RESEARCH REPORT
SWINE HEALTH

Title: B Cell Repertoire Diversification and Class Switch in PRRS – **NPB #05-174**

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Industry summary

Successful PRRSV vaccines and vaccination strategies are best employed when the host's immune response to the pathogen is understood. Since it was generally known that PRRSV caused non-virus specific polyclonal B cell proliferation, we choose to verify and characterize this phenomenon using germfree isolator piglet so that the effect of PRRSV could only be attributed to the viral infection. We choose to address two features of the antibody response that could characterize this effect: the development of the variable region antibody repertoire (which determines specificity) and class switch recombination (CSR) that determined antibody function.

Studies on repertoire development further characterized the pre-immune VH repertoire (Butler et al 2006) and we used this information to strongly confirm the non-specific polyclonal activation of pre-immune B cells (Butler et al 2007, 2008). These findings suggest that PRRSV produces or stimulates the host to produce a "B cell superantigen" that results in a massive expansion of B cells and non-specific antibodies which subvert the host's immune system from its normal role of making effective anti-viral antibodies (Butler et al 2008). The industrial/therapeutic implication should be to encourage the production of a vaccine strain which does not cause polyclonal B cell activation but rather allows the energy of the host to be used for an anti-viral response.

The second goal, to investigate CSR, has not yet shown that there is any preferential use of an IgG subclass in PRRSV-infected piglets versus littermates infected with swine influenza (SIV). Thus, the delay in an effective neutralizing response to PRRSV is unlikely to be due to CSR to a non-neutralizing IgG subclass antibody. In addressing the subclass issue, we first completed the characterization of the six IgG subclasses of

swine, analyzed their predicted functional capabilities and their relative usage. The studies have generated two major publications (Butler and Wertz, 2006; Butler et al 2008) and one submitted manuscript. The importance of these studies is that they will allow future investigators to better understand the potential of the porcine IgG subclass antibodies in responding to important and emerging epizootic pathogens.

Scientific Abstract

Germfree isolator piglets were used to determine the effect of PRRSV on the B cell repertoire. These studies showed that PRRSV causes proliferation of pre-immune B cells and by-passes antigen-driven repertoire diversification. This can explain the high levels of Igs of which few are PRRSV-specific and also the autoantibodies that characterize PRRSV-infection of isolator piglets. We propose that PRRSV accomplishes this by supplying or stimulating the production of a B cell superantigen. A vaccine strain lacking this property would be desirable.

Research into IgG subclass usage in PRRSV-infected piglets revealed a new major IgG subclass, IgG3 and resulted in the near completion of the characterization of the IgG subclass genes; there are six subclasses and at least two alleles for all but IgG3. Preliminary data indicate no preferential CSR to an IgG subclass in PRRSV-infected piglets that might explain the delay in the appearance of VN antibodies. Thus it is unlikely that the delay is due to the use of an ineffective IgG subclass and more likely due to the lack of repertoire diversification that would normally result in high affinity VN antibodies.

The results obtained when addressing both objectives strongly indicates the value of NPB-funded research in basic immunology of swine. It resulted in five peer-reviewed publications in high profile journals and contributed to three reviews. It is hoped that the discoveries made will have long term benefit in vaccine development, monitoring immune response and in swine management.

Introduction

PRRSV uses non-virus specific B cell proliferation as a virulence factor to produce immune dysregulation. Successful PRRSV vaccines and vaccination strategies should be designed to prevent viral disruption of the immune system. This can be managed by two separate approaches: Engineer a vaccine that stimulates T-cell dependent anti-viral immunity *without* causing polyclonal B cell proliferation or administer a therapeutic such as antagonistic CpG oligos that separately block the proliferation. However, before undertaking either

approach, unambiguous proof at the molecular level is needed that the immune response to PRRSV by-passes both T cell dependent repertoire diversification (first objective) and Class Switch Recombination (CSR; second objective). These are independent criteria for testing this hypothesis and are proposed herein.

Objectives.

First Objective. Monitor the degree of V_H antibody repertoire diversification in PRRSV-infected piglets by determining V_H gene usage in blood, bronchial lymph nodes, tonsil and MLN and comparing usage to that in germfree piglets (negative control) and parasite-infected animals (positive control). If PRRSV *does not* cause V_H repertoire diversification of either IgM transcripts or switched isotypes (represented by IgG) as in parasite-infected piglets, it would mean that PRRSV is elevating Ig levels by proliferation of the B cell population that comprises the pre-immune repertoire without specific refinement (see below) so the available energy for adaptive immunity is diverted away from producing highly specific "protective" anti-PRRSV antibodies.

Second Objective. The proportional transcription of six IgG subclass genes in various lymphoid tissues of PRRSV-infected piglets, will be monitored as a second criteria of T cell-dependent, antigen-driven CSR that would normally yield IgG neutralizing antibodies. Preliminary data has shown that naive piglets and fetuses spontaneously and preferentially transcribe the putative IgG6 subclass whereas antigenized piglets favor IgG1. This change is thought to be mediated by T cells in germinal centers (GC) that are also the ones believed to drive V_H repertoire diversification through somatic hyper mutation (SHM). If PRRSV causes *no shift* in subclass expression the naïve condition when compared to antigenized controls, it would confirm the hypothesis that PRRSV-induced B cell proliferation by-passes T- cell and GC-mediated events that cause both CSR and V_H antibody repertoire diversification primarily through SHM.

Material and Methods

a. Source of tissues. A great number of the tissues required have already been acquired in ongoing collaborations with Drs. Kelly Lager and Joseph Urban, Jr, (see Table 1) that were partially supported by the NPB. Additional tissues are being generated in ongoing NPB-supported studies on the TCRβ repertoire in viral diseases of swine (NPB 05-143). Isolator piglets are primarily used because the four factors controlling neonatal

development, i.e. *diet, maternal passive immunity, normal gut flora* and *pathogen exposure*, can be tightly controlled reducing ambiguity and the number of animals needed.

Table 1 summarizes the number of animals and tissues to be examined.

Table 1. Treatment Groups and Tissue to be Analyzed

		Tissue Analyzed			
Animal Group	Number[a/]	MLN[e/]	PBL	BLN[e/]	Tonsil[e/]
Newborn	4	+	+	+	+
5-week GF[b/]	4	+	+	+	+
3-week PRRSV[b/]	4	+	+	+	+
3- week SHAM [b]	4	+	+	+	+
5-week PRRSV[b/]	4	+	+	+	+
5-week PRRSV-SHAM[b/]	4	+	+	+	+
Conventional PRRSV[c/]	12	+	+	+	+
Conventional helminthes control[d/]	7	+	+	+	+

a/ Previous data have shown that four piglets/group are adequate to reveal any significant changes when isolator piglets are used. Fewer isolator piglets are needed because environmental factors are tightly controlled.
b/ Inoculation is done on week 1. Isolator piglets are usually reared on SPF-lac.
c/ Conventional piglets inoculated at 10 days with WT-PRRV and sacrificed 4 week later.
d/ Samples provided by Dr. Joseph Urban, Jr., USDA-ARS, Beltsville, MD.
e/ BLN and tonsil are target tissue for PRRSV and the MLN is the target for helminthes infection.

b. Preparation of RNA, cDNA and cloned transcripts. RNA recovery from tissues frozen in liquid nitrogen follows the procedures routinely used in the laboratory. cDNA preparation and the recovery of isotype-associated VDJ, i.e. IgM, IgG, IgA, IgD, IgE and IgG transcripts, will be prepared as described previously. IgG subclass transcripts are recovered using a similar procedure. Clones are individual picked, transferred to the wells of 96-well culture plates, grown overnight, the plasmid DNA prepared in-well, and transferred to nylon membranes and cross-linked.

c. Quantitation of V_H and Cγ subclass usage. The sequential hybridization of cloned cDNA bound to nylon membranes with dual probes for the CDR1 and CDR2 regions of V_HA, V_HB, V_HC, V_HE and V_HF or Cγ1, Cγ2a, Cγ2b, Cγ3, Cγ4, Cγ5 and Cγ6 (CSR study) allows the number of each V_H gene, V_H gene hybrid or Cγ subclasses to be determined. Membranes containing cDNA are finally hybridized with a pan-specific FR2 probe or a pan-specific Cγ probe (CSR studies) to determine the total number of clones containing VDJ and Cγ inserts, respectively. This allows proportional usage to be calculated. In the case VDJ clones, non-

hybridizing clones constitute "other" . Sequence analysis reveals that "other" is comprised primarily of highly mutated variants (up to 100 mutations/kb) of the major V_H genes (e.g. VHA, VHB, etc) and occasionally the use of certain less-frequently encountered porcine V_H genes. In any case, the increase use of "other" and the decrease in use of IgG6, are ***independent indicator*** of normal T-cell driven maturation of porcine adaptive immunity.

d. Alternative approaches. Since there are no subclass specific mAb available, CSR cannot be analyzed by immunochemistry. Real-time PCR cannot be used for V_H expression and repertoire diversification studies. Subclass expression can theoretically be done by real-time PCR but cloning is still needed to provide standards. However, real-time PCR provides much less information because it does not allow the individual clones to be preserved and sequenced. It also does not allow new IgG gene sequences to be identified. Our method allows subclass transcripts to be recovered with their VDJs so repertoire diversification in each subclass can be studied. No other laboratory possesses the experience, the defined probes and methods to definitely address the hypothesis described

e. Statistical analyses. Data will be plotted in a scattergram or as mean values connected by trend lines. Appropriate statistical criteria will be employed through the advise and assistance of Dr. Kathryn Chaloner, Chairman, Department of Biostatistics, The University of Iowa.

Results

First objective: We first undertook a more detail analysis of the VH repertoire of newborn piglets. We now show that seven VH genes account for >95% of the pre-immune repertoire whereas in parasitized control pigs, > 80% were mutated beyond identification (Butler et al 2006). Using isolator piglets we demonstrated the lack of VH repertoire diversification in PRRSV-infected piglets in variable regions associated with both IgG and IgM (Lemke, 2005; Butler et al 2007). Furthermore we showed that undiversified B cells with hydrophobic binding sites preferentially proliferated. Thus an unexpectedly high proportion of “antibodies” (immunoglobulins= Igs) responsible for the hypergammaglobulinemia in these piglets [35% in PRRS versus 7% in conventional] had very hydrophobic binding sites. A somewhat less hydrophobic population comprised 60% of the Igs in PRRS but only 25% in conventional animals and 30% in SIV-infected littermates (Butler et al

2007). Over 90% of the very hydrophobic binding sited used DHA in reading frame 3 and 75% contained the AMVLV motif.

Further analysis of these Igs demonstrated they came from B cells that comprised the pre-immune repertoire, i.e. B cells that were not yet de-selected or that had altered their repertoire by antigen-driven somatic mutation (Butler et al 2008). In fact, the apparent selective proliferation of B cells with hydrophobic binding sites (Butler et al 2007) is more accurately an expansion of the fetal pre-immune repertoire in which such binding sites are over-represented. We further showed that B cells transcribing all three major Ig classes were expanded thus resulting in the 10-30 fold elevation of IgM, IgG and IgA in both serum and bronchial alveolar lavage (Butler et al 2008).

Second objective: Since B cells secreting IgG and IgA are also expanded in PRRSV-infected piglets, it suggests that CSR: (a) occurs as a result of a viral-antigen driven response, (b) occurs without a specific viral antigen or (c) piglet B cells have undergone CSR in utero. Our earlier study demonstrated the latter point (Butler et al 2001). However the very high levels of IgG and IgA after infection mean that PRRSV induces further CSR (Butler et al 2008). Perhaps most notable is that switch occurs independent of repertoire diversification, i.e. B cells of the pre-immune repertoire switch, and proliferates but do not diversify their repertoire. It would have been of some interest to know if they develop germinal centers.

The question of whether CSR favors certain porcine IgG subclasses, requires that the porcine IgG subclasses first be characterized. This delayed the completion of the second objective but yielded some valuable information and two major publications. First we discovered that a major and most ancestral IgG (IgG3) had been overlooked in earlier studies. IgG3 is the second most expressed IgG subclass in swine and interestingly is the major IgG transcribed by the ileal Peyers patches and mesenteric lymph nodes of neonates (Butler and Wertz, 2006). The same study showed that CSR to IgG1 occurs as early as 50 days in utero and it is the major IgG transcribed during fetal life.

Before proceeding to an analysis of IgG subclass usage in PRRSV-infected piglets, we undertook a project to characterize all the porcine IgG subclass genes. This is now complete and demonstrated that there are six IgG subclasses and five occur as two allelic variant (Butler et al 2008 in press). The deduced subclass

proteins were also analyzed for their potential biological function through analysis of sequence motifs. As a result, IgG is most likely to activate complement and bind Fcγ receptors.

Most recently we have used "hemizygous" pigs, i.e. piglets with one disabled heavy chain locus to determine the linkage of the various IgG allotypes and the allotypes of IgA (Butler, Wertz, Mendicino, submitted).

We initially proposed the use of cloning and hybridization to characterize porcine IgG subclass usage in PRRSV-infected piglets. Preliminary studies revealed there to be no significant CSR to an IgG subclass in PRRS versus those infected with SIV. This would be consistent with antigen-independent CSR and would agree with our failure to see antibody repertoire development in PRRS (Butler et al 2007, 2008).

Most recently we have employed real-time PCR to address this issue but have found the method unreliable compared to cloning and hybridization in the case of subclass genes. This observation will be further addressed in the final report o the companion grant on T cells (05-143).

Discussion

Research supported buy NPB 05-174 significantly expands our understanding of the effect on PRRSV on the humoral immune system of piglets and on the IgG subclass antibodies of swine. First it re-enforced the view that PRRSV is a polyclonal B cell activator, not merely because it greatly elevated Ig levels and B cells numbers, but because it does so without diversification of the pre-immune B cell repertoire. The fact that the expanded B cell population in PRRSV-infected piglets resembles the pre-immune fetal repertoire, suggests that PRRSV targets features of this B cell population. The unifying feature is their hydrophobic binding sites. Since few viral epitopes are hydrophobic, we suspect PRRSV subverts the piglet immune system by producing or stimulating the production of an endogenous B cell superantigen. To avoid this subversion, a vaccine strain lacking this superantigen property should be the goal of those who develop vaccines.

The second contribution of the NPB-supported research has been the nearly complete characterization of the porcine IgG subclass genes. This achievement goes well beyond PRRS and has relevance for addressing immunological response issues that are important in other disease of swine including those that are/will be emerging.

As regards IgG subclass usage in PRRSV-infected piglets, we have no evidence that CSR is preferential to a certain subclass that might effect viral neutralization. It would seem that the delay in the appearance of neutralizing antibodies in PRRS is due to other reason, perhaps the subversion caused by a B cell superantigen as we have speculated. The ability to monitor IgG subclass responses will depend on the availability of subclass specific mAb, the topic of a separate NPB-sponsored project with Dr. Lunney.

Publications

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