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Antibody Repertoire Development in Fetal and Neonatal Piglets. I. Four \( V_H \) Genes Account for 80 Percent of \( V_H \) Usage During 84 Days of Fetal Life$^{1,2}$

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VDJ rearrangement and \( V_H \) gene usage during fetal development in 35 outbred piglets was examined by PCR amplification of VDJs; VDJs were subsequently characterized by hybridization with \( V_H \)-specific gene probes and by sequencing. VDJ rearrangement was first seen in the fetal liver on day 30 of a 114-day gestation. Four \( V_H \) genes (\( V_H^A \), \( V_H^B \), \( V_H^C \), and \( V_H^E \)) accounted for \~80\% of all \( V_H \) gene usage regardless of gestational age, choice of piglet, or lymphoid tissue tested; \( D_H^A \) and \( D_H^B \) were used in >90\% of the fetal VDJs examined. Evidence of somatic hypermutation during fetal development was not found. The proportion of the four prominent fetal \( V_H \) genes did not differ significantly between cDNA and DNA, suggesting the absence of selective B cell differentiation. A comparison of recombination signal sequences, flanking sequences, and framework sequences of these fetal genes with other germine \( V_H \) genes of swine offered no clue as to their selective usage. N-region additions were prominent on day 40 but not on day 30, suggesting that the onset of terminal deoxynucleotidyltransferase activity occurs after 30 days of fetal development. These collective findings indicate that the preimmune, “natural Ab” repertoire of the fetal piglet is largely restricted to the use of four nonpolymorphic and nonmutated \( V_H \) genes and two nonmutated \( D_H \) segments. This suggests that the preimmune repertoire of swine is either highly restricted or almost entirely determined by junctional diversity in complementarity-determining region-3. The Journal of Immunology, 1998, 161: 5070–5078.

Characterization of the Ab repertoire that develops during fetal life could lead to a better understanding of natural Abs and the role they may play in the fetal immune system and in protective immunity for the newborn. Although the Ab repertoire in adult animals is highly diversified, and \( V_H \) gene usage more or less reflects the complexity of the locus, the fetal and neonatal response is characterized by a limited and preferential usage of certain \( V_H \) genes (1–4). The repertoire that develops during fetal life develops before exposure to environmental Ags and is referred to as the preimmune repertoire. This repertoire is the subject of this report.

In fetal and neonatal mice, B cell subset distribution differs from that seen in adults, as does the use of \( V_H \), \( D_H \), and \( J_H \) segments (5). In the fetus, a significant portion of the B cells are CD5$^+$ (6); in addition, at least in mice and humans, the preimmune repertoire is characterized as having low affinity, connectivity, and multiple reactivity (7). The bias toward the use of certain \( V_H \) genes in the formation of the preimmune repertoire could be the consequence of the proximity of rearranged components, the advantageous accessibility of \( V_H \) genes to recombinases, common recombination signal motifs, the presence of \( V_H \) gene-specific promoter or enhancer sequences, or the selection of B cells expressing certain VDJ gene products by self Ags or B cell superantigens. Whatever the mechanism that determines the selective usage of certain \( V_H \) genes (or VDJs), it may have evolved because such VDJs encode binding sites that recognize pathogenic bacteria; such bacteria threaten all members of the species at birth or encode nonconventional Fv epitopes (8) that are recognized by stro- malign ligands. In any case, these VDJs encode the “natural Ab” of the preimmune repertoire and might even be considered part of innate immunity (9).

Another distinctive feature of fetal VDJ rearrangements that distinguishes them from those in adults is \( D_H \) usage and complementarity-determining region-3 (CDR3)$^3$ length. Fetal mice preferentially use their most 5’ \( D_H \) segments, and the average length of their CDR3 is significantly shorter than the CDR3 of their neonatal or adult counterparts (5, 15, 16). N-region diversity appears early and steadily increases with age (17). Although fetal rabbits do not preferentially use their DQ52 homologue, which lies 800 bp upstream of the most 5’ \( J_H \), they do use Df (located 32 kb upstream) in 40% of the VDJ rearrangements (18).

The pig is being used to study the development of the Ab repertoire because, unlike humans, mice, and rabbits, there is no transfer of maternal Abs or regulatory proteins via the placenta in utero.

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4 Abbreviations used in this paper: CDR, complementarity-determining region; FR1, framework 1; FR2, framework 2; UTR, untranslated region; RSS, recombination signal sequence.
This means that the fetal Ab repertoire develops in isolation from the impact of maternal regulatory factors that have been shown to affect immunoontogeny at least during the last third of gestation (reviewed in Refs. 19–21). Such studies are considered to be particularly relevant, since there are now extensive maternal vaccination schemes designed to influence the perinatal immune repertoire (reviewed in Refs. 22 and 23). The swine system offers several practical advantages for studying fetal/neonatal repertoire development. First, pigs have ~20 VH genes, all of which appear to share nearly identical leader, adjacent 5′ untranslated region (UTR), and framework 1 (FR1) sequences (Ref. 24 and Fig. 1) and possess only one JH (25), so that a single primer set (FR1 and antisense JH) amplifies all the VDJ s. Second, gene-specific oligonucleotide probes are available for the major fetal/neonatal VH genes. Third, fetal material from many outbred animals is abundant. Finally, neonatal piglets can be reared gnotobiologically (26) or in “autosows” (27), so that the influence of maternal factors, gut flora, and dietary factors on Ab repertoire development in the neonate can be examined.

Studies in rabbits suggest that the fetal repertoire does not diversify until after birth, perhaps stimulated by bacterial colonization of the gastrointestinal tract (28). In contrast, Reynaud et al. (29) found no difference between conventional and germfree lambs; both display somatic hypermutation. Furthermore, fetal lambs are immunocompetent despite a lack of bacterial colonization (29). Since both swine and sheep are artiodactyls and since fetal pig lymphocytes, like those of sheep, can respond to mitogenic and antigenic stimulation (30, 31), we wondered when, where, and to what extent this Group III mammal (32) diversifies its preimmune repertoire.

Our previous observations (33) on the expression of VH genes in a neonatal piglet indicated that only five VH genes and two DH segments were used; this is a much more restricted pattern than is observed in neonatal mice and humans. However, these data were generated from the mesenteric lymph node mRNA of primarily one animal, so such preferential VH and DH use may not authentically reflect the general pattern in all piglets or reflect VH and DH usage in the DNA. The present study reports on the preimmune VH repertoire in various lymphoid tissues in 35 outbred fetuses ranging in age from 23 to 110 days. Data indicate that VDJ rearrangement is first seen on day 30, that four nonmutated VH genes account for 80% of VH usage, and that there is little evidence for individual or tissue variation in VH usage.

Materials and Methods

Animals

White cross-bred gilts (1/4 Yorkshire, 1/4 Large White, 1/4 Chester White, and 1/4 Landrace) from the Roman L. Hruska U.S. Meat Animal Research Center and Yorkshire × Meishan F1 crosses from Iowa State University were used in the study. Animals were hand-mated and scheduled for the slaughter and collection of 24-, 27-, 30-, 40-, 60-, 70-, and 110-day-old fetuses. Gestation in swine is 114 days. All gilts were healthy and normal at slaughter, and fetuses were immediately removed from the gravid uterus; fetal liver samples were collected at all slaughter dates. Fetal spleens were collected from 40-, 60-, 70-, and 110-day-old fetuses. Mesenteric lymph nodes, ileal Peyer’s patches, and bone marrow were obtained from 110-day-old fetuses only.

Synthesis of first-strand cDNA and cloning of the amplified VDJ cDNAs

Total RNA was purified using Trizol according to the manufacturer’s instructions (Life Technologies, Gaithersburg, MD). First-strand cDNA was synthesized as described previously (21) using an antisense Cα1 primer (5′-tacagaggttagagac-3′). Next, 2 μl of the first-strand cDNA product was used for the initial round of PCR. The 30-μl PCR mixture contained 3 μl of 10× buffer, 0.1 mM deoxynucleoside triphosphate, 1 μl of Pfu polymerase (Stratagene, La Jolla, CA), 10 pmol of a primer for the 5′ UTR (Fig. 1), and 10 pmol of an antisense JH primer (5′-tggagggagcagcagtccacc-3′). After 2 min of initial denaturation at 94°C, the samples were subjected to 30 cycles of amplification (1 s of denaturation at 94°C, 10 s of annealing at 63°C, and 10 s of extension at 72°C). PCR products of the predicted length were then excised from the ethidium bromide-stained gel. The gel blocks were transferred to 1.5-ml tubes and an approximately equal volume of water was added to each. The tubes were incubated at 65°C for 2 h. The aqueous phase, containing the DNA that had diffused out of the gel, was then used for the second round of PCR. The second round of PCR was conducted under the same conditions as the first round except that: 1) the 5′ UTR primer was replaced by an internal FR1 primer (Fig. 1), and 2) the templates were replaced by 2 μl of the aqueous solution obtained from the first round of PCR (see above). In all cases, the second-round PCR products were purified using a PCR purification kit (Promega, Madison WI) and were then directly ligated into EcoRV-digested pBluescript phagemids. The ligation mixture was used to transform XL-1 Blue competent cells.

Amplification and cloning of VDJ s from DNA

DNA was purified using DNAzol (Life Technologies) and VDJ rearrangements amplified in the same manner as for cDNA (see above) except that: 1) 200 ng of DNA was used, and 2) both rounds of PCR were conducted using the sense FR1 primer (Fig. 1) instead of the 5′ UTR primer. To validate the genomic DNA amplification, a 250-bp fragment of porcine Ca was used as a positive control for the PCR (see Fig. 2). The second-round PCR products from genomic DNA were ligated into EcoRV-digested pBluescript as described above for VDJ cDNAs.

Determination of VH gene usage by screening VDJ clones

The first round of screening was performed with a pan VH probe as described previously (24). Positive clones were selected and grown overnight in individual wells of 96-well microtiter plates in 200 μl of Luria-Bertani-ampicillin medium. Half of the culture volume in each well was then transferred to a corresponding well in a new microtiter plate for plasmid preparation, while the remainder was stored at −70°C for further analysis. The bacterial cultures for plasmid preparation were pelleted and resuspended in 50 μl of resuspension solution. The cells were then lysed with 70 μl of lysis solution, and the mixture was neutralized with 70 μl of neutralization buffer. The composition of these solutions is described in the instructions.
 provisioned with the Promega Miniprep kit. Cell lysates were then pelleted, and 100 µl of each supernatant was transferred to the corresponding well of a new microtiter plate. Subsequently, 100 µl of 20× SSC solution was added to each well. Finally, the plasmid-containing solutions were transferred to a nylon membrane using a 96-well membrane manifold (Pierce, Rockford, IL) connected to a vacuum pump. The membranes were dried, and the plasmid DNA was immobilized by cross-linking with UV radiation. The porcine V<sub>H</sub> genes have been named in order of their abundance in cDNA from newborn piglets (V<sub>H</sub>A→V<sub>H</sub>E; Ref. 33) and/or in order of their discovery (V<sub>H</sub>F→V<sub>H</sub>O); these genes were not named according to chromosomal location. The porcine V<sub>H</sub> genes were originally cloned using anchored PCR and were shown to share nearly identical leader and FR1 cDNA from newborn piglets (V<sub>H</sub>A and V<sub>H</sub>C probes). The hybridization time was usually 4 h for the V<sub>H</sub>A and V<sub>H</sub>D probes. The hybridization of PCR products with VH-specific oligonucleotide probes. The amount of the amplified VDJs that had been adsorbed to the membrane in each sample was measured by quantifying the radioactive signal generated when a FR2 probe was hybridized with the same immobilized VDJs. This FR2 probe hybridized with all V<sub>H</sub> genes to the same degree. This method was used so that the data obtained with V<sub>H</sub> gene specific oligos could be normalized to the total VDJ content. The validity of the direct method has been verified in two ways (35). First, when empirical data obtained with known mixtures of V<sub>H</sub> gene DNAs (observed) were compared with the actual content of these mixtures (expected), the deviation between the observed and expected results was <10%. Second, the results obtained using differential PCR product hybridization were in agreement with results obtained by conventional cloning and hybridizing procedures.

**Results**

**VDJ rearrangement is first seen in the 30-day fetus**

Although rearranged VDJs could not be amplified from the DNA of 24- and 27-day fetal livers, they could be amplified from some of the 30-day liver DNAs (Fig. 2). Failure to amplify VDJ was not due to the loss of DNA or to technical problems, since the Cβ segment could be amplified in all samples. Failure to identify VDJ rearrangements in most 30-day samples suggested that rearrangements might be infrequent at this timepoint.

**Specificity of CDR-specific oligonucleotide probes**

The left panels of Figure 3 illustrate the specificity of representative CDR-specific oligos. cDNA containing V<sub>H</sub>A, V<sub>H</sub>B, V<sub>H</sub>C, V<sub>H</sub>D, and V<sub>H</sub>E was transferred to nylon membranes as described in *Materials and Methods*. These clones, as well as several other hybridizing hybrids of V<sub>H</sub>A→V<sub>H</sub>E, have been sequenced and described previously (33). When oligonucleotide probes specific for the unique CDR1 or CDR2 regions of these V<sub>H</sub> genes or a pan V<sub>H</sub> probe were sequentially hybridized with the membrane, no cross-hybridization was observed, except when plasmids containing hybrid V<sub>H</sub> genes were used. The latter result further confirms the CDR specificity of the oligos used. This same specificity was obtained with all of the remaining CDR-specific oligos. The right panels of Figure 3 illustrate that 11 of 64 clones (17%) use V<sub>H</sub>E.

**Proportional usage of V<sub>H</sub> genes in VDJs from DNA and cDNA**

Previous results obtained with a newborn piglet indicated that V<sub>H</sub> gene usage in cDNA from the mesenteric lymph nodes followed the order V<sub>H</sub>A>V<sub>H</sub>B>V<sub>H</sub>C>V<sub>H</sub>D>V<sub>H</sub>E, and that V<sub>H</sub>A accounted for half of the total sequenced clones. Since these data came from cDNA clones, we wondered whether there was any discrepancy between V<sub>H</sub> gene usage in genomic DNA and V<sub>H</sub> gene transcripts. We also questioned whether the preferential usage of V<sub>H</sub>A in the cDNA of the newborn piglet was characteristic of V<sub>H</sub> usage in DNA at any time during development. These questions were addressed by preparing DNA and cDNA from the same

![FIGURE 2. The number of piglets in which VDJ rearrangement could be amplified from fetal livers as a function of fetal age. Data are expressed as the number of fetuses from which VDJ could be amplified at each timepoint. Amplification of a 250-bp segment of porcine Co served as a positive control and corresponds to the number of fetuses tested at each timepoint.](http://www.jimmunol.org/)

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and hybridized with a pan VH probe. The with VH A-, VH C-, and VH E-specific probes, respectively. The obtained when the same blot was stripped and subsequently hybridized VH genes. All data were obtained by the cloning and hybridization method of 40- and 60-day-old fetuses. “UN” designates clones that contained other VH B, VH C, and VH E in DNA and in cDNA prepared from the fetal liver. The proportion of VDJ rearrangements containing VH A, VH B, VH C, and VH E in the spleens of 40-, 60-, 70-, and 110-day fetal piglets was compared (Fig. 6). Except for VH D, which was seen only in one 40-day sample, all of the remaining genes (VH A, VH B, VH C, and VH E) were detected in each sample. There appeared to be no obvious trends in the usage of VH A and VH C, although VH B usage progressively increased during days 40 to 70 and VH B was not among the VH genes cloned from 30-day-old fetuses (Table I); VH B is the most 3′ functional VH gene in the swine VH locus (33). The sum of VH A, VH B, VH C, and VH E accounted for ~80% of total VH gene usage at all four timepoints studied.

VH gene usage is independent of fetal age

Usage of VH A→VH E in the spleens of 40-, 60-, 70-, and 110-day fetal piglets was compared (Fig. 6). Except for VH D, which was seen only in one 40-day sample, all of the remaining genes (VH A, VH B, VH C, and VH E) were detected in each sample. There appeared to be no obvious trends in the usage of VH A and VH C, although VH B usage progressively increased during days 40 to 70 and VH B was not among the VH genes cloned from 30-day-old fetuses (Table I); VH B is the most 3′ functional VH gene in the swine VH locus (33). The sum of VH A, VH B, VH C, and VH E accounted for ~80% of total VH gene usage at all four timepoints studied.

VH gene usage among major lymphoid tissues does not differ in late gestation

Lymphoid microenvironments have been shown to influence B cell development. To determine whether fetal VH gene usage differed among lymphoid tissues, late-term fetuses were examined with the belief that differences in organ-specific microenvironments would be maximal at that time. At day 110 of gestation, the major lymphoid tissues were well developed and can be easily and clearly removed for study. Four fetuses from the same sow were chosen for this purpose, and the results are shown in Figure 7. There was no notable variation in the pattern of VH usage among the tissues tested.

Nonhybridizing VH genes are not a result of somatic mutation

The use of CDR1- and CDR2-specific oligos to study VH gene usage does not permit differences in CDR3 regions to be accessed or explain why ~20% of the total VDJ failed to hybridize with the five VH specific probes. These 20% could represent the VH A→VH E genes with mutated CDR1 or CDR2 regions or may prevent bias in the VH gene frequency studies. To rule out the possibility of contamination of genomic DNA in cDNA samples, a 5′ primer from the 5′ UTR was chosen for the first round of PCR. If the cDNA product was contaminated by DNA, the PCR product should be larger, because the genomic DNA has an intron between the leader and the FR1 region. Using this criterion, no evidence of DNA contamination was found. Furthermore, the results of this experiment suggest that VDJ amplification from DNA is unlikely to be a consequence of differences in the efficiency of amplification due to gene location.

Animal variation in VH gene usage is surprisingly small

Since it was convenient to use outbred animals in our research, we were especially concerned with VH polymorphism. In work reported elsewhere (34), the frequently cutting restriction enzyme TaqI was chosen to study RFLP in unrelated animals, and a surprising lack of animal variation was observed. To determine whether this lack of polymorphism in genomic blots would be reflected in variations in VH usage during fetal development, eight fetuses from three different sows were selected, and the variation in VH A→VH E usage was determined. Since we were concerned only with the variations among fetuses and not with the absolute content of VH A→VH E, comparisons were expressed as the ratio of the radioactive intensity obtained with a particular VH specific probe to that obtained with a pan-specific FR2 probe. These comparisons revealed that the variation in VH gene usage among animals was generally <20% for all of VH genes studied, except for one animal (S27-1; Fig. 5). This animal behaved differently when tested using probes for VH A and VH E. It is noteworthy that another animal, S27-2, which was a littermate of S27-1, did not show a similar deviation in VH gene usage.

spleen samples collected at 40, 60, and 110 days. Since the prediction of an exact farrowing date can be in error by 2 days, collecting fetuses at 110 days assures that they will be collected before the physiologic events associated with parturition occur. Figure 4 indicates that VH expression in the cDNA from 40- and 60-day fetal piglets was compared (Fig. 6). Except for VH D, which was seen only in one 40-day sample, all of the remaining genes (VH A, VH B, VH C, and VH E) were detected in each sample. There appeared to be no obvious trends in the usage of VH A and VH C, although VH B usage progressively increased during days 40 to 70 and VH B was not among the VH genes cloned from 30-day-old fetuses (Table I); VH B is the most 3′ functional VH gene in the swine VH locus (33). The sum of VH A, VH B, VH C, and VH E accounted for ~80% of total VH gene usage at all four timepoints studied.

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indicate that other V<sub>H</sub> genes had been used. Therefore, 35 nonhybridizing clones were sequenced (Table I). Sequence data revealed that all but one of the nonhybridizing clones used other V<sub>H</sub> genes, of which the most frequently encountered were germline V<sub>H</sub> F (8), V<sub>H</sub> O (4), and V<sub>H</sub> B2 (7). There was only one instance in which a V<sub>H</sub> C was found among the nonhybridizing clones that could represent somatic mutation, genetic polymorphism, or a Taq polymerase error.

Among the 39 sequenced VDJs, 36 used either D<sub>H</sub> A or D<sub>H</sub> B. Two of the three unknown D<sub>H</sub> sequences were from 30-day-old fetuses. All six of the VDJs, which showed no evidence of N-region diversity, were found in DNAs from 30-day-old fetuses. Although the sample size was too small for statistical analyses, there was a tendency for junctional diversity to increase with age. Finally, unlike the data obtained using cDNA from a neonatal pig (which showed that all of the rearrangements were productive), a significant proportion of the VDJ rearrangements amplified from DNA were nonproductive (out of frame).

Table I also provides information on the four hybridizing V<sub>H</sub> genes cloned from 30-day-old fetuses. Three of the four clones were V<sub>H</sub> C, and only one showed any evidence of N-region addition. All four of the hybridizing 30-day VDJs used D<sub>H</sub> A.

**Discussion**

The data presented here indicate that VDJ rearrangement can first be detected on day 30 in the fetal livers of piglets, thus providing an 84-day window in which to examine the preimmune repertoire of this species in the absence of maternal or environmental influences. Our finding of VDJ rearrangements in nearly all 40-day livers is in temporal agreement with the developmental studies of...
Trebichavsky et al. (36). These investigators reported that hemopoiesis begins on day 16, that some CD45$$^+$$ cells appeared on day 22, that yolk sac function was lost by day 24, and that lymphoid cells first appeared in the liver on day 28. Since the earliest detection of B cell or B cell activity in fetal piglets was reported for day 44 fetal liver cells that were cultured and stimulated in vitro, and because we have been unable to immunohistochemically detect IgM$$^+$$ cells in 30- and 40-day-old fetuses (our unpublished observations), the earliest VDJ rearrangements we report here are probably from pro- or pre-B cells. Whether the liver is the actual site of VDJ rearrangement in fetal piglets cannot be determined from this study, although 30-day-old piglets lack both bones and a spleen.

The initial reason for undertaking the current study was to determine whether the very restricted V$$\text{H}$$ and D$$\text{H}$$ gene segment usage seen in transcripts of the newborn piglet was a consequence of a selective expansion/activation of B cell clones bearing these rearrangements or whether such restriction was also seen at the DNA level. We observed that, throughout fetal life, four of the same five V$$\text{H}$$ genes (V$$\text{H}$$A, V$$\text{H}$$B, V$$\text{H}$$C, and V$$\text{H}$$E) predominated in DNA rearrangements (Figs. 4–7). Moreover, when V$$\text{H}$$ usage in cDNA and DNA from the same tissue was compared, only small differences were observed at days 40, 60 (Fig. 4), and 110 (data not shown), indicating that it was unlikely that B cells expressing certain V$$\text{H}$$ genes were being selectively stimulated to differentiate to plasma cells in fetal life. Thus, our previous cDNA data from the newborn (33) most likely reflected what was occurring at the DNA level. In addition, as reported for the newborn piglet, >90% of all VDJs used during fetal and neonatal life use D$$\text{H}$$A or D$$\text{H}$$B.

Since single nucleotide changes in CDR1 and CDR2 result in nonhybridization with our V$$\text{H}$$-specific probes, the nonhybridizing VDJs encountered (Figs. 6 and 7) must represent either mutations in CDR1 or CDR2 or the usage of V$$\text{H}$$ genes for which gene-specific oligonucleotides were not available. This was clarified by sequence analyses of 35 nonhybridizing clones (Table I); only one of these sequences could be identified as a V$$\text{H}$$A→V$$\text{H}$$E gene. The sequence in question was a V$$\text{H}$$E from a 110-day spleen that might represent a polymorphism, a mutation, or a Taq polymerase error. Otherwise, all of the nonhybridizing V$$\text{H}$$ genes represented other known germ line V$$\text{H}$$ genes as well as V$$\text{H}$$ genes for which no germ-line sequences are available for comparison (Table I; Unk). Compared with the germ line sequences available for V$$\text{H}$$F, V$$\text{H}$$O, and V$$\text{H}$$G (J.S and J.E.B., manuscript in preparation), no evidence of somatic mutation could be found. Since all V$$\text{H}$$B2 genes had identical sequences in CDR1 and CDR2, we suspect that V$$\text{H}$$B2 is an allele of V$$\text{H}$$B and not a somatic mutation of V$$\text{H}$$B. Thus, no evidence was found that somatic hypermutation was occurring at any time during the 84-day fetal period in which VDJ rearrangements were recovered.

Since the data presented were obtained from 35 outbred fetuses, it is both surprising and noteworthy that variations in the usage of V$$\text{H}$$A, V$$\text{H}$$B, V$$\text{H}$$C, and V$$\text{H}$$E among so many genetically different animals were not observed (Fig. 5). Only one animal (S27-1) deviated from an otherwise regular pattern and from its littermate

**FIGURE 6.** The proportion of V$$\text{H}$$A→V$$\text{H}$$E usage in VDJ rearrangements amplified from fetal spleens at various times during gestation. Data are presented as the percentage of the total amount of rearranged VDJ recovered. The left panel provides data on individual V$$\text{H}$$ genes, whereas the right panel expresses the data as the combined usage of the five V$$\text{H}$$ genes. Data are the average of four fetuses collected at each timepoint as determined by differential hybridization of amplified VDJs.

**FIGURE 7.** The proportion of VDJ rearrangements amplified from four different 110-day lymphoid tissues containing V$$\text{H}$$A→V$$\text{H}$$E. Data were obtained and are expressed in the same manner as those given in Figure 6. BM: bone marrow; IPP: ileal Peyer’s patches; MLN: mesenteric lymph node; and SPL: spleen. Data are the average of four fetuses.
S27-2. Since all three sows were unrelated cross-bred animals, it has been concluded that the predominant usage of four V_H genes is a developmental rather than an individual feature of this species. Because a single nucleotide change would prevent hybridization of the CDR1 or CDR2 gene-specific oligonucleotides, the data also suggest that polymorphism among these four genes is generally lacking (with the possible exception of S27-1). Further exceptions may be V_H D or V_H B2, which could be alleles of V_H C and V_H B, respectively. These findings are consistent with the idea that conserved and highly monomorphic V_H genes are used to generate the preimmune repertoire in swine; this possibility is consistent with the overuse of monomorphic V_H 6 in fetal humans (37). Although V_H gene usage in fetal mice and humans has been studied and shows a selective use of certain V_H genes, there is a paucity of information that addresses the issue of whether fetal V_H usage is consistent among individuals or strains of mice. Perhaps studies in rabbits, another outbred species, will provide the greatest insight into this matter. Rabbits use their most 3′ V_H (V_H 1) 90% of the time (38); in addition, the Alicia rabbit, which has a mutation of V_H 1, uses a V_H 1-like upstream gene to eventually compensate for the lack of V_H 1. Recently, Pospisil et al. (39) proposed that selective V_H 1 usage may be a result of positive selection by some Fv-like stromal ligand. Rather, recombinases may favor V_H genes with certain recombination signal sequences (RSSs) or sequence motifs that flank the RSSs. Chen found that frequently expressed human V_H 3 family genes such as Humhv3005 and V_H 26 share a unique 32-bp, \( \kappa_B \) enhancer-like sequence that is located 16 to 17 bp downstream of the nonomer as well as many enhancer-like motifs in the 5′ UTR (44). We aligned a few of the genomic V_H gene sequences and RSSs, including those that are frequently and infrequently used (Fig. 8), but no clue was found that might explain the frequent usage of V_H A

Although the observations reported here offer no clues to explain the preferential usage of V_H genes and the almost exclusive use of two D_H segments, it is important to realize that, unlike mice, serum IgS of all isotypes can be detected by day 44 of gestation; the thymus is already active in switch recombination to IgA and IgG synthesis as early as day 67 (30); and IgM, IgG, and IgA transcripts can be readily recovered from newborn piglets (33). Furthermore, IgM and trace amounts of IgG and IgA have been routinely detected in the sera of newborn piglets using radial diffusion (45, 46); Abs reactive with Igs, thymocytes, myosin, trimi-trophenyl, follicle-stimulating hormone, thyroglobulin, Escherichia coli, ssDNA, dextran, and tetanus toxin can be detected by ELISA (30). Thus, the contention that Abs in fetal piglets are the result of maternal contamination (47) appears unfounded. Furthermore, if contamination was responsible, IgG should predominate rather than IgM. Therefore, preimmune natural Abs appear to be a legitimate developmental event in the fetus of this species and

![FIGURE 8. RSSs (indicated by boldface) and 3′-flanking sequences of some V_H genes used during fetal development. The sequences of the V_H genes preferentially used by the fetus (V_H A→V_H E) were aligned adjacent to each other to allow the identification of possible shared motifs.](http://www.jimmunol.org/)}
probably at least all antidiotyks. Since fetal levels of these natural Abs are rapidly increased by mitogenic stimulation (48), they typify the rapid kinetics of innate immunity as displayed by broadly specific cell receptors (e.g., macrophage bacterial receptors and γδ T cells) (49). Natural Abs with similar specificities have been reported in humans and mice (50, 51). Recently Reid et al. have shown that natural IgM Abs from B1 cells protect newborn mice from endotoxin shock (52). Thus, the conserved and apparently limited preimmune Ab repertoire of the piglet may have evolved to recognize a small number of pathogens that threaten the newborn of this species.

CDR3 is generally believed to contribute most to the diversity of the Ab repertoire. In initial studies of early development in humans and mice, CDR3 was characterized by few N-region additions (53, 54), nonrandom D<sub>β</sub>H<sub>β</sub> recombination (55), and a bias for a particular reading frame (56). Although N-region additions were absent in 30-day fetal VDJs, they were routinely found on and after day 40 (Table I); more recent reports indicate that N-region additions are routinely observed in fetal mice and humans (13, 14, 17). Similar to mice and humans, D<sub>β</sub> usage is also nonrandom in fetal and newborn piglets, since two D<sub>β</sub> segments account for D<sub>β</sub> usage in >90% of fetal VDJ rearrangements.

The data presented here provide valuable information about Ab repertoire development in yet another species. Although swine and sheep are both Group III artiodactyls (19), we found no evidence of somatic hypermutation in fetal life as has been reported for fetal sheep (29). Rather, our findings are more similar to fetal rabbits in both the lack of hypermutation during this stage of development and the use of very few V<sub>H</sub> genes (28, 28, 39). The pattern in rabbits and swine clearly differs from the pattern in rodents and rabbits and swine clearly differs from the pattern in rodents and sheeps (29). Rather, our findings are more similar to fetal rabbits in both the lack of hypermutation during this stage of development and the use of very few V<sub>H</sub> genes (28, 28, 39). The pattern in rabbits and swine clearly differs from the pattern in rodents and primates, in which many V<sub>H</sub> genes are used (57). Nevertheless, the fetal piglet appears immunocompetent (30, 31) and displays switch recombination (30, 33), and natural fetal Abs are common (30, 45, 46). The fact that hypermutation is absent and combinatorial diversity provides only limited possibilities in the fetal piglet suggests that junctional diversity in CDR3 is the primary source of Ab diversity and specificity in this species. This finding is reminiscent of what has been described for the TCR (58) and for transgenic diversity and specificity in this species. This finding is reminiscent of what has been described for the TCR (58) and for transgenic diversity and specificity in this species.

References


