Antibody Repertoire Development in Fetal and Neonatal Piglets. XX. B Cell Lymphogenesis Is Absent in the Ileal Peyer's Patches, Their Repertoire Development Is Antigen Dependent, and They Are Not Required for B Cell Maintenance

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Antibody Repertoire Development in Fetal and Neonatal Piglets. XX. B Cell Lymphogenesis Is Absent in the Ileal Peyer’s Patches, Their Repertoire Development Is Antigen Dependent, and They Are Not Required for B Cell Maintenance

John E. Butler,* Kristina Santiago-Mateo,† Xiu-Zhu Sun,* Nancy Wertz,* Marek Sinkora,‡ and David H. Francis†

The continuous ileal Peyer’s patches (IPP) of sheep are regarded as a type of mammalian bursal equivalent where B cells diversify their repertoire in an Ag-independent fashion. Anatomically and developmentally similar IPP occur in swine. Resection of ~90% of the IPP in piglets at birth did not alter Ig levels in serum and secretions or retard diversification of the Ab repertoire when animals were maintained in isolators and colonized with a defined gut flora. Resection or sham surgery elevated IgG and IgA in serum and in lavage fluid from the gut, lung, and in saliva. No changes in the frequency of IgG-, IgA-, and IgM-containing cells in the spleen and peripheral lymph node were observed. Using an index that quantifies diversification of the VDJ repertoire, no differences were seen in three secondary lymphoid tissues between piglets lacking IPP and colonized controls, whereas both groups displayed >10-fold greater diversification than did late-term fetal piglets or piglets maintained germ-free. Somatic hypermutation was very low in fetal IPP and the IPP of germ-free piglets but increased 3- to 5-fold after colonization. D–J signal joint circles were not recovered in IPP, and V–DJ signal joint circles were 5-fold lower than in bone marrow and similar to those in thymus and spleen. We conclude that the porcine IPP are not a site of B cell lymphogenesis, do not undergo Ag-independent repertoire diversification, and are not primary lymphoid tissue since they are not required for maintenance of Ig levels in serum and secretions. The Journal of Immunology, 2011, 187: 5141–5149.

The site of B cell and Ab repertoire development and diversification differs among vertebrate classes. In chickens, and perhaps all birds, this occurs when B lymphocytes formed in embryonic hematopoietic tissues migrate to the hindgut organ known as the bursa of Fabricius (1–3). In mice and humans, B cell lymphogenesis occurs in fetal liver and thereafter in bone marrow (BM) throughout life. In rabbits, B cell lymphogenesis also occurs in BM but appears to be determinant, ceasing after a few months (4). Diversification of the B cell repertoire continues in lymph nodes and other secondary lymphoid tissues (5). Lymphoid tissues that are required for development of B cells and Abs or T cells are traditionally called primary lymphoid tissue, that is, their absence or removal results in the lack of or severe systemic deficiency of B and T cells and their products. Bursectomy in chickens or BM depletion in mammals results in B cell and Ab deficiency.

Issues regarding B cell lymphogenesis and the definition of primary lymphoid tissue for B cells in artiodactyls, that is, hoofed mammals, are unresolved and may present yet another pathway. Artiodactyls are important because they account for ~50% of all mammals, are a major world food source, and have become valuable models in biomedical research (see below). Thirty years ago Reynolds and Morris (6) proposed that the ileal Peyer’s patches (IPP) of sheep played a role similar to the avian bursa, that is, they were primary for B cell development. The equivalent of an IPP is not found in mice or humans and appears to be largely a feature of artiodactyls, although there are similar structures in some cetaceans (7) and some carnivores (8); the sacculus rotundus in rabbits may belong to the same category (9). The lymphoglandular complexes of the colon may act analogously. Similar to sheep, swine are artiodactyls with prominent IPP. The IPP are a series of continuous follicles that occupy ~2 m of terminal ileum and are sometimes referred to as “continuous” PP (6, 10–13). In addition to their anatomical similarity, the IPP of swine share many other features with those of the sheep. They develop at 70 d gestation (DG; gestation, 114 d) and 110 DG in sheep (gestation, 150 d), are dominated by B cells (12, 14–16), and appear to involute several weeks after birth (6, 12, 17). Age-related loss of GALT is also seen in chickens (3) and in dolphins (7). In any case, both the IPP and bursa of Fabricius develop in the Ag-free environment. Also similar to the bursa, the IPP of sheep and swine are characterized by a high rate of apoptosis; only
5–13% of the B cells generated enter the circulating pool (18–20). In piglets maintained germ-free (GF), the length of the IPP actually increases, suggesting that without environmental stimuli, their possible status as a primary B cell organ may continue (12). In conventional or colonized piglets IgA production becomes dominant (21). The observation that hindgut lymphoid tissues change their function during postnatal development has also been reported in rabbits (22).

The jejunal PP (JPP), which are homologous to the conventional PP in mice and humans, are also present in artiodactyls and are found along the upper ileum and jejunum, but their development depends on colonization of the gastrointestinal tract as it does in rodents (6, 17, 23–25). There are differences in T cell trafficking and occurrence between the IPP and JPP; there is less T cell trafficking into the IPP during development than in the JPP (26). There are also many more T cells in the IPP of swine and sheep than in the IPP (12, 27). Thus, it is not surprising that most cells emerging from the IPP in sheep are B cells and not T cells (16). It appears then that JPP and “conventional” PP are especially dependent on CD 4 helper T cells, consistent with the concept that 1) the IgA response is T cell-dependent (28, 29), 2) IgA responses are dependent on T cell cytokines (30), and 3) rescue from apoptosis depends on CD40/CD40L interactions (31). The T-independent response of mouse peritoneal B1 cells has not been confirmed in other species (32).

The observation that the IPP of swine and sheep differ from the JPP and classical PP of rodents, humans, and other species, combined with their developmental similarity to the bursa of Fabricius, was the basis for their classification as a primary lymphoid tissue. These criteria are summarized in Table I and are discussed below. Reynolds and Morris (6) described lymphopoiesis in the IPP, but without distinguishing lymphogenesis from proliferation-associated diversification. Reynaud and colleagues (33, 34) pursued the Reynolds and Morris hypothesis and subsequently presented evidence that in sheep 1) B cell repertoire diversification was extreme in the IPP, 2) diversification was by somatic hypermutation (SHM), and 3) the process was Ag-independent. This is consistent with data from the chicken bursa, that is, the IPP are a site of diversification with no reference to B cell lymphogenesis. Further studies by Griebel et al. (35) supported this view by showing that B cells in the IPP underwent rapid negative selection similar to BM B cells and those in the avian bursa. These and other observations concerning the IPP, rabbit appendix, and the bursa of Fabricius have been molded into a paradigm regarding a role for hindgut lymphoid tissue that is embedded in textbooks and reviews (36, 37).

The chicken bursa and the rabbit appendix are not anatomical homologs of the IPP of artiodactyls, but the anatomical and developmental similarities of the IPP in sheep and swine indicate they are homologous. Thus, using the piglet as a model to study the role of the IPP of artiodactyls is appropriate but more importantly permits improvements to the experimental design. The fetal B and T cell repertoires of both species develop without maternal influence and regulatory factors such as maternal IgG that are known to influence fetal immunological development in mice and rabbits (37–41). However, swine have multiple offspring (6–20), weigh <2 kg at birth, and can be conveniently reared in GF isolators after their recovery by cesarean surgery where all environmental and maternal factors are thereafter controlled by the experimenter (41, 42). Furthermore, the V_{H} repertoire of swine is well characterized, partially mapped, and has been followed during development using methods that quantify repertoire development and frequency of SHM (43–47).

We reasoned that if the conclusions from the studies of Reynaud et al. (33, 34) and the hypothesis of Reynolds and Morris (6) were correct and the IPP of swine served the same role as those in sheep, they would diversify their repertoire in the absence of Ag and their removal at birth should significantly affect B cell development, Ig levels, and Ab repertoire diversification. We included tests for repertoire diversification with the thought that the IPP may not be needed for systemic B cell levels but could be needed for allowing IPP-derived B cells to diversify in secondary lymphoid tissues. To address these issues, we developed surgical methods to perform IPP resection of swine at birth under GF conditions, colonized them with a defined gut flora, and then reared them for 5 wk in isolator units (48). We could then employ our methodology for evaluating repertoire development and diversification and for characterizing Ig and B cell levels. This approach would also permit the effect of IPP resection on the number and phenotype of T and B cells to be studied (49).

Addressing the role of the IPP has implications beyond comparative and veterinary immunology because swine are used as models for human disease in cases where mouse models fail (50–52), in studies on xenotransplantation (53), as a model for immuno-ontogeny (41), and in the generation of B cell knockout pigs as the first step toward preparing humanized Abs in a large xenogeneic species (54, 55).

Our studies show that resection of the IPP increases the levels of lgs in serum and secretions and does not affect diversification of the Ab repertoire. This is supported by immunohistological studies showing that the number of IgG, IgA, and IgM containing cells in the draining mesenteric lymph node was unaffected. Using the occurrence of signal joint circles (SJC) as a criterion for B cell lymphogenesis, only trace amounts of SJC equivalent to those seen in spleen and thymus were recovered. Our findings do not fit to the hypothesis of Reynolds and Morris (6) and the findings of Reynaud et al. (33, 34) that the IPP are primary lymphoid tissue as traditionally defined, and we challenge the paradigm that was developed from this hypothesis. Although refuting older data, our present studies have not identified a unique role for this organ, but we hypothesize that it may act as “first responder” mucosal immune tissue.

### Materials and Methods

#### Experimental design

Table I reviews the parameters measured in this study and those previously measured in sheep. In this study, piglets were recovered by cesarean section at DG 112 in the manner previously described (56). Piglets were distributed in four experimental groups: those with surgically resected IPP; sham-operated piglets with a transected lower ileum; untreated, colonized controls; and untreated, GF controls (Table II). After 5 d, piglets in the first three groups were then colonized with a defined probiotic gut flora (57). All animals were maintained under these conditions for an additional 4 wk and monitored for the unwanted appearance of pathogenic bacteria. Piglets were

<table>
<thead>
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<th>Criterion</th>
<th>Sheep Current Study</th>
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<tr>
<td>L chain repertoire diversification</td>
<td>+</td>
</tr>
<tr>
<td>H chain repertoire diversification</td>
<td>-</td>
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<tr>
<td>Test for SHM</td>
<td>+</td>
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<tr>
<td>Tests for B lymphogenesis</td>
<td>-</td>
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<td>Measurement of serum Ig</td>
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<tr>
<td>Measurement of Ig in secretions</td>
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<td>Immunohistochemical studies</td>
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Table II. Experimental design and piglet performance

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Contamination</th>
<th>Weight Gain (%)</th>
<th>Survival</th>
<th>Health Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resected IPP</td>
<td>4</td>
<td>0/4</td>
<td>5.95</td>
<td>4/4</td>
<td>4.5</td>
</tr>
<tr>
<td>Sham surgery</td>
<td>4</td>
<td>0/4</td>
<td>5.47</td>
<td>3/4</td>
<td>5.0</td>
</tr>
<tr>
<td>Colonized</td>
<td>4</td>
<td>0/4</td>
<td>7.48</td>
<td>4/4</td>
<td>5.0</td>
</tr>
<tr>
<td>GF control</td>
<td>2</td>
<td>0/2</td>
<td>6.73</td>
<td>2/2</td>
<td>5.0</td>
</tr>
</tbody>
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Percentage weight gain was determined by final weight gain divided by initial weight. Data are from Santiago-Mateo et al. (48). For health scores, observations on feeding behavior, stool conditions, and overall activity were scored from 1 to 5, with 5 being the most healthy condition.

A total of 150 cloned VDJ sequences expressed on IgG transcripts were recovered using PCR from a variety of lymphoid tissues, including the MLN, spleen, BM, IPP, and TBLN in the manner previously described (44). We chose to study IgG transcripts on the assumption that 1) all IgG-producing cells were derived from IgM precursors, and 2) any effect of IPP resection on repertoire diversification of any meaningful consequence should be amplified in the IgG response. The resulting product was cloned into pCR4-TOPO and used to transform Escherichia coli Top Ten cells (Invitrogen, Carlsbad, CA). Individual colonies were transferred to 96-well plates and their plasmid DNA was purified and transferred to nylon membranes. The membranes were then hybridized with two [32P] probe mixtures: one comprised of probes that recognize the CDR1 regions, and the second the CDR2 regions of the six VH genes that comprise >80% of the preimmune VH repertoire of swine (45) (Supplemental Table II). A probe specific for the BH region common to all five VH genes was employed to identify all VDJ-containing clones (Supplemental Table II). The validity of using a mixture instead of individual CDR probes has been previously established (47). Hybridization results were used to calculate a repertoire diversification index (RDI) using the following equation that is a modification of one previously used (45): RDI = (UNK + (CDR1 + CDR2/2))/full repertoire. Where UNK indicates unknown and includes VH, VN, and other minor nonmutated VH genes; CDR1 and CDR2 indicate partially hybridizing clones (hybridization with one CDR mixture only); and full repertoire indicates complete hybridization with a probe mixture that recognizes both CDR1 and CDR2 of VH1A, VH1B, VH1C, VH1E, and VH1F (see Supplemental Table II).

This equation is based on the principle that as the VH repertoire diversifies, either 1) other minor VH genes are used (those not indicated in the denominator of the RDI equation), or 2) VH genes that dominate the preimmune repertoire become somatically mutated. In either case, the proportion of so-called unknown or partial unknown clones increases and thus represents diversification of the VH repertoire in this species.

Measurement of B cell lymphogenesis by recovery of SJC

Ongoing B cell lymphogenesis was evaluated by recovery of V-Dep and D-J SEP. DNA was prepared as previously described (61, 62). The primers employed for recovery of SJC and VDJ and the expected product size are given in Supplemental Table II. This procedure is simplified in swine since they possess only two functional DH segments and one functional JH segment (46, 61–63). Furthermore, all swine VH genes belong to the ancestral Vβ3 family so that only one Vb gene primer is needed (37, 43) (Supplemental Table II). In these studies, two rounds of PCR using heminested primers and 30 and 20 cycles, respectively, were employed. Target DNA was used at an initial concentration of 200 ng and in using a one-fifth serial dilution sequence out to 1:15,600. All products were cloned and sequenced to confirm their identity. Titration end points for the PCR dilution assay for V-Dep and D-J SEP were compared with end points for VDI recovery in the same sample (Fig. 4). The abundance of rearranged VDI was considered to reflect the total B cell content of the sample.

Bacteriology

Rectal cultures were obtained periodically to assess for contamination prior to colonization with the defined gut flora and after colonization. A change would suggest environmental or pathogenic contamination. Specimens were cultured on blood agar and cultured aerobically and anaerobically. Examination for change in flora included assessment for hemolytic bacteria (not present in the defined probiotic gut flora employed) and unusual colony morphology.

Repertoire diversification

Rearranged VDJ sequences expressed on IgG transcripts were recovered using PCR from a variety of lymphoid tissues, including the MLN, spleen, BM, IPP, and TBLN in the manner previously described (44). We chose to study IgG transcripts on the assumption that 1) all IgG-producing cells were derived from IgM precursors, and 2) any effect of IPP resection on repertoire diversification of any meaningful consequence should be amplified in the IgG response. The resulting product was cloned into pCR4-TOPO and used to transform Escherichia coli Top Ten cells (Invitrogen, Carlsbad, CA). Individual colonies were transferred to 96-well plates and their plasmid DNA was purified and transferred to nylon membranes. The membranes were then hybridized with two [32P] probe mixtures: one comprised of probes that recognize the CDR1 regions, and the second the CDR2 regions of the six VH genes that comprise >80% of the preimmune VH repertoire of swine (45) (Supplemental Table II). A probe specific for the BH region common to all five VH genes was employed to identify all VDJ-containing clones (Supplemental Table II). The validity of using a mixture instead of individual CDR probes has been previously established (47). Hybridization results were used to calculate a repertoire diversification index (RDI) using the following equation that is a modification of one previously used (45): RDI = (UNK + (CDR1 + CDR2/2))/full repertoire. Where UNK indicates unknown and includes VH, VN, and other minor nonmutated VH genes; CDR1 and CDR2 indicate partially hybridizing clones (hybridization with one CDR mixture only); and full repertoire indicates complete hybridization with a probe mixture that recognizes both CDR1 and CDR2 of VH1A, VH1B, VH1C, VH1E, and VH1F (see Supplemental Table II).

This equation is based on the principle that as the VH repertoire diversifies, either 1) other minor VH genes are used (those not indicated in the denominator of the RDI equation), or 2) VH genes that dominate the preimmune repertoire become somatically mutated. In either case, the proportion of so-called unknown or partial unknown clones increases and thus represents diversification of the VH repertoire in this species.

Frequency of SHM

A total of 150 cloned VDJ transcripts from 95 DG fetal tissues and isolator piglets were sequenced in the DNA Core Facility of the University of Iowa using the four-color ABI Prism DNA analyzer (Applied Biosystems). VH genes were identified by alignment with their germline counterparts and the number of mutations was recorded. Mutations are expressed as changes per kilobase.

Statistical analysis

All statistical analyses were done after consultation with Dr. Kathryn Chaloner (Department of Biostatistics, University of Iowa College of Medicine) and with the assistance of Shuhui Wang. Data obtained using sections were stained with H&E. Results were imaged on a model 710 Zeiss confocal microscope (see Fig. 3).
ELISA analysis (64) were treated so that each value obtained for points in the linear titration plot was used in generation of the mean values. Mean difference were examined using the Student t test and ANOVA programs provided in the statistical package of the Prism program.

Results

Eighty percent of piglets survived without contamination or surgical complications

Table II provides data on the 14 animals used in the study, 13 of which survived for the 6-wk study period. One sham-operated animal developed peritonitis and was euthanized. Pathogenic bacteria could not be cultured from any piglet during the study period, only those from the defined flora used for colonization. General health was scored and found to not differ significantly from those in the nonsurgical control group. However, surgical piglets showed a significantly lower weight gain (48).

Resection of the IPP caused elevation of especially serum IgG and IgA levels

Fig. 1 shows that surgical manipulation or removal of the IPP at birth does not hinder the production of serum IgGs. Rather, surgery results in a significant elevation of serum IgG and IgA. In transected sham controls, the elevated IgG levels returned to those of the colonized control by day 35. Serum IgA levels in transected control animals were higher than in piglets with resected IPP, but this increase was marginally significant. IgM levels were higher on day 7 after surgery in all surgically manipulated animals but thereafter levels paralleled those in colonized controls. Ig levels in GF piglets remained within the range for newborn levels throughout the study (58).

Resection of the IPP does not affect Ig levels in secretions

Absolute concentrations of IgGs in secretion and lavages vary as a result of changes in secretion rate or by dilution during the collection process. Raw data are given in Supplemental Table I, and the level of IgGs in secretion relative to total protein concentrations are shown in Fig. 2. Data show that IgA is the major Ig isotype recovered in all secretions, including those from GF piglets. Compared to colonized controls, resection resulted in a significant elevation of IgA in IW and saliva but not in the BAL (Fig. 2). Sham controls with a transected ileum had elevated IgG levels in IW (Fig. 2). Thus, neither differences in absolute nor relative Ig levels provide evidence that resection of the IPP reduces Ig levels in secretions.

Resection of the IPP does not alter the lymph node anatomy or the abundance of Ig-containing cells

We examined a variety of lymphoid tissues for frequency of Ig-containing cells and found no apparent effect of IPP resection. Because the MLN are the draining lymph nodes for the ileum, and because B cell and dendritic cells migrate to the MLN from the gut (65, 66), we especially focused our studies on the MLN. We observed no changes in the microanatomy of this organ (data not shown) or the frequency of cells containing IgG, IgM, and IgA in piglets with resected IPP and their colonized controls (Fig. 3). Although not quantified, there appeared to be more IgG-containing cells in the MLN of piglets with resected IPP than in colonized controls.

Only traces of V–DJ SJC are found in the IPP

Fig. 4A shows the titration of the PCR reactions used to recover VDJ rearrangements and SJC in late fetal BM and IPP. D–J SJC were not recovered in IPP, and V–DJ SJC were recovered at a 125-fold lower dilution than in BM. Fig. 4B summarizes the comparative titration end points for these recoveries in various tissues. Relative to the BM, the detection of SJC in fetal or neonatal IPP is similar to the trace detection seen in spleen and thymus. The titration end point for V–DJ SJC in the IPP, spleen, and thymus was 1:5, whereas it was 1:125 in BM. The titration end point for VDJ recovery was nearly equivalent (∼1:625) in all tissues sampled.

The RDI is similar between piglets lacking an IPP and colonized controls

Fig. 5 compares the degree of Ab repertoire diversification in various lymphoid tissues of piglets reared GF, those colonized only, and those with resected IPP. Data show that repertoire development in piglets maintained for 5 wk GF is >10-fold lower than in colonized isolator piglets but higher than in 95 DG fetal tissues (note log scale). The RDI in all tissues examined in piglets with resected IPP does not differ significantly from their colonized control litters. TBLN are not developed at DG 95, and of course the IPP from IPP-resected piglets were not available.

FIGURE 1. The concentration of IgG, IgA, and IgM in the sera of isolator piglets with resected IPP and their controls. Ig levels that are significantly higher or lower than for colonized control piglets are indicated. *p < 0.05.
SHM in the IPP of fetal, GF, and colonized piglets is low and similar to that in fetal BM and spleen

Fig. 6 compares the frequency of SHM in three fetal lymphoid tissues at DG 95 with the frequency in the IPP and MLN of GF isolator piglets and 5 wk colonized piglets. This comparison provides no evidence for a uniquely high frequency of SHM in the IPP of any group studied. Specifically, the frequency of SHM in fetal BM (primary) and spleen (secondary) is not different from in the IPP. GF isolator piglets, which develop in the absence of environmental Ag, have a lower frequency of SHM than that found in tissues from fetal piglets ($p < 0.001$). The frequency of SHM in the IPP of 5 wk colonized piglets is the same as in fetal IPP and only marginally greater than in GF piglets ($p = 0.047$). In contrast, and for references, the frequency in the MLN of 5 wk colonized piglets is greater than all other samples compared ($p < 0.001$). These results do not support the contention that a high rate of SHM occurs in the IPP of fetal or neonatal animals (33, 34).

Discussion

Interest in lymphoid tissues associated with the gut peaked with the discovery that bursectomy in chickens resulted in Ig-deficient birds (67). The search for a bursal equivalent in mammals followed with the identification of a bursa-like structure in insectivores, rabbits, sheep, and swine (6, 68–70). However, these observations were preceded by nearly 100 y by anatomical studies that suggested such structures were lymphoid in nature (10).
Rabbit studies became focused on the sacculus rotundus and the appendix (68, 69) and those in sheep and swine on the IPP (6, 70). The first molecular studies on B cell and Ab repertoire development in a “mammalian bursa” were those of Reynaud et al. (33), Griebel et al. (35), and Motyka and Reynolds (19), who focused on the IPP of lambs (Table I). The high rate of B cell apoptosis was suggestive of primary lymphoid tissues (18, 35) and was also seen in swine (20). Reynaud et al. (33, 34) presented evidence that diversification in the IPP was by SHM. Because of their fetal location, the authors reasoned that development was Ag-independent. This work followed on the heels of their own seminal studies of Ab repertoire development in the chicken bursa (1, 71). These studies, combined with those of Reynolds and Morris (6), who showed the PP to be the largest lymphoid organ of the newborn sheep, which involuted after birth similar to the avian bursa, led to the paradigm that the IPP was the most important primary lymphoid organ for newborn lambs. Support for this paradigm came from studies by Gerber et al. (72), who resected the sheep IPP and reported that B cell deficiency was the consequence.

In these studies and in a companion article (49) we investigated the role of the IPP on B cell development and Ab repertoire development in piglets after their surgical removal at birth. We also comparatively analyzed Ab repertoire diversification, including SHM in the IPP of DG 95 fetuses. We found no evidence to support the conclusion of Reynaud and colleagues (33, 34) that fetal IPP or that in GF newborns was uniquely involved in Ag-independent repertoire diversification, including a high rate of SHM (Figs. 5, 6). The surgical removal of 90% of the IPP neither reduced B cell numbers in the draining lymph nodes (Fig. 3) (49) nor Ig levels in serum and secretions (Figs. 1, 2). Resection failed to affect the degree of repertoire diversification in secondary lymphoid tissues (Fig. 5). The failure of resection to reduce B cell levels is inconsistent with the pivotal report of Gerber et al. (72). These investigators resected 1–2 m ileum of fetal, newborn, and 6-mo-old sheep and manually recorded the number of Ig+ cells per 500 small lymphocytes in the draining lymph using a primitive slide smear method. Only resection in fetal sheep reduced the number of Ig+ cells in the lymph, but the difference was marginally significant and no sham controls were included. However, this deficiency in one animal persisted 14 wk. Surprisingly, a subsequent, non–peer-reviewed report by the same group stated that serum Ig levels were unaffected would lead one to conclude that resection had no effect on serum Ig levels (73), essentially in agreement with the finding we report in this study for swine (Fig. 1). Considering the primitive techniques used, the marginal significance of the observation based on this technique, and the fact that serum Ig levels were unaffected would lead one to conclude that the IPP are not required for B cell development or maintenance after birth in sheep. Because B cell replacement following BM grafts can occur in 3–6 wk (74), the effect of resection might be obscured. Because we euthanized our animals after 4 wk, this could explain why both we and Gerber et al. (73) saw no differences in Ig levels at the time of necropsy. However, our companion study (49) indicates that B cell numbers in blood did not

![FIGURE 4. Titrations of PCR assays for the recovery of VDJ rearrangements, V-DJ (VD) and D-J (DJ) SJCS, in various tissues. A. Actual titration pattern in agarose gels for 95 DG BM and IPP (95 DG corresponds to late-term fetuses at a time when both BM and IPP are well developed). Titrations are expressed as the log to base 5 in which only the exponent is shown (e.g., 4 = 5^4). Data are based on three replicates, which yielded the same end points for VDJ, VD, and DJ in BM and VDJ and DJ in the IPP. One of the three replicates for DJ in the IPP was 0. A poly-nucleotide size ladder is provided. B. Histogram comparing the titration end points for VDJ, V-DJ (VD), and D-J (DJ) in various tissues.](http://www.jimmunol.org/)

![FIGURE 5. Scattergrams of RDI values for 5-wk-old GF piglets, piglets with resected IPP colonized controls, and DG 95 fetuses for VDJs recovered from the MLN, spleen, and TBLN. Note that the x-axis is a log scale. RDI values for colonized controls are significantly higher (p < 0.05) but lower than for resected piglets (p < 0.01) except for TBLN. RDI for all fetal tissues are significantly lower than GF piglets (p < 0.001), colonized controls (p < 0.03), and IPP-resected piglets (p < 0.001). Among fetal tissues, IPP and spleen were significantly lower than BM (p < 0.04).](http://www.jimmunol.org/)
change as a consequence of IPP resection. Thus, we have no evidence that at 5 wk B cell levels were being hemostatically replaced by the remnant (10%) of the IPP that escaped surgical resection.

In our studies, we deliberately colonized our GF isolator piglets with a defined gut flora, thinking that maintaining piglets GF with resected IPP would not stress their immune system and the effect of IPP resection might be overlooked. However, we chose to avoid the use of a pathogen that could seriously injure isolator piglets that lack any maternal Ab protection. As shown in Fig. 1, serum Ig levels were elevated above controls as early as 2 wk after surgery; the same elevation was seen in sham control animals, suggesting that this could be the result of surgically induced inflammation and/or the translocation of gut flora at the surgical site.

A possible explanation for our findings is that the IPP acts as an important primary B cell organ only in late gestation when it first appears. This would be consistent with bursectomy experiments in the chicken (75). If this is true and the Ag-independent concept of Reynaud et al. (33) is added to the equation, there should be vigorous Ab repertoire development including SHM in the IPP of late-term fetuses and also in piglets maintained GF. Fig. 5 shows that the RDI of the DG 95 IPP is lower than in BM of the same animals and that the frequency of SHM in the IPP of fetal and GF piglets remained low, as in BM and spleen (Fig. 6). This level of SHM was significantly lower than in tissues from colonized and parasite-infected piglets.

Rothkötter et al. (76) resected the IPP in conventional piglets. They indicated that resection did not affect T cell subpopulations in other lymphoid tissues, although these investigators did not study the effect of resection on Ig levels, B cell development, or repertoire diversification. Their data on T cells are consistent with findings based on FCM (49). The phenotypic analyses of Sinkora et al. (49) showed that IPP resection does not alter the number, distribution, or phenotype of B and T cell subsets in any lymphoid tissue examined.

Fig. 4 shows that SJC can be detected in the IPP, but only at the level seen in thymus and spleen. Compared to the nearly equal titration end point for VDJ in IPP (considered a measure of the total B cells present), SJC recovery in IPP, spleen, and thymus is 125-fold less. The recovery of SJC from these tissues is clearly distinct from recovery from fetal BM and neonatal BM, which are universally considered primary lymphoid tissue. The presence of B cells and even partial B cell development in the thymus of both ruminants and swine (77–79) may explain finding low levels of SJC in this organ. Alternatively, recovery of small amounts of SJC in spleen, thymus, and IPP could result from a few BM-derived B cells that have recently immigrated and contain SJC as undergraded nuclear relics. We interpret the data on lymphopoiesis (6, 18) to merely indicate proliferation, not the formation, of new B cells. Rapid proliferation and vigorous apoptosis was considered by Reynolds to support the view that the IPP of lambs was a primary lymphoid tissue (19). We observed the same phenomenon in piglets, so it alone is an unreliable criterion (49). Based on SJC recovery, the porcine IPP are not involved in B cell lymphogenesis to any greater extent than the spleen and thymus, which are not considered primary lymphoid tissue for B cell lymphogenesis. Failure to find evidence for B cell lymphogenesis would not in itself rule out the IPP as primary lymphoid tissue because B cell lymphogenesis does not occur in the chicken bursa, yet its removal results in B cell deficiency. We included these data because the proliferation and apoptotic activity in sheep IPP (6, 18) could have been due to this process.

Our studies do not support the 25- y-old paradigm described in reviews (23, 35, 37) that the IPP is a “mutant breeding organ” (80) and as such a bursal equivalent, as proposed by Morris and Reynolds (6) and then reinforced by the studies of Reynaud et al. (33, 34) and others (14, 35). Our studies on SHM and repertoire diversification in the IPP of DG 95 fetuses and in GF animals revealed that the magnitude of SHM and repertoire diversification were the same or lower than in other lymphoid tissues examined (Figs. 5, 6). Additionally, resection had no effect on the RDI in the secondary lymphoid tissues that were presumably seeded by B cells from the IPP (65). In both IPP-resected piglets and colonized controls the RDI is >10-fold higher than in 5-wk-old GF controls. Any notion that the IPP is a primary lymphoid tissue affecting Ig levels in the gut is dispelled by our data on Ig levels in the I&W and on Ig-containing cells in the MLN and the phenotypic characterization of intestinal lymphocytes (49). Evidence that late-term fetal IPP are unique in the rate of repertoire diversification or the frequency of SHM is not supported. Although it appears early and before colonization, its B cell repertoire diversification appears to be Ag dependent in the manner of secondary lymphoid tissues, which refutes the Reynaud et al. (33, 34) concept.

Our studies reveal the danger of extrapolating among species in cases where hindgut lymphoid tissues are not anatomical homologs, that is, the chicken bursa and the IPP of artiodactyls. To avoid this caveat in the current study, we used a species in which the IPP is clearly an anatomical homolog of the IPP of sheep. Although the artiodactyl IPP and chicken bursa both involute after birth, this is at most a tertiary criterion and is not sufficient to conclude that they have the same function (Table I). Realizing that the pig cecum and rabbit appendix are homologs, we also conducted an experiment in which both the IPP and cecum in newborn piglets were resected. This was a limited study but produced the same effect as removal of the IPP alone (data not shown). Because the IPP of swine and sheep are homologs, the disparity between our findings and the paradigm that was established from the work of Reynaud et al. (33, 34) is unlikely to be species-dependent. As shown by Gerber et al. (73), the resection of the IPP of sheep had the same effect on Ig levels as did the resection of the IPP of swine. Importantly, the Reynaud et al. (33, 34) studies were restricted to the V& locus, so a head-to-head comparison of repertoire diversification is not possible (Table I). However, there is no precedent showing that SHM can occur in L chain loci independently from SHM in the H chain locus. Subsequent studies by Jenne et al. (81) have, in any case, challenged the V& SHM data of Reynaud et al. (33).

Refuting the claim that the IPP is a bursal equivalent does not explain the true function of the continuous PP. The IPP appears to be a specialized secondary lymphoid tissue that appears during late fetal life in the absence of environment influences in contrast to the JPP or conventional PP. Certain features of the IPP in fetal and newborn piglets distinguish it from secondary lymphoid tissues like spleen, such as the dominant expression of IgG3, the most primary IgG3 (also the most 3C& gene) is important in marginal zone B cells and seems targeted to bacterial polysaccharides in a T-independent fashion (83, 84). Thus, B cells of the fetal IPP that have already switched to IgA and IgG3 might be poised to secrete “natural” Abs (85) to intestinal bacteria. Therefore, we speculate that the IPP is a type of “first responder” gut (mucosal) immune tissue but not primary lymphoid tissue. It is noteworthy that the IPP is strategically located at a site that separates the high levels of colonic bacteria from the lower levels in the ileum. Whereas the IPP appears to be an artiodactyl specialty, other mammals that lack an IPP have prominent lymphoglandular complexes in the colon and the appendix that are located at the same strategic point in the intestinal anatomy. Except for studies...
in rabbits, the role of these other hindgut tissues has not been rigorously explored.

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References


necessary for systemic B cell development and maintenance and do not contribute significantly to the overall B cell pool in swine. J. Immunol. 187: 5150–5161.


