Antibody Repertoire Development in Fetal and Neonatal Piglets: XIX. Undiversified B Cells with Hydrophobic HCDR3s Preferentially Proliferate in the Porcine Reproductive and Respiratory Syndrome

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Antibody Repertoire Development in Fetal and Neonatal Piglets: XIX. Undiversified B Cells with Hydrophobic HCDR3s Preferentially Proliferate in the Porcine Reproductive and Respiratory Syndrome

John E. Butler,2* Caitlin D. Lemke,† Patrick Weber,* Marek Sinkora,‡ and Kelly M. Lager§

Porcine respiratory and reproductive syndrome virus (PRRSV) causes an extraordinary increase in the proportion of B cells resulting in lymphoid hyperplasia, hypergammaglobulinemia, and autoimmunity in neonatal piglets. Spectratypic analysis of B cells from neonatal isolator piglets show a non-Gaussian pattern with preferential expansion of clones bearing certain H chain third complementary region (HCDR3) lengths. However, only in PRRSV-infected isolator piglets was nearly the identical spectratype observed for all lymphoid tissues. This result suggests dissemination of the same dominant B cell clones throughout the body. B cell expansion in PRRS was not associated with preferential VH gene usage or repertoire diversification and these cells appeared to bear a naive phenotype. The B cell population observed during infection comprised those with hydrophobic HCDR3s, especially sequences encoded by reading frame 3 of DHA that generates the AMVLV motif. Thus, the hydropathicity profile of B cells after infection was skewed to favor those with hydrophobic binding sites, whereas the normally dominant region of the hydropathicity profile containing neutral HCDR3s was absent. We believe that the hypergammaglobulinemia results from the products of these cells. We speculate that PRRSV infection generates a product that engages the BCR of naive B cells, displaying the AMVLV and similar motifs in HCDR3 and resulting in their T-independent proliferation without repertoire diversification. The Journal of Immunology, 2007, 178: 6320 – 6331.

Viral infection impacts the immune system in many different ways. Some infections evoke a robust Ab response that neutralizes further infectivity. Others promote a more delayed cytotoxic response that eliminates infected cells whereas some do both. Many viral pathogens have evolved mechanisms to down-regulate MHC class I presentation of viral Ags. Some produce mimic FcRs and others alter cytokine profiles (1–3). A few viruses elaborate T cell superantigens (SAgs)3 that expand T cell subpopulations independent of virus specificity that can result in cytokine shock and cause clonal deletion (4, 5). Some viruses elaborate B cell SAgs that expand nonvirus-specific B cell subsets (6). This effect may explain why lymphocytic choriomeningitis virus and other viruses evoke polyclonal B cell activation that can result in autoantibodies (7–11).

We reported that infection of Caesarian-derived isolator piglets with porcine respiratory and reproductive syndrome (PRRS) virus (PRRSV) resulted in polyclonal B cell activation, autoimmunity, extreme lymphoid hyperplasia, and hypergammaglobulinemia but few virus-specific Abs (12). A similar polyclonal syndrome is produced in mice by lactate dehydrogenase-elevating virus, a related virus to PRRSV (9, 11–13). We used PRRSV infection in isolator piglets as a model to understand polyclonal B cell activation and autoimmunity associated with viral infections.

PRRSV is a positive-strand RNA virus of the arterivirus group (14–17). The disease syndrome (also called swine infertility and respiratory syndrome, mystery swine disease, and “blue ear” disease) initially manifested in sows and was characterized by increased rates of reproductive failure, including anorexia, abortions, early farrowings, stillbirths, mummified fetuses, weak newborn piglets, and a delayed return to estrus (reviewed in Ref. 18). PRRSV Ags are localized primarily in porcine alveolar macrophages in infected piglets (19, 20), suggesting that PRRSV replicates in vivo in porcine alveolar macrophages and in other macrophages and dendritic cells throughout the body (21, 22). On average, 40–80% of herds are infected by PRRSV in the United States and Europe (18, 23). PRRS is currently considered the most important infectious disease of swine worldwide, resulting in an estimated $560 million annual loss in the United States (24).

The adaptive immune response to PRRSV has not been well defined and is both controversial and contradictory. PRRS is associated with a relative decrease in CD4+ cells and an increase in CD8+ cells in conventional and specific pathogen-free piglets (25). CD4+ cell numbers in blood remained unchanged for 35 days postinfection (dpi), whereas after 21 dpi both CD8+ and IgM+ cell numbers proportionally increased. An increase in the numbers of B
cells and CD8$^+$ cells in bronchoalveolar lavage (BAL) can be seen as early as 5 dpi without effect on CD4$^+$ cells (26, 27). PRRSV-specific T cell responses appear delayed and are not detected until 28 days dpi (28, 29). Blocking studies indicate the delayed T cell response is largely due to a delayed response of CD4$^+$ T cells (28, 29). Depletion of CD8$^+$ cells had little effect on clinical symptoms and, not surprisingly, on PRRSV-specific Abs (30). By comparison swine influenza (SIV)-specific T cells are detected within 7 dpi (31). Although SIV induces a significant inflammatory response in the respiratory tract that appears related to viral clearance (32), no such inflammation is associated with PRRS (32, 33). Despite a delay in virus-specific T cell responses, Ab responses to PRRSV of all major isotypes occur 6–14 dpi in blood or BAL (34–36). IL-10 is up-regulated (37–39), but the appearance of virus-neutralizing (VN) Abs and CD4$^+$ antiviral responses is delayed until 28 dpi (40). When present, VN is often mediated by Abs to open reading frame (ORF)5 glycoprotein (41, 42) and to a lesser extent epitopes on the M protein (membrane protein, ORF6; 43), even through robust responses to this highly immunogenic nucleocapsid protein (N protein, ORF7) are seen (44, 45). Because PRRSV causes polyclonal B cell activation, the early non-VN Abs in PRRS may represent a T-independent preadaptive response triggered through pathogen-associated molecular patterns. These may nevertheless play a role in reducing the viral load (34). Of major concern to animal health is that PRRSV 1) results in persistent infections that may require 150 days to clear (46) and 2) infections increase susceptibility to secondary infection and may interfere with the success of vaccines (47–49).

Our recent observation of hypergammaglobulinemia (100- to 1,000-fold increase in serum IgG; 10- to 100-fold increase in IgA and IgM in PRRSV-infected isolator piglets (12)) is consistent with immune dysregulation. This hypergammaglobulinemia and the delay in Th2 responses may play a role in virus persistence.
Although B cell expansion during PRRSV infection is polyclonal, spectratypic analyses consistently showed that BCRs with certain H chain CDR3 lengths (H chain third complementary region; HCDR3) often dominate the profile (12). Because of the limited combinatorial diversity in swine (50), HCDR3 length is a quasi-clone marker. Therefore, it suggests that PRRSV causes selective expansion of certain B cell clones, although the low antiviral titer suggests that few of these clones are virus specific. We show here that the expanded B cell population in PRRS is not associated with preferential usage of a VH gene or typical repertoire diversification, but that >80% selectively used reading frame (RF)3 of diversity segment A (RF3-DµA) in germline configuration yielding an unusually hydrophobic binding site motif, AMVLV. Meanwhile, the normal neutral HCDR3 hydrophobicity profile fails to develop. We propose that PRRSV targets for proliferation B cells of the preimmune repertoire that bear this and very similar HCDR3 motifs. We further suggest that PRRSV infections are persistent because they dysregulate the immune system through T-independent expansion of a naive B cell subpopulation that is not virus-specific, which in turn delays stimulation of the virus-specific CD4 T cells needed for effective VN Abs. It is also possible that Igs with hydrophobic HCDR3s may form aggregates that accumulate in the glomeruli of PRRSV-infected piglets and may even contribute to the autoantibodies we have described (12), collectively causing damage that weakens resistance to opportunistic pathogens.

Materials and Methods

Animal studies

All animal studies were conducted at the National Animal Disease Center (NADC; Ames, IA). Piglets were recovered by closed hysterectomy from 112-day gravid outbred swine, placed in groups of four in rigid tub isolators, and reared on ESPLac (PetAg) as described previously (12, 51). All studies were approved by the Animal Care and Use Committee of the NADC (Ames, IA). Most animals remained germfree whereas others were colonized with benign Escherichia coli as described previously (52), although we previously showed that E. coli colonization did not affect our results (12). Groups of three to five piglets from the same litter were used and were treated one of three ways: 1) inoculated intranasally with 10^4 TCID50 PRRSV; 2) inoculated intranasally with 10^4 TCID50 SIV; or 3) given a sham inoculum. All inoculations took place on day 7 (0 dpi). Animals were maintained for up to 46 days postinoculation (dpi) but most were necropsied at 10 and 25 dpi. A number of piglets were euthanized at delivery to obtain tissues for determining baseline data. Mesenteric lymph nodes (MLN) and PBLs from conventional young pigs were provided by Dr. J. Urban, Jr. (U.S. Department of Agriculture-Agricultural Research Service, Beltsville, MD).

Sample collection

Blood samples were collected weekly from the time of birth (~7 dpi) for preparation of serum and for recovery of PBLs. The former were used for

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**FIGURE 2.** Comparative spectratype analyses of B cells from various tissues of PRRSV-infected piglets at 25 dpi. A. Spectratypic analyses of HCDR3 from piglet 49D. The abbreviations for the different tissues are the same as in Fig. 1. Arrows call attention to prominent pnts. Unlabeled lanes are blanks. B. Comparative spectratypic profiles of three PRRSV-infected animals in four different lymphoid tissues. Arrows indicate prominent HCDR3 pnt lengths that are responsible for the tissue-wide spectratype of each animal.
measurement of Ig levels as described previously (12, 51). The latter were used for CBC analyses and for flow cytometric analysis (FCM), and the remainder were frozen in liquid nitrogen for eventual transcript analysis. Animals were euthanized with pentobarbital (Sleepaway; Fort Dodge Laboratories). At necropsy, lungs were lavaged and used to recover cells for FCM and transcript analysis. Various lymphoid tissues were also collected, frozen in liquid nitrogen, and stored at $-70^\circ C$ for transcript analysis. These tissues included the following: tonsil (TON), tracheal-bronchial lymph nodes (BLN), lung, inguinal lymph nodes, MLN, spleen (Spl), ileal Peyer’s patches (IPP), and jejunal Peyer’s patches.

FIGURE 3. Spectratypic analysis of HCDR3 from SIV-infected and sham control piglets at 25 dpi. A, HCDR3 spectratype of SIV-infected piglet 48A. B, HCDR3 spectratype of a sham control piglet. Legend is similar to Fig. 1. The brackets emphasize spectratypes that are similar among tissues. The spectratype of newborns (data not shown) resembles the Gaussian spectratype of IPP and thymus shown in this figure (50). Lg, lung; jPP, jejunal Peyer’s patches.

Flow cytometric analysis

FCM to obtain preliminary phenotypic data was done using a FACSCalibur flow cytometer (BD Biosciences). Three-color FCM was performed as described previously (53).

Spectratypic analysis

The HCDR3 plays a significant role in Ag recognition and binding (55, 56). HCDR3 is especially important in swine because of their limited combinatorial diversity (50, 57, 58). Because single B cell clones display a single-length HCDR3 (Ref. 59 and Fig. 1A; L23 and L35 cells), separation of HCDR3 regions on polyacrylamide sequencing gels provides a clonotypic analysis known as spectratyping. Rearranged VDJs and HCDR3 were recovered from solid tissue preserved at $-70^\circ C$ and those stored in TRIzol. These were processed as previously described for the preparation of total RNA and subsequently cDNA (12, 60). VDJ is expressed on IgM and IgG transcripts were recovered as described previously (60, 61). HCDR3 regions for spectratypic analysis of Ig were amplified using a FR3-JH primer set as described previously (50). However, in this study, acrylamide gels were not totally dried but coated in saran wrap and incubated overnight at 4°C with Kodak BioMax MR film. The autoradiograph was subsequently aligned with the partially dried original gel, and prominent HCDR3 polynucleotides (pnt) (Figs. 1–3) were then excised from the gel, cloned, and sequenced.

Sequence analysis

HCDR3 pnts of predominant HCDR3 lengths (e.g., 27, 39, 42, 45, 59 and Fig. 1) were recovered by removal of gel segments using a scalpel and placed into 100 µl of dH2O. Because pnt bands having a total length of 36–45 were often dominant in the spectratype of PRRSV-infected piglets and in controls (Figs. 1–3), these were targeted for recovery and sequence analysis as well as a few other prominent lengths that were occasionally observed. These were incubated overnight to permit diffusion of the DNA, and the fluid phase was targeted for 15 cycles of PCR using the same primer set used for original amplification of HCDR3. PCR products of the expected length were verified by agarose electrophoresis, and the resulting products were cloned into pCR2 TOPO (Invitrogen Life Technologies). Plasmid DNA was sequenced in the University of Iowa DNA core using FIGURE 3.
Table I. Molecular characteristics of major HCDR3 lengths recovered from different treatment groups

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<th>Duplicates (%a)</th>
<th>% Dm Usage</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>RF3 %d</th>
<th>H.L.</th>
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<td>36 (10)</td>
<td>86 0 3 83 0.5</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<td>48–51</td>
<td>50</td>
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<td>64 34 2 82 0.4</td>
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<td>66 25 9 34 0.3</td>
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<td>57 43 0 27 0.2</td>
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1. Percentage of duplicates.
2. Usage of Dm is significantly higher in all groups than Dm,B; p < 0.014.
3. Data from BLN, jejunal, lung, BAL, BM, MLN, inguinal lymph node, and PBL collected at 10, 25, and 46 dpi.
4. Data from spleen, BAL, MLN, and BM collected at 25 dpi.
5. Data from MLN, splen, jejunum, and BAL collected at 25 dpi.
6. Data from MLN of seven adult animals.
7. Data from MLN, splen, and PBL of 10 newborn piglets.

Results

PRRSV infection results in a spectratype shared among tissues

Fig. 1A compares the HCDR3 spectratype of B cells in various tissues of PRRSV-infected piglet 49B at 10 dpi. Although the profile is polyclonal like that in PIC MLN (Fig. 1A and B), certain pnt bands are pronounced, e.g., pnt 27,24 and 57, in nearly all tissues examined. A similar HCDR3 spectratype profile, in which pnt 42 is pronounced, is also shared among tissues from PRRSV-infected piglet 49C (Fig. 1B). The spectratype of the PBLs at both 7 and 10 dpi in both animals is somewhat less similar to the pattern in other tissues, but the pronounced bands are still apparent. The pattern at 0 dpi (Fig. 1B) resembles the polyclonal pattern of the control MLN. Fig. 2A shows the spectratype of a third PRRSV-infected piglet. Data show that at 25 dpi five HCDR3 bands are prominent in nearly all tissues: pnts 66, 54, 39, 27, and 17. Even the IPP and thymus, primary lymphoid tissues in newborn piglets, have lost their typical unselected Gaussian pattern (compare with Fig. 3, A and B) and resemble other tissues in spectratype. The PBL spectratype was least biased. The examples shown in Figs. 1 and 2 are representative of every PRRSV-infected piglet that we have examined at 10 and 25 dpi.

All PRRSV-infected piglets do not share the same spectratype

Although a common spectratype among different tissues of the same piglet is characteristic of PRRSV infection, the same spectratype is not observed in each animal (Fig. 2B). Although pnt 42 is expanded in 49C and 49B, a major pnt of 27 is pronounced in tissues of 49B and 49D but is absent from 49C (Figs. 1 and 2B). The prominent 42 pnt in 49B and 49C seems replaced in 49D with a 39 pnt (Fig. 2, A and B). Piglet 49D has also uniquely expanded the 66 pnt band, whereas pnt 45 is expanded in 49B. Thus, infection with PRRSV results in a similar spectratype among tissues, but spectratypic patterns are individually specific.

Spectratypes in sham and SIV-infected piglets are not shared among all tissues

A shared tissue-wide HCDR3 spectratype has not been observed in animals infected with other pathogens. Fig. 3 shows that unlike PRRSV-infected piglets, only anatomically adjacent tissues, e.g., colon vs jejunal Peyers patches and BAL cells vs lung share a common spectratype in SIV-infected piglets (Fig. 3A). Otherwise, each tissue from the SIV-infected animal displays its own unique spectratype. In both SIV-infected (Fig. 3A) and sham controls (Fig. 3B), primary lymphoid tissue (IPP and thymus) display a near Gaussian polyclonal pattern, whereas this Gaussian pattern is not seen in PRRSV-infected piglets. In sham piglets, anatomically adjacent tissues also share a common spectratype, e.g., colon, jejunal, and MLN. This same spectratype is also shared with BAL cells and PBL but not others. Spl, TON, and lung have their own unique spectratypes. Unlike PRRSV-infected piglets, the spectratype of PBL from sham animals often resembles that of peripheral lymph nodes, e.g., MLN.

Statistical analysis

Statistical analysis of data when appropriate was done using the stats program of Prism (GraphPad), with the help of Dr. K. Chaloner (Department of Biostatistics, University of Iowa, Iowa City, IA). ANOVA was used to compare the frequency of recovery of identical HCDR3 sequences. Simple mean differences were compared by two-sided Student’s t analysis.

VH gene usage

VH gene usage by IgM and IgG transcripts from PRRSV-infected piglets and sham controls was determined as described elsewhere (58, 61, 65). Briefly, rearranged VDJs transcribed with IgM and IgG were recovered from cDNA by PCR. The size of the product was verified by agarose electrophoresis, and the appropriate pnt band was removed for secondary amplification. Following secondary amplification the resulting product was cloned into pCR2 TOPO (Invitrogen Life Technologies). VH gene usage was determined by clonal hybridization using probes specific for the CDR1 and CDR2 region of the major porcine VH gene (58, 61, 65).

VH usage does not significantly differ between PRRS and control piglets

In mouse and humans, B cell SAgs that can potentially expand certain populations target the framework region of certain VH genes (6). Thus, the tissue-wide spectratype among PRRSV-infected piglets that was observed (Figs. 1 and 2) might reflect the selection and expansion of a small number of B cell clones that use...
a common $V_H$ gene. Fig. 4 shows that $V_H$ usage by IgM and IgG transcripts from blood PBMCs of PRRSV-infected piglets does not differ from sham-inoculated controls. Because spectratypic analyses indicated that the repertoire of B cells in blood was least representative of those from infected tissues (Figs. 1 and 2), we also compared $V_H$ usage among transcripts recovered from blood and those recovered from sites associated with infection. Data show that the pattern observed for BLN and TON was the same as the pattern observed for PBLs (Fig. 5). Thus, data in Figs. 4 and 5 show that B cell hyperplasia in PRRSV-infected piglets is not associated with selective expansion of cells expressing a particular $V_H$ gene either in blood or at the site of infection.

**Repertoire diversification does not occur in PRRSV-infected piglets**

In the swine system, 70–80% of the naive preimmune repertoire uses the combination of $V_{H\alpha}$, $V_{H\beta}$, $V_{H\gamma}$, and $V_{H\epsilon}$ in germline configuration (58). Maturation/diversification of this repertoire after Ag encounter results in somatic hypermutation of these major genes and in the use of other minor $V_H$ genes (61). This shifts the usage profile (Figs. 4 and 5) to increased usage of “other” so that in a fully diversified repertoire, 80–90% of $V_H$ usage would be found in the “other” category (61). Most $V_H$ genes in the “other” category are mutated versions of $V_{H\alpha}$, $V_{H\beta}$, $V_{H\gamma}$, and $V_{H\epsilon}$ that no longer hybridize with their gene-specific CDR1- and CDR2-specific probes (see Materials and Methods and Ref. 61). Fig. 4 shows that IgM transcripts from the majority of B cells use the four major $V_H$ genes at all three time points, whereas the remainder use “other” $V_H$ genes. There was a greater usage of both $V_{H\alpha}$ and “other” by IgG transcripts and proportional usage changed with age. However, the pattern of usage was the same for PRRSV-infected piglets and sham controls. These data for PBMCs and our failure to see any evidence for repertoire diversification when tissues at the site of infection were compared (Fig. 5), indicate that infection with PRRSV does not result in Ab repertoire diversification.
Representative HCDR3 sequences of different length from PRRSV-infected and control animals. Cloned sequences are organized in groups of five and aligned according to the 3’ end of FR3 and the beginning of FR4 anchored by the invariant tryptophan. DH segments and their frame (RF) are identified and underlined within the region that also contains N and P additions. The sequence of the often shortened and mutated 5’ JH region is also shown.

H.I. were calculated over the region between cysteine 104 and the invariant tryptophan that starts FR4. The cysteine and tryptophan residues are in bold. The deduced amino acid sequences and H.I. for DHA and DHB in all RFs is given at the bottom. Brackets indicate RFs containing stop codon that are indicated with dashes.

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<th>CDR3 Length</th>
<th>Treat Group</th>
<th>Clone Name</th>
<th>FR1 Gene</th>
<th>RF</th>
<th>DH + N/P</th>
<th>5’ JH</th>
<th>H.I.</th>
<th>FR4</th>
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<td>RTEDTARYCAI B 3</td>
<td>EVA</td>
<td>L</td>
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FIGURE 6. Representative HCDR3 sequences of different length from PRRSV-infected and control animals. Cloned sequences are organized in groups of five and aligned according to the 3’ end of FR3 and the beginning of FR4 anchored by the invariant tryptophan. DH segments and their frame (RF) are identified and underlined within the region that also contains N and P additions. The sequence of the often shortened and mutated 5’ JH region is also shown. H.I. were calculated over the region between cysteine 104 and the invariant tryptophan that starts FR4. The cysteine and tryptophan residues are in bold. The deduced amino acid sequences and H.I. for D1α and D1β in all RFs is given at the bottom. Brackets indicate RFs containing stop codon that are indicated with dashes.
CDR3 sequences from PRRSV-infected piglets have a skewed hydropathicity profile

Because we could not find evidence that B cell expansion in PRRSV-infected piglets results from SAg-like selection of certain $V_H$ genes or clones with certain HCDR3 lengths, we shifted our focus to the sequence of HCDR3s in PRRSV-infected piglets. Examples of HCDR3 sequences from piglets in different treatment groups are given in Fig. 6. Boldface underlines denote the sequence of known DH segments and these are identified in a column to the left along with their RF. The complete germline DH segment sequences translated in all RFs are shown at the bottom of the figure. Values for the hydropathicity index (H.I.) of all translated sequences are given. Fig. 6 shows that the H.I. of PRRSV HCDR3 sequences was often higher than for HCDR3s from other treatment groups. Thus, we examined HCDR3 sequences of various lengths from PRRSV-infected piglets and controls. Data obtained were used to prepare hydropathicity profiles. Fig. 7 shows that analysis of 92 HCDR3 sequences from three PRRSV-infected piglets yields a hydropathicity profile with major peaks of 0.4 and 0.7 and a mean H.I. 2- to 3-fold higher than in all other groups. Newborns, SIV-infected, and conventional pigs (PIC) all display a major population (60 – 80% of the HCDR3s) that is neutral to slightly hydrophobic (0.0 – 0.3), whereas this population is nearly absent in the PRRSV-infected piglet profile. In contrast, a hydrophobic population (H.I. > 0.5) was nearly absent in PIC pigs that had been exposed to many environmental microorganisms, although it was present as a minor subpopulation in newborns. Thus, infection with PRRSV selectively results in the expansion of B cell clones that skews the hydropathicity profile of HCDR3 strongly toward the hydrophobic range.

The skewed hydropathicity profile of PRRS results from hydrophobic motifs in the HCDR3

We analyzed the most frequently recovered HCDR3 lengths from piglets in all groups and showed that the HCDR3s from PRRSV-infected piglets used $D_{14} A$ three times more frequently than $D_{14} B$, although this did not differ significantly among treatment groups (Table I). However, RF3 usage in $D_{14} A$ for all lengths of HCDR3 from PRRSV-infected piglets was double or more that seen in other groups ($p = 0.0005$). RF3 of $D_{14} A$ has a H.I. of 1.35 (Fig. 6) and its usage can explain the skewed hydropathicity profile of PRRSV-infected piglets (Fig. 7). We then related DH and RF usage to three major regions of the hydropathicity profiles indicated in Fig. 7. Analyses of the sequences in regions I, II, and III for each animal treatment group are summarized in Table II. First, this comparison shows that only in PRRSV-infected piglets was there a substantial number of HCDR3 sequences in region I (35 vs 7% for PIC). In all other groups, region III contained the majority of sequences. Second, Table II shows that in region I, 92% of PRRSV-infected piglet sequences used $D_{14} A$, i.e., all but two, which is significantly greater than in other groups. Neither usage of $D_{14} A$ nor RF3 in region II of PRRSV-infected animals was greater than in other groups (Table II). The skewing of the hydrophobicity profile in PRRS to yield the 0.7 major peak (Fig. 7) is the combined result of a greater proportion of sequences that group to region I in PRRSV-infected piglets and their use of RF3 of $D_{14} A$. The region II peak in these animals (0.4) is not simply explained by $D_{14} A$-RF3 usage and this subpopulation is discussed below. Examination of 24 sequences from hydropathicity profile region I of PRRS indicated that of the 22 that used $D_{14} A$-RF3, 17 contained the AMMLV motif. Three of the remaining had similar motifs: AMMLV, VLVM, and MPVM. The two non-$D_{14} A$-RF3 sequences in region I had different hydrophobic motifs: IAIVGV and IAILMYL. Thus, the AMMLV motif accounts for 80% of $D_{14} A$ in region I. The AMMLV motif was found in only 10% of sequences in region II, although a number of HCDR3s encoded by RF3 of either $D_{14} A$ or $D_{14} B$ contained related motifs like VLVM, MVLM, IAILMYL, and AVLL. A substantial number of $D_{14}$ segments in the PIC group could not be classified because they were heavily mutated.
Discussion

Polyclonal B cell activation and hypergammaglobulinemia occur in association with various viral infections (7, 8, and 12) that stimulate type I IFNs that in turn enhance B cell proliferation (66). This hypergammaglobulinemia is often associated with autoimmunity (7, 12, 13) perhaps because of nonspecific proliferation of B cells expressing a preimmune-like repertoire that is known to be polyreactive and to recognize autoantigens (67–70). Although previous spectratypic analyses show that B cell HCDR3s from PRRSV-infected piglets were a signature for polyclonal activation, certain HCDR3 lengths were pronounced (12). This result differed from the Gaussian profile seen in primary lymphoid tissue (50; Thymus and IPP; Fig. 3B) and the non-Gaussian pattern of conventional MLN B cells (Fig. 1A). The unusual B cell phenotype (see below) and our original spectratypic observations (12) prompted this study.

Data presented in Figs. 4 and 5 indicate that clonal expansion is neither associated with usage of a certain VH gene nor diversification of the Ab repertoire. Lack of a VH-associated SAg effect is homologous (72), a classical B cell SAg would go undetected. In any event, differences in length, when verified by sequence, is a clone marker (B cell) and the non-Gaussian pattern of conventional activated B cells (see below) may depend on the probability of encountering B cells expressing the most abundant HCDR3 lengths.

HCDR3 sequence analysis initially revealed that 20–50% of the HCDR3s sequences from PRRSV-infected animals were duplicates, suggesting that only a small number of clones were expanded in PRRS (Table I). However, because duplicates were also recovered frequently in newborn, age-matched sham and SIV-infected piglets but not in PIC pigs (Table I), we believe this phenomenon is age, not PRRS-related. Duplicate sequences aside, sequence analysis revealed that HCDR3s in PRRSV-infected piglets are hydrophobic compared with all other groups studied (Fig. 7). This result was due to usage of the very hydrophobic region encoded by RF3 of DHA (Table II and Fig. 6). Eighty percent of hydrophobic HCDR3s encoded by RF3 of DHA and lower (DHB) than for all other groups (p < 0.0001). DHA plus DHB usage does not equal 100 percent in all cases because in highly diversified repertoires, e.g., PIC, the DHA segment cannot be identified due to somatic hypermutation. The percentage of clones in regions I–III is given. A significantly higher proportion of sequences fit to region I for PRRS-infected piglets compared to other treatment groups (p < 0.001).

Table II. DH and RF usage of HCDR3 sequences from different regions of hydropathicity profilesa

<table>
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<th>Hydropathicity Region</th>
<th>Number of Clones (%)</th>
<th>Percentage of Usage</th>
<th>DHA% RF3%</th>
<th>DHB% RF3%</th>
<th>DHA plus RF3%</th>
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<tr>
<td></td>
<td>III</td>
<td>16 (23)</td>
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a Hydropathicity regions I, II, and III are indicated in Figure 7.

b Region I differences: significantly greater (DHA) and lower (DHB) than for all other groups (p < 0.0001). DHA plus DHB usage does not equal 100 percent in all cases because in highly diversified repertoires, e.g., PIC, the DHA segment cannot be identified due to somatic hypermutation. The percentage of clones in regions I–III is given. A significantly higher proportion of sequences fit to region I for PRRSV-infected piglets compared to other treatment groups (p < 0.001).

c Not significantly greater than in other isolator piglets or newborns.

d Based on only two sequences. Most DHB segments in PIC pigs cannot be recognized.

Hydropathicity regions I, II, and III are indicated in Figure 7.

Data presented herein show that so-called “polyclonal B cell activation” in this viral infection is instead selective expansion of B cells with hydrophobic HCDR3s that are normally deselected during development (75) to yield an oligoclonal population with hydrophobic HCDR3s. This biased expansion means that the near neutral (0.0 – 0.3) population (64). Hydrophobic HCDR3s are found in the T1 repertoire and the immature B cells from which they are derived, but this population is selectively lost in mature, circulating B cells (75).

Data presented herein show that so-called “polyclonal B cell activation” in this viral infection is instead selective expansion of B cells with hydrophobic HCDR3s that are normally deselected during development (75) to yield an oligoclonal population with hydrophobic HCDR3s. This biased expansion means that the near neutral (0.0 – 0.3) population (64). Hydrophobic HCDR3s are found in the T1 repertoire and the immature B cells from which they are derived, but this population is selectively lost in mature, circulating B cells (75).
their repertoire in a T-dependent manner (Figs. 4 and 5). Evidence that these remain naïve B cells comes from FCM studies showing that >95% are CD2−, similar to those emerging at birth from marrow whereas only 50% remain CD2− in SIV-infected littersmates (76) (M. Sinkora, K. Kimura, and J. E. Butler, unpublished observations). Failure of PRRSV-infected piglets to develop a normal diversified repertoire with a neutral HCDR3 hydrophobic profile may explain why VN Ab responses are delayed (29), and <1% of the IgG is virus specific in PRRSV-infected isolate piglets (12). This may also explain why PRRSV establishes a persistent infection (40).

Because the hyperimmunoglobulinemia we reported for PRRS elevates serum IgG, IgM, and IgA in proportion to the levels in fetal serum (60) and Ig aggregates of all three isotypes are deposited (12), we had no reason to suspect that B cell expansion is biased to IgG-secreting cells. It is important to remind readers that class shift combination occurs during fetal life in piglets when they have no exposure to environmental Ag and receive no maternal Ig (60, 76–79). We considered it more important to compare >415 sequences of one isotype to avoid criticism that our conclusions were drawn from too small of a sample.

We assume that the Igs responsible for the hyperimmunoglobulinemia in PRRSV-infected piglets result from the B cells with hydrophobic HCDR3s that we report here. The alternative is that the secreted Igs are not derived from the predominant B cells but rather from a minor population within the hydrophathy spectrum (Fig. 7). The antibodytoxins in PRRSV-infected piglets are another matter. If they had hydrophobic HCDR3s, it would create an enigma. The best correlation between HCDR3 sequences and autoantibodies are those to dsDNA and these have charged groups that might fail to support Ab repertoire development, iso-type switch, and consequently effective VN Abs. The molecular basis of hyperimmunoglobulinemia and sometimes autoimmunity in some viral infections remains unexplained. Molecular data presented here provide insight into one of these viral infections.

Disclosures
The authors have no financial conflict of interest.

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