mediated immunity, especially the host IFN-γ response.

In this regard, mRNA encoding IFN-γ, a cytokine known to inhibit PRRS virus replication (Bautista and Molitor, 1999; Rowland et al., 2001), has been detected in the lung and lymphoid tissues of PRRS virus-infected pigs (Lopez Fuertes et al., 1999; Rowland et al., 2001). Additional indirect evidence suggesting a role for IFN-γ in mediating protective immunity against PRRS virus includes (i) the demonstration that pigs which have recovered from a previous PRRS virus infection are at least partially resistant to a secondary challenge by the homologous virus strain for up to 604 days after the initial infection (Lager et al., 1997)—a time as shown in the present study when there was still a strong presence of PRRS virus-specific IFN-γ-SC (450 ± 50 cells/10^6 PBMCs) but not of VN antibodies; (ii) the obtainment of a positive correlation coefficient of 0.61 (P = 0.0013) between the frequency of PRRS virus-specific IFN-γ-SC in the lymphocyte population of previously MLV-immunized sows at the time of virulent PRRS virus challenge (90 days of gestation) and the number of subsequent live-born piglets (unpublished observations); and (iii) the observation that of the routinely vaccinated sows at a commercial facility, only those with a frequency of >150 PRRS virus-specific IFN-γ-SC/10^6 PBMCs did not abort as a result of exposure to a natural outbreak of PRRS during their pregnancies (unpublished observations).

Although the number of virus-responsive, cytokine-secreting cells may be underestimated by using the ELISPOT (Waldrop et al., 1997), the comparative approach used in the current study demonstrated that the initial cell-mediated immune response as defined by the frequency of virus-specific IFN-γ-SC was substantially lower to PRRS virus than to PR virus. In addition, the gradual increase in the frequency of PRRS-virus-specific IFN-γ-SC in the peripheral blood of swine during an interval of 16 to 24 weeks after infection or vaccination confirmed that the initial IFN-γ response was weak. The kinetics of this response is unlike the situation in humans and mice, where the frequency of $CD4^+$ T cells specific for other viruses is usually either fairly constant (Flaño et al., 2001; Rentenaar et al., 2000; Waldrop et al., 1997) or decreasing (Homann et al., 2001) during the several months ensuing infection. One exception is the observed progressive increase in the frequency of Sendai virus-specific $CD4^+$ T cells in mice during the first 6 months postinfection (Topham and Doherty,

1998). In this case the expansion was partially attributed to an accumulation of memory cells as the host aged. Such an explanation is not applicable to the present situation, because swine peripheral blood T cells exhibiting a memory phenotype are not readily detected until the animals are >12 months old (Zuckermann and Husmann, 1996) and the pigs used in the present study were terminated by, at most, 9 months of age. A second variance is the observed late emergence (>1 month) of mouse $CD4^+$ cytotoxic T cells in response to infection by *Mycobacterium tuberculosis* (Orme et al., 1992). This delay was proposed to be the result of clonal expansion and an apparent change in the function of previously generated, cytokine (IL-2)-secreting protective $CD4^+$ cells (Orme et al., 1993).

A mechanism, similar to that proposed for the impedance of mouse anti-*M. tuberculosis* cell-mediated immunity, could be operative in regard to the protracted generation of anti-PRRS virus IFN-γ-SC in swine. Thus, a lag in the transition of virus-specific T cells into a predominate population capable of secreting IFN-γ-SC upon interaction with PRRS virus, and not functional inactivation of the T cells, would be responsible. That there are abundant amounts of active T cells in the host early after PRRS virus infection or vaccination is indicated by the differentiation of B cells into ones secreting characteristic immunoglobulins of the G and A isotypes. Without substantial T-cell involvement with virus-specific B cells in the lymphoid tissues, the robust virus-specific humoral immune response detected in both serum and bronchoalveolar lavage fluids by 3 weeks postinfection (Labarque et al., 2000) would not be apparent. That activated T cells can undergo a functional change is supported by the observation that the frequency of PRRS virus-specific IFN-γ-SC can be increased >twofold by culturing PBMCs with viral antigen in the presence of exogenous IL-12 (unpublished observations). Clearly, direct visualization of PRRS virus-specific T cells with MHC class I or class II tetramers would be necessary for the precise measurement of the frequency of virus-specific T cells, regardless of their cytokine expression profile. Alternatively, the use of ELISPOT assays designed to recognize other cytokines such as IL-2, IL-4, or IL-10 would allow for the enumeration of virus-specific T cells presumed to perform other functions. Likewise, the possibility that sequestration of virus-specific T cells into tissues prevents the rapid induction of the generation of IFN-γ-SC is also not plausible. Such isolation events are not compatible with the demonstrated availability of a relatively strong PRRS virus-specific lymphoproliferative response in the peripheral blood of pigs by 4 weeks post PRRS virus infection (Bautista and Molitor, 1997; Lopez Fuertes et al., 1999). Thus, we hypothesize that exposure to either virulent or attenuated PRRS virus initially fails to provide an adequate amount of host-supplied signals necessary for directing the differentiation of a substantial number of virus-specific T cells into virus-specific IFN-γ-SC.

Generally, the interaction of viruses with the precursors of dendritic cells (DC) causes them to undergo a maturation and activation process that renders such cells competent to secrete cytokines that modulate and direct the differentiation of naïve T cells responding to viral antigens (Banchereau and Steinman, 1998; Kadowaki et al., 2000; Steinman, 1991). Of these secreted entities, IFN-α is now recognized as being one that provides a major stimulus for the differentiation of T cells into IFN-γ-SC during a viral infection (Cella et al., 2000; Cousens et al., 1999; Kadowaki et al., 2000). Interestingly, PRRS virus has been shown to be incapable of inducing significant IFN-α production by porcine alveolar macrophages (Albina et al., 1998a; Buddaert et al., 1998; Van Reeth et al., 1999) or PBMCs (Albina et al., 1998a). Thus, a lack of available IFN-α during the development of the primary immunity could be responsible for the limited, initial IFN-γ response. In support of this notion we have observed that providing an exogenous source of IFN-α (unpublished observations) or IL-12 (Murtaugh et al., 2002; Foss et al., 2002) at the time of immunization with a PRRS MLV vaccine accelerates the development and intensity of a virus-specific IFN-γ response to the vaccine. Since, like other viruses, PRRS virus is susceptible to the anti-viral actions of IFN-α (Albina et al., 1998a), the absence or a very low level of this cytokine combined with the initially inadequate virus-inhibitory immunity could contribute to PRRS virus's propensity for prolonged infections and the elicitation of a weak initial IFN-γ response. Therefore, a plausible explanation for the gradual increase of the host IFN-γ response to PRRS virus is that IFN-γ produced by the initially, infrequent virus-specific IFN-γ-SC could promote, during a period of several months, the differentiation of additional naïve T cells into virus-specific IFN-γ-SC. As the frequency of IFN-γ-SC increased, there would be a microenvironment conducive to T-cell differentiation into IFN-γ-SC, resulting in an eventual enhancement of this type of immune response. In this regard, a positive feedback loop whereby IFN-γ stimulates the expression of the T-box transcription factor, T-bet, that in turn promotes IFN-γ production has recently been postulated (Lighvani et al., 2001).

As to the identity of the porcine IFN-γ-SC, the majority exhibited a $CD3^+/CD4^+/CD8\alpha^+$ phenotype based on the results of the antibody and complement-mediated depletions conducted in the present study. Such CD4/CD8α DP T cells are usually associated with immune memory function (Summerfield et al., 1996; Zuckermann and Husmann, 1996). In fact, in swine this cell subset represents the majority of $CD4^+$ memory T cells (Zuckermann, 2000), which have also been shown to have B-cell helper function (Ober et al., 1998). In addition to the memory T cells, a second group composing less than one-fifth of the IFN-γ-SC was identified as being $CD4^-CD8\alpha\beta^+$ T cells which would be expected to function as cytotoxic T cells. Our demonstration that the majority of cells secreting IFN-γ in response to viral antigens are CD4/CD8α DP extends the results by Rodriguez-Carreño et al., (2002), who found that porcine cells

exhibiting this phenotype compose the main population of IFN-γ-SC PBMCs responding to activation with PMA plus the calcium ionophore A23187. Moreover, a counterpart to the swine DP T cell that is found in the peripheral blood of cynomolgus monkeys (reviewed by Zuckermann, 1999) has also been show to produce large amounts of IFN-γ in response to mitogen or CD3 stimulation (Nam et al., 2000). Based on the elicitation of IFN-γ production by CD4/CD8DP T cells under a variety of conditions as well as the fact that this type of cell, when isolated from PRRS or PR virus-immune pigs, is able to secrete IFN-γ after only a short exposure (20 h) to its relevant antigen, this lymphocyte subset can be considered to be composed of effector memory T cells. However, confirmation of this classification according to the guidelines proposed by Sallusto et al. (1999) awaits verification of L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7) expression by these DP cells.

As shown in this study, both virulent and attenuated forms of PRRS virus are capable of delaying a specific IFN-γ response and also of constraining the production of VN antibodies. In the case of the MLV, this intrinsic ability was not overcome by the inclusion of a conventional adjuvant even though the same treatment was very effective at enhancing the level of virus-inhibitory immunity stimulated by a PR vaccine virus. Apparently, use of the conventional adjuvant did not provide the necessary signals to stimulate the development of cell-mediated immunity against PRRS virus. Thus, the induction of this type of virus-inhibitory immunity with the potential to mediate the purging of PRRS virus from the pig is more problematic than simply increasing the immunogenicity of the vaccine. Rather, it seems likely that cytokines, i.e., IL-12 or IFN-α, produced by cells of the innate immune system are required to direct the differentiation of T cells into ones capable of secreting IFN-γ upon exposure to PRRS virus. Indeed, as stated above, providing exogenous sources of either cytokine can enhance and accelerate the development of IFN-γ-SC, but not VN antibodies (Muratugh et al., 2002; Foss et al., 2002; unpublished observations). Thus, the influence of PRRS virus on accessory cell function and antigen presentation as well as regulation of the host immune response by cytokines must be examined. Our future efforts will be directed toward determining the molecular basis of how PRRS virus infection or vaccination primarily elicits a nonneutralizing humoral response from the host and the mechanisms by which the addition of exogenous agents, namely cytokines, are able to increase the intensity of the initial IFN-γ response.

Materials and methods

Viruses and cells

Virulent PRRS virus strain 12068–96 was isolated in September 1995 from a neonate during an outbreak of the severe reproductive form of PRRS affecting a breeding farm located in Johnson County, Nebraska (Sur et al., 1997). The virulent strain VR-2332 was obtained from ATCC (Hanassas, VA). Quantitation of PRRS virus stocks was performed in MARC-145 cells and the titers were calculated and expressed as $TCID_{50}$/ml (Reed and Muench, 1938). All PRRS virus stocks used either for intranasal inoculation or as a source of recall antigen for cellular assays were passaged three times in MARC-145 cells since isolation from an animal (strain 12068–96) or retrieval from an ATCC ampule (VR-2332). MARC-145 cells were grown in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum, 50 mg/ml gentamicin, 5 μg/ml amphotericin B, 110 U/ml penicillin, and 0.2 mg/ml streptomycin (Sigma Chemical Co., St. Louis, MO). The Kaplan (Ka) strain of PR virus was propagated and titrated in MDBK cells as previously described (Zuckermann et al., 1990). Both PRRS and PR virus stocks consisted of virus-infected cell lysates obtained by freezing and thawing monolayers when they showed 75–80% cytopathic effect. Disrupted cells were centrifuged at 1000*g* and 4°C for 15 min and the resulting virus-containing supernatants were stored at −80°C.

Commercially available MLV vaccines for either PRRS (Ingelvac PRRS MLV, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) or PR (PrV/Marker Gold, Syntrovet, Lennexa, KS), as well as an inactivated PRRS virus vaccine (Promise, Bayer, Shawnee Mission, KS), were used in the immunization studies. Also included was PR MLV that had been inactivated by exposure to binary ethylenimine (BEI) (Bahnemann, 1975; Larghi and Nebel, 1980). For this purpose, 20 ml of reconstituted PrV/Marker Gold (3×10^5 $TCID_{50}$/ml) was combined with 2.2 ml of 0.1 M 2-bromoethylamine (Sigma) in 0.2 M NaOH and the resulting solution was rotated at ambient conditions for 18 h. The reaction was then terminated by the addition of 0.44 ml of 1 M sodium thiosulfate. After this treatment the PR virus titer had been reduced to $<10TCID_{50}$/ml. The inactivated PR virus vaccine was mixed with Imugen at a 20% ratio (volume:volume) prior to injection into pigs.

Animals and housing

For studies involving infection with virulent PRRS virus, 8-week-old Yorkshire × Landrace cross-bred weaned pigs and 5-month-old boars of the same cross-breed were obtained from an unvaccinated, PRRS and PR virus-free herd located at the University of Nebraska (Lincoln, NE). For studies involving immunization with either PRRS or PR virus vaccines, 8-week-old pigs were acquired from the PRRS and PR virus-free herd derived from European-bred pigs obtained originally from Genetiporc (Quebec, Canada) and currently located at the University of Illinois Veterinary Swine Research Farm (Urbana, IL). Prior to the beginning of each study, all pigs were confirmed to be free of PRRS virus based on the inability to detect either humoral (ELISA serology; HerdChek PRRS, IDEXX Laboratories, Inc., Westbrook, ME) or cellular (IFNγ ELISPOT) immunity to

PRRS virus. The PR virus-negative status of the pigs was also verified by using virus-specific IFNγ ELISPOT and VN assays. All animals were maintained in a bio-safety level 2 facility with temperature and light cycles regulated to 20°C and 12 h, respectively, and fed a standard corn-based diet ad libitum.

Animal inoculation with virulent virus

In the first infection study, four 8-week old pigs were inoculated intranasally with 10^5 $TCID_{50}$/2 ml (1 ml per nostril) of the virulent PRRS virus strain 12068–96. Two other age-matched pigs remained as uninfected controls. In a subsequent study involving 11 5-month-old boars, the dosage was decreased to $10^{3.8}$ $TCID_{50}$/2 ml (1 ml per nostril). In both cases, infected and control pigs were maintained in separate isolation rooms. Successful virus infection of the pigs was confirmed by detecting either viremia (virus isolation) or PRRS virus genome (RT-PCR) in their blood.

Animal inoculation with vaccine virus

For the first vaccination study, two groups of 8-week-old pigs (5 pigs per group) were injected intramuscularly with either 2 ml of RespPRRS or PrV/Marker Gold MLV and received a second inoculation of the respective vaccine 3 weeks later. Prior to vaccination and throughout the experiment, each group was maintained in a separate isolation room. In the second study, 40 8-week-old pigs were randomly assigned into 5-member groups whose housing location was based on the type of vaccination protocol being performed. On one side of the isolation facility were four units holding pigs immunized intramuscularly with 2 ml of either RespPRRS MLV alone or supplemented with 20% (volume:volume) oil-in-water adjuvant Imugen (Intervet, Inc., Millsboro, DE); Promise inactivated PRRS virus vaccine; or sterile saline. On the opposite end were the animal groups receiving a similar quantity of PrV/Marker Gold MLV alone or in combination with Imugen, inactivated PR virus mixed with Imugen, or sterile saline. In a third vaccination experiment, aimed at monitoring the short- and long-term kinetics of the cell-mediated immune response to PRRS virus, a group of 35 8-week-old pigs were injected intramuscularly with 2 ml of RespPRRS MLV and received a second inoculation with the same vaccine 3 weeks later. The PRRS virus-specific immune response of all 35 pigs was monitored for 9 weeks. Thereafter, because of logistic and economic considerations the size of the group was first reduced to 12 animals and then at 23 weeks post primary vaccination further decreased to 6 pigs.

Detection of PRRS virus in sera

The presence of infectious PRRS virus in the peripheral blood of pigs was determined as described by Benfield et al., (1992). Briefly, twofold serial dilutions of serum in 0.1 ml MEM were added to duplicate MARC-145 monolayers grown in a 96-well cell culture plate. After 3 days of culture at 37°C in a 5% CO_2 atmosphere, positive scores were based on the observation of a characteristic cytopathic effect (Kim et al., 1993). The presence of PRRS virus nucleic acid in serum and semen was determined by using RT-nested PCR of extracted RNA (Sur et al., 1997). The outer set of primers designed to direct the amplification of a 403-bp fragment of ORF6 of PRRS virus isolate 12068–96 were AGGTGCTCTTGGCGTTCTCTATT and GCTTTTCTGCCACCCAACACG, respectively. Forward and reverse primers for the nested PCR were CCTCCAGATGCCGTTTGTGCT and TGCCGTTGACCGTAGTGGAGC and enabled the generation of a 150-bp amplicon. Cycling parameters for both PCRs were: 95°C, 1 min; 60°C, 1 min; 72°C, 1 min in a standard PCR mix with 4 mM $MgCl_2$ for a total of 30 cycles.

Determination of humoral immunity

To measure the presence of PRRS virus-specific antibodies in swine sera, a commercially available ELISA (HerdChek PRRS) was used. Sample-to-positive (*S*/*P*) ratios greater than 0.4 were considered positive. PRRS virus neutralizing antibody titers were measured using modified fluorescent focus neutralization assay as described by Wu et al. (2001). Briefly, 100 μl of twofold serially diluted pig serum was mixed with an equal volume of homologous PRRS virus at a concentration of 2000 $TCID_{50}$/ml, incubated for 1 h at 37°C, and then transferred to a 96-well plate containing confluent MARC-145 cells. After 24 h, the monolayers were fixed in 80% acetone and incubated for 1 h with fluorescein-conjugated anti-PRRS virus monoclonal antibody SDOW-17 (Wu et al., 2001), diluted 1:100 in phosphate-buffered saline (PBS) containing 5% horse serum. Cells exhibiting a cytopathic effect were visualized as fluorescent masses when viewed with a fluorescent microscope. A standard SN assay was used to assess the PR virus-specific antibody response in pigs (Zuckermann et al., 1998). In this assay cytopathic effects were visualized by light microscopy using an inverted microscope. The end point titer of the SN of either test was expressed as the $\log_2$ of the reciprocal of the highest serum dilution that inhibited the development of virus-induced cytopathic effect.

Isolation of peripheral blood mononuclear cells

Swine PBMCs were isolated from fresh venous blood anti-coagulated with 5 mM heparin. Buffy coats containing PBMCs were collected following centrifugation at 450*g* and 20°C for 30 min through a Ficoll–Hypaque 1077 (Sigma) gradient. PBMCs were then washed twice in 15 ml Hanks' balanced salt solution and suspended in RPMI medium (Life Technologies, Gaithersburg, MD) supplemented with 2% fetal porcine serum (Life Technologies), 100 U/ml pen-

icillin (Sigma), 100 μg/ml streptomycin (Sigma), 100 U/ml gentamicin (Sigma), 4 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Life Technologies), 1× MEM nonessential amino acids (Life Technologies), and 250 mM 2-mercaptoethenol (Sigma).

Antibodies

Purified P2G10 monoclonal antibody (mAb) and biotin-labeled P2C11 mAb, which are specific for different epitopes of porcine IFN-γ (Mateu de Antonio et al., 1998), were purchased from BD/Pharmingen (San Diego, CA). Purified mAbs specific for porcine CD3 (8E6), CD4 (74-12-4), CD8α (76-2-11), CD8β (PG164A), and CD21 (BB6-11C9) were purchased from VMRD, Inc. (Pullman, WA).

Determination of cell-mediated immunity

The intensity of the cellular immune response was quantified by utilizing an IFN-γ ELISPOT assay as previously described (Zuckermann, et al., 1999). Briefly, 96-well Immulon II plates (Dynatech, Inc.) were coated with 50 μl per well of a 10 μg/ml solution of mAb P2G10 in 0.1 M carbonate buffer, pH 9.6. After an overnight incubation at 4°C, each well was washed three times with sterile PBS and then incubated with 50 μl of RPMI supplemented with 5% fetal porcine serum for 2 h at 37°C in a 5% CO_2 atmosphere. PBMC from virus-infected, vaccinated, or control pigs were plated at 5×10^5 viable cells per well. In all samples, PBMC were >98% viable as confirmed by vital dye exclusion.

The in vitro recall response to PR virus or PRRS virus was stimulated by the addition of antigen in the form of live virus corresponding to the respective one used for infection or vaccination. To perform a valid comparison between the immune responses elicited by exposure to these two viruses, the dose of recall viral antigen to be used was titrated to be suboptimal in regard to stimulation of the PBMCs. For both viruses, this amount was ascertained to be at a multiplicity of infection (MOI) of between 0.1 and 1.0.

PBMCs were exposed to viral antigen at 37°C in a 5% CO_2 atmosphere for 20 h. This short incubation time permits a direct determination of the frequency of PRRS virus-specific INF-γ-SC in each sample, since this interval is not of sufficient duration for cell replication. Afterward, the cells were removed by washing the wells six times with PBS containing 0.05% Tween-20. Fifty microliters of 0.5 μg/ml biotin-labeled mAb P2C11 in 0.05% PBS–Tween was then added to each well and the plates were incubated for 1 h at 37°C. After washing, 50 μl of 0.31 μg/ml of streptavidin–horseradish peroxidase (SA-HRP; Zymed; San Francisco, CA) was added to each well and and the plates were incubated for an additional hour at 37°C. Excess SA-HRP was removed by washing the wells three times and then 50 μl of TMB membrane peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. Hydrolysis of this compound results in the development of blue spots whose size and intensity are directly proportional to the amount of bound IFNγ. (Zuckermann et al., 1999). The frequency of virus-specific IFN-γ-SC was determined by enumerating the blue spots.

As negative controls, 5×10^5 PBMCs per well were either untreated or incubated with the reciprocal virus (PR or PRRS virus) to which the pig had not been exposed. In all cases the background did not exceed five spots per well and the obtained values were subtracted from the respective counts obtained from the stimulated cells. To confirm the ability of each PBMC sample to produce IFNγ, 5×10^4 PBMCs per well were cultured in the presence of 10 μg/ml of the mitogen phytohemagglutinin. In all instances a positive response was observed.

Phenotyping of IFN-γ-secreting cells

PBMCs were obtained from pigs which had been vaccinated twice at a 3-week interval with RespPRRS MLV 5 months earlier. A 1-ml volume of RPMI medium containing 2.5×10^6 cells was either incubated in the presence of a saturating concentration of one of five (CD3, CD4, CD8α, CD8β, CD21) anti-porcine CD mAbs or left untreated. After 1 h at 4°C, 1 ml of rabbit complement (HLA-ABC/DR Typing grade; Pel-Freeze, Brown Deer, WI) diluted 1:12 in RPMI medium was added to each tube. After an additional hour at 37°C, the cells were washed twice with PBS and suspended in 1 ml of RPMI medium. One hundred microliters of each cell preparation was then plated in triplicate mAb P2G10-coated wells in a 96-well plate and exposed to PRRS virus. After 20 h, the PBMCs were removed by washing and the bottom surface of each well was tested for IFN-γ deposition using the ELISPOT assay.

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