Antibody repertoire development in fetal and neonatal piglets. XXIII: Fetal piglets infected with a vaccine strain of PRRS Virus display the same immune dysregulation seen in isolator piglets

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Abstract

The Ig levels and antibody repertoire diversification in fetal piglets infected with an attenuated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) were measured. Serum Ig levels were generally elevated in PRRSV-infected fetuses; IgG was elevated >50-fold, IgM > 5–15-fold and IgA > 2-fold compared to control fetuses. Their IgM to IgG to IgA profile was the same as that in isolator piglets infected for the same period with wild-type PRRSV. Fetal animals showed less repertoire diversification than even isolator piglets that were maintained germfree (GF) while the repertoire diversification index (RDI) for PRRSV-infected isolator piglets was 10-fold higher and comparable to littermates infected with swine influenza (S-FLU). However, when expressed as the RDI:lg ratio, infected fetuses appeared 10-fold less capable of repertoire diversification than uninfected littermates and GF isolator piglets. Compared to S-FLU isolator piglets that resolve the infection, the RDI:lg of PRRSV-infected isolator piglets was 100-fold lower. Overall, infection of fetuses with an attenuated virus shows the same immune dysregulation seen postnatally in wild type infected isolator piglets, indicating that: (a) attenuation did not alter the ability of the virus to cause dysregulation and (b) the isolator infectious model reflects the fetal disease.

1. Introduction

Our laboratory is interested in how environmental factors affect the development of the neonatal immune system. Using an isolator piglet model we previously demonstrated that colonization of the GI tract or administration of purified PAMPs (signature molecules for Pathogen Associated Molecular Patterns) was necessary for humoral immune responsiveness to irrelevant thymus independent (TI-2) and thymus dependent (TD) antigens [1,2]. Since viruses also display PAMPs, such as dsRNA, we infected isolator piglets with swine influenza (S-FLU) to see if they would exhibit features of adaptive immunity. These piglets made antibodies to S-FLU and trinitrophenyl (TNP) given as TNP-KLH or TNP-Ficoll (Butler et al., unpublished data). Colonization of isolator piglets with wild-type Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) caused polyclonal B cell activation, autoimmunity, lymphoid hyperplasia and hypergammaglobulinemia [3]. We found that <1% of the IgG in these hypergammaglobulinemic piglets was antibody to PRRSV suggesting an abnormal development of the adaptive immune response. A similar polyclonal syndrome is produced in mice by lactate dehydrogenase-elevating virus (LDHV), a related virus to PRRSV [4–6]. We also showed that viral infection with PRRSV was associated with expansion of B cell clones with especially hydrophobic HCDR3 sequences that are characteristic of the pre-immune repertoire [7,8]. This observation suggested that PRRSV infection failed to stimulate robust diversification of the antibody repertoire but merely expanded the pre-immune repertoire in polyclonal fashion. We have since expanded our knowledge of VH gene usage during development allowing us to quantify the degree of antibody repertoire diversification [9]. This quantitative tool allows us to re-address the issue of how PRRSV infection affects the development of adaptive immunity. Instead of employing germfree isolator piglets, we conducted the current study using fetal piglets because: (a) like germfree isolator piglets they have had no contact with bacteria, PAMPs or maternal antibodies, (b) PRRSV naturally infects fetal piglets while infection of isolator piglets is considered contrived by some critics and (c) the design allowed a larger numbers of animals to be studied in a more cost-effective manner.
PRRSV is an enveloped, positive-sense, single-stranded RNA virus that is a member of the Arteriviridae family in the order Nidovirales [10, 11]. It was first discovered in 1991 and within a few years became pandemic resulting in major economic loss to the swine industry [12]. Like many other viral infections, PRRSV and LDHV are associated with immune dysregulation in conventional animals. This includes polyclonal B cell activation, the appearance of autoantibodies, vasculitis and deposition of complexes in the kidney [13–21]. As we reported, this immunopathology is exacerbated in isolate piglets, whether bacteria-free or colonized with benign *Escherichia coli* [3]. For this reason, we consider the isolate piglet model to be a sensitive indicator of virus-induced immunopathology. Because events are not compromised by other environmental and maternal influences, identifying the factor(s) responsible for the immune dysregulation should be easier in fetal and isolate piglets than in conventionally reared animals. PRRSV is highly infectious with pigs being infected with less than 50 cell culture infectious units. The virus can persist in pigs for 150 days [22] whereas S-FLU infection, which also produces lung lesions in piglets, is resolved within 7–10 days [23]. Since S-FLU does not cause immune dysregulation (Butler et al., unpublished data), the immune disorder induced by PRRSV constitutes a virulence factor. Therefore we wanted to test whether inoculation of fetal piglets with an attenuated vaccine strain of PRRSV had the same features as postnatal infections with wild type PRRSV. This is biologically relevant since PRRSV crosses the placenta resulting in fetal loss [24].

We addressed events in the systemic portion of the fetal immune system by studying the spleen. The spleen is an important organ regulating lymphocyte recirculation with about 2.7 × 10^11 cells passing through it daily, about 30 times the blood lymphocyte pool of the animal [25, 26]. In systemic infections like hog cholera, splenitis reflects the response of the host to viruses that has reached the blood. This also occurs with other viral diseases, bacterial infection and parasitosis [27–30]. In a natural PRRSV infection the virus has a tropism for the respiratory tract that develops into a systemic infection of the pig and the immune response can be monitored by collecting cells from a bronchoalveolar lavage and tracheal bronchial lymph nodes (TBLN). Presumably, a natural fetal infection is through a hematogenous route or the ingestion of amniotic fluids (the virus crosses the placenta and enters the fetal circulation or amniotic fluids) that results in a systemic infection that is typically fatal. Monitoring the fetal immune response is difficult because of limited quantities of tissues and the lung is not functional and filled with amniotic fluid. Moreover, the TBLN are under-developed in the fetus. Lymphoid tissue in the small intestine of swine occurs as single lymphoid folicles (jejunal Peyers patches, JPP) or as groups of aggregated and continuous lymph follicles called the ileal Peyers patches (IPP). In the large intestine lympho-glandular complexes with undefined function are also found [31]. The IPP is well developed in late gestation, while the JPP do not develop until the gut is colonized. The role of the IPP in pig development is not yet clarified. They retain their typical morphology only for a short period after birth [32] and postnatal resection of the JPP suggests they are not primary lymphoid tissue [33]. We have proposed they represent a type of first responder mucosal lymphoid tissue and selected them here to represent the local immune response.

We report here on serum Ig levels and repertoire diversification in infected and control fetuses and compare these to germfree (GF) isolate piglets, isolate piglets infected with wild type PRRSV, S-FLU or colonized with commensal gut flora. We show that infected fetal piglets behave like PRRSV-infected isolate piglets having elevated serum Ig levels without a concomitant diversification of the antibody repertoire.

### 2. Materials and methods

#### 2.1. Fetal piglets

At day of gestation 70, two Gilts underwent a cesareanotomy to inoculate fetuses with an attenuated PRRSV (Inelvac PRRS, St. Joseph, MO) following previously published procedures and the animal use guidelines of the National Animal Disease Center, Institutional Animal Care and Use Committee. Ten fetuses were inoculated in each litter beginning with the fetus located next to the tip of the left uterine horn, allowing the others to serve as controls (Table 1). Each fetus was located by palpation of the uterus and received a trans uterine intramuscular inoculation with 0.2 mL of 5 × 10^7 CCID50/mL of virus. Control fetuses received a sham inoculum of 0.2 mL of MEM. Gilts were euthanized 28 and 35 days post infection (dpi) to collect fetal tissues. Blood was collected from each gilt at the time of surgery and necropsy, and from each fetus at the time of necropsy. Gilt and fetal sera were tested for infectious virus, for PRRSV-specific antibody by ELISA (HerdChek PRRS 2XR ELISA: IDEXX Laboratories, Westbrook, ME) and Ig levels by sandwich ELISA. Control animals that became sero positive were moved to the immunized category (Table 1). The condition of each fetus was evaluated and spleen and IPP were collected from each fetus and frozen in liquid nitrogen for subsequent molecular biology studies.

#### 2.2. Isolate piglets

Piglets used in isolate studies were recovered by cesarean surgery and reared in germfree isolators as previously described [34, 35]. Briefly, treatment groups of 4–6 piglets were obtained from three gilts and distributed so that each group contained offspring from all three gilts (Table 1). At 7 days of age, piglets in appropriate groups were inoculated intranasally with 10,000 CCID50 of PRRSV (strainVR-2332) or S-FLU (Iowa 1930 H1N1) or maintained germfree. An additional group was colonized with a defined probiotic flora [36]. Only small numbers of isolate animals were used since data from large numbers of these animals have been previously published. Various tissues and blood were collected at necropsy.

#### 2.3. Measurement of serum IgM, IgG, and IgA

The serum levels of Iggs were quantified by sandwich ELISA as previously described [1, 34]. Briefly, IgA was captured using rabbit anti-swine IgA (B53768H) and detected using mAb 1459 (provided by Klaus Nielson, Nepean ONT CA), followed by goat anti-mouse IgG conjugated to alkaline phosphatase (AP) (Sigma–Aldrich, St. Louis, MO). Rabbit anti-swine IgM (4984) was used to capture IgM and mAb 160 was used for its detection (also provided by

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^a S-FLU, infected with swine influenza virus; PRRSV, infected with Porcine Reproductive and Respiratory Syndrome Virus.
^b All piglets were infected.
^c Only small numbers of isolate piglets were used since the data obtained are consistent with previous studies obtained using 36 S-FLU: 10 controls; and 20 PRRSV piglets (see Section 2). In previous studies all virus-infected isolate piglets were seropositive.
Klaus Nielson), followed by goat anti-mouse IgG conjugated to AP. IgG was captured using rabbit B78 (3 × Abs on F(ab)₂) and detected using biotinylated B78 prepared as described by Pierce Chemical (Rockford, IL). Extravidin conjugated to AP (Sigma–Aldrich, St. Louis, MO) was then applied. Para nitrophenyl phosphate (Thermo Scientific #34045) was the substrate used in all assays. Methods for the preparation of all polyclonal Abs and purified IgM, IgG, and IgA reference standard sera have been previously described [34,37].

2.4. Preparation of DNA and cDNA

IPP and spleen that had been stored in liquid nitrogen were pulverized and DNA extracted into Danazol as previously described [38]. RNA was extracted from the same tissues and then converted to cDNA [39]. V(D)J rearrangements from cDNA were recovered using a semi-nested PCR using 5’ FR1 and anti-sense JH followed in the second round by 3’ FR1 and anti-sense JH [40]. The products were tested for size on an agarose gel, cloned into pCR4 TOPO and propagated in Top 10 cells following manufacturer’s recommendations.

2.5. Determination of VH usage and the repertoire diversification index (RDI)

Bacterial colonies were transferred to 96-well plates and grown overnight. Bacterial cells were then lysed and plasmid DNA was recovered and transferred to a nylon membrane [38,40]. Plasmid DNAs transferred to the membranes were then sequentially hybridized with VH gene specific probes for sequences in CDR1 and CDR2 of the major genes [9,40]. In swine, seven major VH genes account for >90% of the repertoire. Those that hybridized with both a CDR1 and a CDR2 probe are recorded as full hybridization (see below). Those that hybridized only with a pan-specific probe for FR2 are designated as unknown (UNK) clones or “non-hybridizing” VH clones. Clones that hybridized with only one CDR probe (either CDR1 or CDR2) were separately enumerated and are included in the RDI equation (see below). Since sequential hybridization of the VH-containing clones on membranes with up to eleven probes is labor intensive, we developed a cocktail system in which one cocktail comprised a mixture of CDR1 gene-specific probes and a second mixture of CDR2 gene-specific probes. The hybridization methods, including the use of cocktails have been repeatedly analyzed and tested [9,38].

Hybridization results were used to calculate a repertoire diversification index (RDI) using the equation below. The UNK category contains a few seldom-encountered VH genes but mostly comprises mutated versions of frequently used VH genes [9,40]. Mutations increase in frequency with environmental antigen exposure causing an increase in the number of UNK clones as well as clones that bind only one CDR cocktail.

\[
\text{RDI} = \frac{\text{UNK}^A + [\text{CDR1} + \text{CDR2}]^B}{V_{\text{H}A}, V_{\text{H}B}, V_{\text{H}C}, V_{\text{H}E}, V_{\text{H}F}}
\]

where superscript a represents UNK which are non-hybridizing Vᵢ gene clones; b represents clones hybridizing with only one CDR-specific probe; c represents full hybridization with any of these major VH genes.

2.6. Statistical analysis

Mean differences were examined using the Student t test and ANOVA programs provided in the statistical package of the Prism program.

3. Results

3.1. Virus isolation and serology

Both gilts were seronegative prior to surgery but developed antibody to PRRSV by the time of necropsy. PRRSV was not isolated from the pre-surgery or necropsy serum samples. In the first litter, one fetus died and was undergoing mummification (a common occurrence in swine). Fetuses #3–11 were alive and normal in appearance. PRRSV was isolated from all live fetuses. Three fetuses tested positive by ELISA (piglets #1, 4 and 9) and #10 was suspect. In the second litter necropsied 35 days post surgery, all 10 inoculated and 5 sham-inoculated fetuses were alive and normal in appearance. Virus was isolated from all inoculated fetuses and from fetus #11 (the sham-inoculated fetus adjacent to fetus #10). Virus was not isolated from fetuses #12–15 and these served as controls. Four fetuses (piglets #2, 4, 6, and 8) were ELISA positive and two other were suspect. Altogether samples were recovered from 22 infected fetuses, seven of which were seropositive and four controls that remained virus free and seronegative (Table 1). One sham inoculated fetus in each litter became infected and was removed from control status.

3.2. Serum IgG and IgM levels were significantly elevated in PRRSV-infected fetuses

Serum IgG and IgM were >50-fold and >5–15-fold higher in animals 35 dpi than in control fetuses (Fig. 1A). IgA levels were similar to control fetuses. This finding differs somewhat from the elevated IgA levels observed in 5-week PRRSV-infected isolator piglets [8]. The Ig levels of control fetuses closely resemble those in 5-week GF isolator control piglets except that IgM levels were marginally higher in the GF isolator group at 28 dpi (P=0.065).

IgG levels in PRRSV-infected fetuses were the same as in colonized and S-FLU infected isolator piglets but significantly lower in PRRSV-infected isolator piglets (P=0.0001). Fetal IgM levels were higher than in S-FLU isolator piglets but lower than in 5-week PRRSV-infected piglets or those colonized with the probiotic flora (Fig. 1A). Only a small number of isolator piglets were used in this study since data on large numbers of colonized piglets, GF piglets and those infected with PRRSV and S-FLU have been previously published [1,3,8,9] (Butler et al., unpublished data). Levels reported here are consistent with those previous studies.

3.3. The immunoglobulin profile of PRRSV-infected fetuses was similar to that in 5-week PRRSV-infected isolator piglets

Fig. 1B compares the profile of Ig levels in the sera of piglets in the various treatment groups by showing the relative proportion of the three major Ig isotypes. Results show that the profile seen in infected fetuses was statistically indistinguishable from the patterns in PRRSV-infected isolator piglets; both infected for the same period. The proportion of IgM in infected fetuses and 5-week PRRSV-infected isolator piglets was significantly lower than that in controls (P=0.01; P=0.017; P=0.047; at 28 and 35 dpi and in 5-week isolator piglets respectively). Thus, fetal and GF isolator controls differed from PRRSV-infected fetuses especially because of difference in the profile of IgM (P=0.07). The S-FLU and colonized piglets were the most different; S-FLU animals had a profile that greatly favors IgG (P=0.0024) while colonized piglets had a profile that favored IgM (P=0.0016).
3.4. Viral infection and age are associated with repertoire diversification

Fig. 2 shows that fetal PRRSV-infection for 28 or 35 days results in increased repertoire diversification compared to controls ($P \leq 0.001$ and $P \leq 0.01$ respectively). The total number of clones examined from animals in a particular group is indicated in the figure; this was on average 70 clones per animal. The RDI for spleen and IPP is the same. A two-sided ANOVA indicated that the RDI for GF isolate piglets was significantly higher than in all fetal piglets ($P < 0.05$) and that S-FLU and PRRSV pigs had a significantly higher RDI than GF isolate piglets ($P < 0.04$ and $P < 0.03$, respectively). Since the RDI is higher in GF isolate controls than in all fetal animals, this must be either age-related or the consequence of exposure to dietary antigens. Again, IPP and spleen do not differ ($P \geq 0.2$). Fig. 2 also shows that the RDI for S-FLU and PRRSV isolate piglets is 10-fold higher than that for PRRSV-infected fetuses ($P \leq 0.01$). Furthermore, all PRRSV-infected isolate piglets were seropositive [3] whereas only seven of 22 infected fetuses were seropositive (Table 1).

3.5. Repertoire diversification in PRRSV-infected piglets is retarded relative to the increases in serum Ig.

An increase in serum Ig levels, especially IgG, typically occurs as a result of an adaptive immune response. In fetal piglets a ratio was calculated involving RDI and total Ig (including IgG, IgM and IgA) levels while with PRRSV-infected isolate piglets, only IgG levels were used for the ratio. Compared to S-FLU, colonized, GF and control fetal piglets, the degree of repertoire diversification in PRRSV-infected piglets does not parallel the increase in Ig levels (Fig. 3). The ratio in PRRSV-infected fetal piglets was lower than that in controls ($P \leq 0.018$) and the ratio in PRRSV isolate piglets was lower than for GF controls, S-FLU piglets and colonized piglets ($P \leq 0.02$; $P \leq 0.03$; and $P \leq 0.001$, respectively).

4. Discussion

B cell development in fetal piglets begins in the yolk sac at DG 20, can be detected in the liver by DG 30 and B cells appear in the spleen by DG 45 [41,42]. The bone marrow is active by DG 60
and serum Ig levels of major isotypes progressively increase from this time until birth [43]. During the fetal period in swine the antibody repertoire is poorly diversified and somatic hypermutation (SHM) is <10 mutations per kilobase [9]. In postnatal piglets exposure to infectious agents can result in a 5-fold increase in SHM and a 2–3 log increase in the RDI [33]. It is generally accepted there is an age-dependence in immune responsiveness which may explain why not all fetuses were seropositive. In humans, this belief partially shapes the strategy for childhood vaccinations. This may, in part, result from interference by passively acquired maternal antibodies [35,44]. Fetal and isolator piglets differ in age but neither receive maternal antibodies, yet behave similarly to PRRSV. However, this is not a perfect comparison, since we used an attenuated vaccine strain of PRRSV derived from the VR-2332 isolate to inoculate fetal piglets. The attenuated virus was chosen to study the fetal immune response because wild-type parental virus produces a fatal infection in the fetus and the attenuated virus is able to replicate in fetuses for at least 6 weeks without clinical effects such as abortion (unpublished observations). The challenge dose (1000 infectious units) was selected to mimic the challenge dose we used for wild-type virus in isolator piglets that develop hypergammaglobulinemia, express autoantibodies and suffer the accumulation of immune complexes in the kidney and vessels [3]. The hypergammaglobulinemia appears to result from the expansion of particular B cell clones of the pre-immune repertoire but with little diversification of the pre-immune repertoire [8].

Since our earlier publication we have developed methods to quantify repertoire diversification, a key feature of adaptive immunity [9]. Adaptive immunity in higher vertebrates is characterized by antibody repertoire diversification such as can occur by somatic hypermutation (SHM) or somatic gene conversion (SGC), the latter being the major mechanism in chickens [45,46]. SHM is increased in the CDRs of rearranged VDJ which hinders or prevents the binding of CDR-specific probes. Quantitation of an RDI which we have done strengthens the data. Furthermore SHM, SGC and class-switch recombination (CSR) occur in parallel because all are mediated by activation-induced cytidine deaminase (AID) [47,48].

Here we report that the hypergammaglobulinemia seen in PRRSV-infected isolator piglets infected with wild type PRRSV was also seen in fetal infected piglets infected with an attenuated virus (Fig. 1A). Furthermore, the profile of Igs in serum is identical to that seen in PRRSV-infected isolator piglets (Fig. 1B). This indicates that: (a) our previously published data are not simply artifacts of the isolator system and (b) attenuated PRRSV produces the same immune dysregulatory effects as wild type virus. Since PRRSV is also a fetal disease and fetal animals also lack gut flora and passive antibody like those reared in isolators, studies reported here are biologically relevant to the etiology of the disease in conventional swine fetuses. They are also relevant since the attenuated virus used is also a vaccine strain, meaning it can also cause the same immune dysregulatory effect we saw in isolator piglets [3].

Specifically, serum Ig levels are elevated without a concomitant increase in the RDI (Fig. 3) supporting our previous conclusion that hypergammaglobulinemia in PRRSV-infected piglets is largely the results of the expansion of the pre-immune B cell repertoire rather than diversification of that repertoire [3,8]. This contrasts with the pattern seen with S-FLU infection and with colonization of the GI tract (Fig. 3). This may be a virulence feature since infection with S-FLU in isolator piglets is resolved in 7–10 days and lesions in <3 weeks whereas PRRSV infections are persistent. If fetal (or neonatal) PRRSV infections continue to cause an increase in serum Ig levels without stimulating repertoire diversification that should lead to highly specific anti-viral antibodies, it could explain the unexpectedly small proportion of PRRSV-specific antibodies [3]. Furthermore the hypergammaglobulinemia caused by PRRSV can compromise further B cell differentiation by FcγRIIB-mediated feedback inhibition [49,50]. In addition, hypersecretion of autoantibodies encoded in the pre-immune repertoire can exacerbate the immune pathology of PRRSV infections [3].

The response of conventional young pigs to infection with PRRSV has not been evaluated using the same criteria that we have used for fetal and isolator pigs. However, numerous studies characterizing the humoral PRRSV immune response in pigs have shown a rapid antibody response to PRRSV beginning 7–10 days post infection when measured by ELISA. Despite this rapid onset of antibody, the development of virus neutralizing antibody is slow occurring 5–6 weeks post-infection. Although the magnitude of the neutralizing response appears low, it typically precedes the clearance of detectable viremia suggesting the humoral response can play a role in the eventual clearance of the virus. Similar to isolator
pigs, conventional‐raised animals also experience polyclonal B cell activation, lymphoid adenopathy and renal lesions [19–21]. Preliminary data cited in reviews suggest that the immune dysregulation we have described is partially ameliorated in colostrum-deprived piglets that are naturally delivered and reside with surrogate mothers [51]. This could suggest that immune regulatory cytokines and other factors in colostrum might be responsible for reducing immune dysregulation. The possible immune regulatory effect of lacteal secretions has been reviewed [52]. We do know that colonization with benign E. coli has no effect on the immune dysregulation [3] but other members of normal gut flora might have different effects. In any case, the isolator piglet model permits both of these possibilities to be experimentally tested with a minimum of confounding factors.

Our findings are especially interesting because of the similarity of results obtained with the wild-type PRRSV isolate used in the isolator pigs (VR-2332) and the attenuated VR-2332 virus used in the fetus, which is also used in a commercial vaccine. Whatever the viral property that produces the immune dysregulation in isolator pigs, it seems unaffected by the attenuation process that required successive passages of wild-type virus through cell culture. This could compromise the efficiency of the vaccine. We have argued in USDA grant proposal to no avail that it would be wise to develop a PRRSV mutant for vaccine use that does not produce immune dysregulation. The lower degree of repertoire diversification seen in fetal piglets is consistent with the conventional paradigm regarding the age‐dependence of immune competence. In any case, the similarities between the fetal and isolator pig response indicate that the isolator pig model is relevant to the natural fetal infection and can be utilized to characterize the porcine immune response in the well controlled environment provided by the isolator piglet model.

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References


