

## COMPARISON OF THE FETAL AND ADULT FUNCTIONAL B CELL REPERTOIRES BY ANALYSIS OF $V_H$ GENE FAMILY EXPRESSION

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One of the most fundamental questions in immunology is how the B lymphocyte immune repertoire develops and diversifies. During ontogeny, there is a temporal appearance of B cells responsive to given antigens during ontogeny (1-8). For example, in the BALB/c strain, B cells responsive to DNP appear first in ontogeny followed by fluorescein, 4-hydroxy-3-nitrophenol and phosphorylcholine-responsive B cells (3-8). Importantly, within a given strain, every individual acquires the ability to respond to particular antigens at roughly the same time (4). This suggests a developmental program for immunocompetence.

One of the ways in which diversity of the antibody response is created is by the combinational joining of gene segments that encode the variable regions of the antibody molecule. During development, the B cell selects one each of many variable ( $V_H$ ), diversity (D), and joining ( $J_H$ ) gene segments to make the active heavy chain variable region gene (9, 10). A similar event occurs for the light chain (9, 10). The exact mechanism of the rearrangement process and how it is regulated remain unclear.

Based on nucleotide sequence similarity, the estimated 100-1,000 murine  $V_H$  gene segments have been categorized into nine distinct families (11-14). Recombination studies have suggested that the families map as discrete units within the heavy chain locus on chromosome 12 (11-13, 15). However, there is increasing evidence for some degree of interspersion among the families indicating that  $V_H$  genes in the mouse are encoded in overlapping clusters (16, 17). The clustered organization of  $V_H$  gene families permitted the following ordering of families:  $J_H D V_H 7183$ ,  $V_H Q52$ ,  $V_H S107$ ,  $V_H J558$ ,  $V_H J606$ , and  $V_H 36-60$  (11, 12, 16, 17). The order was assigned by deletion analyses (16) or studies of recombinant strains (11, 12, 17), and most  $V_H$  gene families have not been physically linked.

An ordered rearrangement of variable region gene segments could be involved in the preprogrammed-like appearance of B cell specificities during development. Recently, it has been shown that BALB/c fetal pre-B cell lines preferentially rearrange  $V_H$  gene segments belonging to the  $V_H 7183$  family (18, 19), the family most proximal to D in BALB/c. The fetal pre-B cell lines studied had either been trans-

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formed with the Abelson murine leukemic virus (AMuLV)<sup>1</sup> (18) or fused with the murine nonsecreting plasmacytoma Ag8653 (19). These pre-B cell lines used the V<sub>H</sub> 7183 gene family almost exclusively with one of its members, V<sub>H</sub> 81X, the most frequent (18). A recombination mechanism dependent upon the position of V<sub>H</sub> gene families could partially explain the patterned appearance of specificities observed during development (18–20). However, it was also pointed out in these studies that the majority of the rearrangements analyzed in the transformants were not productive, and that V<sub>H</sub> 81X is rarely found in an expressed antibody (20, 21). Therefore, the functional significance of the high frequency of V<sub>H</sub> 7183 rearrangements was questioned (20, 21).

In this report it has been possible to assess the functional B cell repertoire of the fetus and neonate directly by using the sensitive technique of *in situ* hybridization. The B cell repertoire in these studies is probed with the mitogen LPS and DNP, an antigen chosen because of its ability to stimulate B cells as early as 14 d of gestation (17, 22). The APCs that develop as a result of mitogen and antigen stimulation are analyzed for V<sub>H</sub> gene expression by *in situ* hybridization of single cells using prototype radiolabeled V<sub>H</sub> family probes. Therefore, only B cells that have undergone productive rearrangements are being analyzed. Moreover since the immunocompetent B cells express surface Ig (sIg), they have also been exposed to any selective influences involving self-tolerance or idiotype networks (23–25).

The results indicate that the functional fetal B cell repertoire expressed after LPS stimulation is distinct from the adult repertoire. There is a greater expression of V<sub>H</sub> 7183 and V<sub>H</sub> Q52 and a lower expression of V<sub>H</sub> J558 in the fetal repertoire. The increased expression of D proximal families observed in the fetus is lost by day 7 after birth, and V<sub>H</sub> gene family expression is essentially identical in B cells from adult spleen and adult bone marrow. Therefore, the prevalent use of D proximal families appears to be associated with developmental age and not a particular maturational stage in the B lineage. In addition, stimulation of fetal B cells with DNP results in a pronounced increase in the expression of member(s) of the V<sub>H</sub> 36–60 gene family. Therefore, the very early appearance of DNP-responsive B cells, compared with other hapten-specific responses that appear later, cannot be explained solely on the basis of preferential rearrangement of V<sub>H</sub> 7183 or V<sub>H</sub> Q52 in fetal B cell precursors. However, it appears that a considerable proportion of fetal pre-B cells that rearrange 3' V<sub>H</sub> gene families become part of the functional repertoire.

### Materials and Methods

*Animals.* Inbred BALB/c mice were purchased from Harlan Sprague-Dawley, Inc., Indianapolis, IN. Livers were dissected from fetuses of dated gestational age ranging from 14 to 19 d. The age of gestation was determined by using a 24-h mating period with day 0 of gestation being the day of mating. Adult spleens were removed from BALB/c mice at 8–12 wk of age. All mice maintained at UTHSCSA are routinely tested for pathogens including mouse hepatitis, Sendai, *Mycoplasma pulmonis*, *Salmonella*, endoparasites, and ectoparasites. Mice used in these experiments have tested negative for the above pathogens.

As a source of KLH-specific T cells, BALB/c adult mice (6–8 wk) were injected with 10

<sup>1</sup> *Abbreviations used in this paper:* ABC, antigen-binding cells; AMuLV, Abelson murine leukemia virus; DNBS, dinitrobenzene sulfonic acid; HRBC, horse red blood cells; MGG, mouse gamma globulin; s, surface.

$\mu\text{g}$  of KLH emulsified in CFA followed 3–4 wk later by a second injection of KLH in HBSS. Mice were routinely used 6–8 wk after the second injection.

**Hapten-carrier Conjugates.** The hapten DNP was coupled to KLH as described (26). Approximately 8–10 moles of DNP were conjugated per 100,000 daltons of KLH.

**Isolation of DNP-antigen-binding Cells.** The rosetting method used to isolate hapten-specific, antigen-binding cells (ABC) has been described in detail previously (27, 28). Briefly, 1 ml of packed horse red blood cells (HRBC) were conjugated with 20 mg dinitrobenzene sulfonic acid (DNBS) for 30 min at 37°C, washed, and resuspended at 1.5% packed cell volume. Either adult splenocytes or 18–20 d fetal liver cells ( $60 \times 10^6/2$  ml) were mixed with 1 ml of DNP-HRBC suspension. The rosettes formed were isolated on a Percoll gradient as described (27). The isolated rosettes were resuspended in a mixture of 1.5 mg/ml trypsin and 1.5 mg/ml pronase and incubated for 30 min at 37°C in order to disrupt the rosettes. To remove the detached HRBC the suspension was layered onto a Ficoll-Hypaque gradient and centrifuged at 2,000 *g* for 20 min at room temperature. The cells were then washed and cultured as described below.

**Source of KLH-specific T Cells.** The KLH specific T cells were depleted of B cells by incubating splenocytes derived from KLH-primed mice on petri dishes coated with anti-mouse gamma globulin (anti-MGG) as previously described (29, 30). Nonadherent cells were then incubated on a second set of anti-MGG coated petri dishes. Nonadherent cells incubated  $2 \times$  in this manner were shown to be free of contaminating B cells by the inability to respond to LPS (30).

**Stimulation of Lymphocyte Cultures with LPS.** Spleens or fetal livers were removed, dispersed into single cell suspensions, and plated into 24-well Costar (Cambridge, MA) dishes at  $2 \times 10^6/\text{ml}$  in DME containing 10% FCS (Grand Island Biological, Grand Island, NY), 10% NCTC 109 medium (Inland Laboratories, Austin, TX), 50  $\mu\text{g}/\text{ml}$  gentamycin, 2 mM glutamine,  $5 \times 10^{-5}$  M 2-ME, 1 mM oxalacetate,  $3 \times 10^{-6}$  M glycine, 0.2 U/ml insulin, and 0.1 mM nonessential amino acids (M. A. Bioproducts, Walkersville, MD). This medium is referred to as DME enriched. Cultures were incubated in 10%  $\text{CO}_2$  for 5–6 d in the presence or absence of 10–40  $\mu\text{g}/\text{ml}$  bacterial LPS (*Escherichia coli* 0111:B4 phenol/water extracted; List Biological Laboratories, Campbell, CA). Cultured cells were harvested, counted, and cytospun onto slides for analysis by immunocytochemical staining (31) and in situ hybridization (32).

**Stimulation of Lymphocyte Cultures with DNP-KLH.** Adult or fetal DNP-ABC isolated as described above were plated in 96-well culture trays at  $5 \times 10^5/\text{ml}$  in DME-enriched medium. After a 16–24-h incubation period, KLH-specific T cells obtained as described were added at  $4 \times 10^5/\text{ml}$  in DME-enriched medium to the wells containing DNP-ABC. The resulting cell mixtures were incubated in the presence or absence of DNP-KLH at  $10^{-7}$  M DNP. Cultures were incubated for  $\sim 7$  d in 10%  $\text{CO}_2$ . Cultured cells were harvested, counted, and cytospun onto slides for analysis by immunocytochemical staining and in situ hybridization.

**Probes.** The  $\text{C}_\mu$  and  $\text{V}_\text{H}$  gene family probes were kindly provided by Drs. Hood, Brodeur, Riblet, and Riley and subcloned into pT7/T3-18 (Bethesda Research Laboratories, Gaithersburg, MD) so that radiolabeled single-stranded RNA probes could be prepared (30, 33). The probes used were *pV36<sup>21</sup>* (36–60), *pV14RI* (J606), *pVJ558* (J558), *pVS107* (S107), *pVQ52* (Q52), *pVSAPC-15* (7183), and *pVX24* (X24) and have been described elsewhere (11, 34, 35). The recombinant plasmids were linearized with the appropriate restriction enzyme and radioactive RNA probes were generated using T3 or T7 polymerase (Bethesda Research Laboratories) and [<sup>35</sup>S]UTP (New England Nuclear, Boston, MA) (36).

**In Situ Hybridization.** The in situ hybridization technique of Harper et al. (37) and Berger (38) was used as modified by Pardoll et al. (39). Slight modifications of this procedure were carried out for the specific analyses of  $\text{V}_\text{H}$  gene expression (32). Briefly, cells were cytocentrifuged onto precleaned slides and fixed in freshly made 4% paraformaldehyde for 1 min. Slides were transferred directly to 70% ethanol and stored at 4°C until used. Slides were then removed from the 70% ethanol and prepared for hybridization by incubating successively in 2X SSC twice for 1 min, 0.1 M triethanolamine, pH 8, containing 0.25% acetic anhydride (10 min), 2X SSC (twice for 1 min), 0.1 M Tris, pH 7.0, 0.1 M glycine (30 min), 2X SSC (1 min), 70% ethanol (1 min), 80% ethanol (1 min), and 95% ethanol (1 min). The slides were then allowed to air dry. A hybridization mix (10  $\mu\text{l}$ ) was pipetted directly onto

the cell button of each slide; it contained 5  $\mu$ l of deionized formamide (EM Science, Cherry Hill, NJ), 1  $\mu$ l 20X SSC/100 mM DTT, 1  $\mu$ l 10 mg/ml *E. coli* tRNA, 1  $\mu$ l denatured, sheared salmon sperm DNA at 10 mg/ml, 0.4  $\mu$ l of nuclease-free BSA at 50 mg/ml and 0.6  $\mu$ l of  $^{35}$ S-labeled  $V_H$  gene family probes or the  $C\mu$  probe ( $2-4 \times 10^6$  cpm/slide). Cover slips previously siliconized and baked were gently placed on top of the cell buttons and sealed with rubber cement. Slides were then incubated in a humidified chamber overnight at 50°C.

After incubation, the slides were washed by successively incubating in 2X SSC containing 40% formamide for 3 min at 54°C, 2X SSC containing 40% formamide at 54°C for 5 min, 2X SSC containing 40% formamide at 54°C with shaking (1 h), 2X SSC (twice for 1 min), 2X SSC containing 100  $\mu$ g/ml RNase A (Sigma Chemical Co., St. Louis, MO), 1  $\mu$ g/ml RNase T1 (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37°C (30 min), 2X SSC containing 40% formamide at 54°C (3 min), 2X SSC containing 40% formamide at 54°C (5 min), and 2X SSC containing 40% formamide at 54°C with shaking (1 h). To improve specificity for  $V_H$  probes X24, J606, and S107, 50% formamide was used in cell wash buffers instead of 40% formamide. The slides were dipped in 2X SSC, 70% ethanol, 80% ethanol, and 95% ethanol and allowed to dry. The slides were then dipped in NTB2 emulsion (Eastman Kodak Co., Rochester, NY) for autoradiography, developed after  $\sim$ 6 d, and subsequently stained with hematoxylin & eosin.

## Results

*Specificity of  $V_H$  Gene Probes.* Because the degree of nucleotide sequence similarity between two different  $V_H$  gene families can be as high as 70% (11, 12), it was important to establish the specificity of the probes when used under conditions of in situ hybridization. In agreement with previous results (32), when each of the radioactive probes was tested on myelomas and hybridomas expressing known  $V_H$  genes from a number of different families, autoradiographs resulted in substantial grain numbers on only the appropriate cell lines (Table I). The only exception was the slight crosshybridization of the X-24 probe with the 7183-expressing hybridoma. Problems of crosshybridization of X-24 and 7183 have been reported previously (34). Attempts to increase the stringency resulted in no labeling. Therefore, only those cells that were heavily labeled with  $V_H$ X-24 were counted as positive.

*Cells Detected by In Situ Hybridization.* It was also important to determine which cells were being detected by in situ hybridization after stimulation with LPS or an-

TABLE I  
Specificity of  $V_H$  Gene Family Probes

$V_H$ probes	Number of grains/cell counted using the following cell lines*						
	TF2-76 (7183)	25-9 (Q52)	139C1.3 (36-60)	28-120 (X-24)	S31.L1 (S107)	J606 (J606)	B1-8 (J558)
7183	>120	8 $\pm$ 1	3 $\pm$ 1	10 $\pm$ 1	7 $\pm$ 1		2 $\pm$ 1
Q52	4 $\pm$ 1	>120	7 $\pm$ 1		9 $\pm$ 1		4 $\pm$ 1
36-60	3 $\pm$ 1	9 $\pm$ 1	>120	5 $\pm$ 1	2 $\pm$ 1	6 $\pm$ 1	
X-24	13 $\pm$ 1	5 $\pm$ 1	5 $\pm$ 2	43 $\pm$ 4	2 $\pm$ 1		
S107	16 $\pm$ 1	5 $\pm$ 1	6 $\pm$ 1	2 $\pm$ 1	>120	7 $\pm$ 1	4 $\pm$ 1
J606			5 $\pm$ 1	3 $\pm$ 1	1 $\pm$ 1	100 $\pm$ 4	
J558	6 $\pm$ 1	6 $\pm$ 1	4 $\pm$ 1		6 $\pm$ 1	7 $\pm$ 1	>120

Each  $V_H$  gene family probe was used for hybridization to cell lines expressing known  $V_H$  gene families. The cell lines used as negative controls for each probe were chosen on the basis of having the highest homology with a given probe as reported by Brodeur and Riblet (11).

\* Data shown are the mean grain count  $\pm$  SEM of 8-16 randomly chosen cells.

TABLE II  
*Analysis of LPS-stimulated Cells Detected by In Situ Hybridization Protocol*

Population	Percent of total cells of indicated morphology found positive by immunocytochemical staining			Percent of cells containing RNA by in situ hybridization with C $\mu$
	B cell	Plasmablast	Plasma	
Adult spleen	23.5 $\pm$ 4.0	8.2 $\pm$ 2.1	26.6 $\pm$ 2.7	38.2 $\pm$ 4.0
Fetal liver	43.1 $\pm$ 12.4	ND	7.3 $\pm$ 6.5	15.2 $\pm$ 6.5

Adult spleen or fetal liver cells were cultured in the presence or absence of LPS for 5–6 d. After incubation, cell cultures were harvested and cytocentrifuged onto slides for analysis by both immunocytochemical staining with anti-MGG and in situ hybridization with <sup>35</sup>S-labeled C $\mu$  probe. The results represent the mean  $\pm$  SEM for five to eight separate experiments.

tigen. Therefore, all cell populations were routinely analyzed by immunocytochemical staining with anti-mouse gamma globulin (MGG) or anti-mouse  $\mu$  and compared with the proportion of cells labeled with the control C $\mu$  probe. The proportion of cells staining positive could be categorized into small B cells, B cell blasts, plasmablasts, and plasma cells on the basis of size, morphology, and intensity of staining as described previously (32). Under the conditions indicated, it was concluded that the cells detected by in situ hybridization were plasma cells and plasmablasts, which stained more intensely than the remainder of B cell blasts. (Table II, reference 32). In addition, the vast majority of cells stimulated with either LPS (or DNP-KLH shown below) were producing IgM since the percentage of cells staining with anti- $\mu$  vs. anti-MGG did not vary by more than 10%.

The data generated with the in situ hybridization protocol described here have been expressed as the percent of positive cells. Specific grain numbers have not been given since the positively labeled cells usually had too many grains to count, as shown in Fig. 1. With exposure times of 6–7 d, the only labeled cells were strongly labeled, suggesting that the described protocol was only detecting cells with high levels of message, i.e., plasma cells and plasmablasts. The number of total cells counted/slide in all experiments ranged from 500–1,500, depending on the frequency of positive cells.

*LPS-induced Expression of V<sub>H</sub> Gene Families in Adult vs. Fetal B Cells.* To determine if the early functional B cell repertoire was biased in terms of V<sub>H</sub> gene family expression as was observed with fetal pre-B cell transformants, V<sub>H</sub> gene expression as a result of LPS stimulation was compared using adult vs. fetal B cells (Fig. 2). The results are presented as the percent of cells containing detectable  $\mu$ -specific RNA that are expressing each of seven different V<sub>H</sub> gene families. As shown previously (32), the predominant V<sub>H</sub> gene family expressed by LPS-stimulated B cells obtained from adult mice is the V<sub>H</sub> J558 family, the family containing the most members (11, 12, 14). Also consistent with previous results with adult BALB/c splenocytes (32, 40, 41), the level of expression of each of the other families studied approximates the complexity or size of that family. Furthermore, similar results were obtained when the source of B cells was either adult spleen or adult bone marrow (Table III). In contrast, V<sub>H</sub> J558 is not the predominant family expressed by LPS-stimulated fetal liver B cells. Instead, there is a preferential expression of V<sub>H</sub> Q52 and V<sub>H</sub> 7183. Therefore, a nonrandom utilization of V<sub>H</sub> gene families appears to extend to the functional fetal B cell repertoire. However, the degree of bias was considerably less

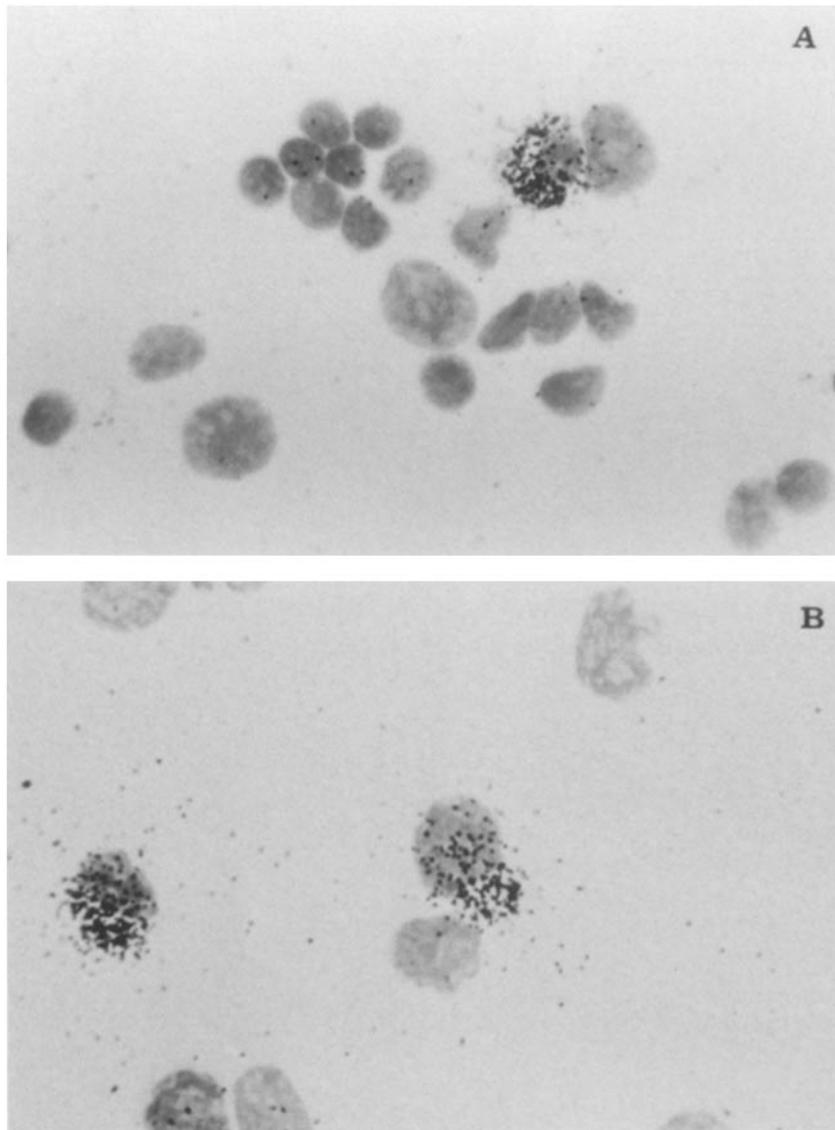


FIGURE 1. Autoradiographs of LPS-induced adult or fetal B cells. (A) Adult spleen cells were stimulated with LPS and hybridized with  $V_H$  36-60 probe. (B) Fetal liver cells were stimulated with LPS and hybridized with  $V_H$  J558 probe. Slides were exposed for 6-7 d.  $\times$  900.

than the rearrangement biases observed with fetal pre-B cell transformants in which the vast majority of precursors had rearranged to  $V_H$  7183 (18, 19).

*$V_H$  Gene Family Expression at Various Stages in Ontogeny.* To determine the age at which BALB/c mice express  $V_H$  genes in an adult-like fashion,  $V_H$  gene family expression was compared among LPS-stimulated B cells obtained from 18-d fetal liver and neonatal spleen (1, 4, and 7 d). The results for expression of  $V_H$  gene families J558, 7183, and Q52 are shown in Table IV. These same data are compared to results

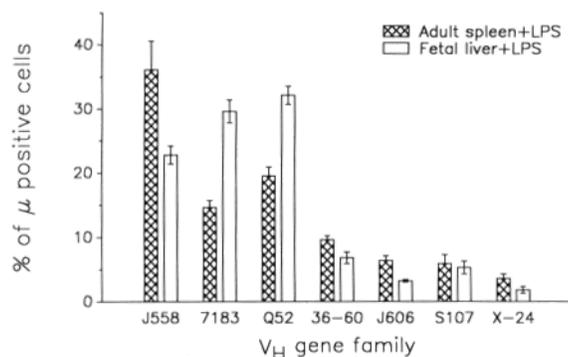


FIGURE 2. LPS-induced expression of V<sub>H</sub> gene families in adult vs. fetal B cells. Adult spleen or fetal liver cells were cultured in the presence of LPS for 5-6 d. After incubation, cell cultures were harvested and cytocentrifuged onto slides for analysis by in situ hybridization with <sup>35</sup>S-labeled C<sub>μ</sub> and <sup>35</sup>S-labeled V<sub>H</sub> gene family probes. Data are expressed as percent of  $\mu$ RNA-containing cells expressing each of seven different V<sub>H</sub> gene families. The data represent the mean  $\pm$  SEM of three to four complete experiments. Each experiment was done by pooling two adult spleens or two to four fetal livers.

obtained with adult spleen and are graphed as percent difference from adult levels (Fig. 3). The data suggest that the transition from fetal to adult-like V<sub>H</sub> gene family expression begins at about day 4 after birth, and by day 7 the LPS-induced V<sub>H</sub> gene family expression by B cells is essentially the same as that of adult B cells.

*V<sub>H</sub> Gene Family Expression After Stimulation with DNP.* Because DNP-responsive B cells are detectable as early as 14 d of gestation (22) and the fetal B cell repertoire appears somewhat skewed in terms of V<sub>H</sub> gene family utilization, it was of interest to examine V<sub>H</sub> gene family expression in fetal B cells as a result of stimulation with DNP. This was accomplished by first enriching for DNP-binding cells using the rosetting technique of Snow et al. (27) and Myers et al. (28). The DNP-specific B cells isolated from either 18-20 d fetal liver or adult splenocytes were cocultured with a source of KLH-specific T cells in the presence or absence of the antigen DNP-KLH. The level of stimulation using the DNP-enriched cells is presented in Table

TABLE III  
*V<sub>H</sub> Gene Family Expression by LPS-stimulated Adult Spleen and Adult Bone Marrow B Lymphocytes*

V <sub>H</sub> gene family	Percent of $\mu$ RNA-containing cells expressing each of the following V <sub>H</sub> gene families		Relative complexity of V <sub>H</sub> gene family %
	Spleen	Bone marrow	
J558 (60)*	37.1 $\pm$ 4.1 <sup>†</sup>	37.0 $\pm$ 3.0	55.6
7183 (12)	15.9 $\pm$ 0.8	17.7 $\pm$ 1.7	11.1
Q52 (15)	19.6 $\pm$ 1.9	19.7 $\pm$ 2.3	13.9
36-60 (5)	10.0 $\pm$ 1.7	8.1 $\pm$ 1.4	4.6
J606 (10)	7.6 $\pm$ 0.4	6.2 $\pm$ 1.9	9.3
S107 (4)	4.4 $\pm$ 0.4	6.2 $\pm$ 1.1	3.9
X-24 (2)	3.5 $\pm$ 1.2	2.6 $\pm$ 1.6	1.9

Spleen or bone marrow cells from BALB/c mice were cultured in the presence or absence of LPS for 5-6 d. After incubation, cell cultures were harvested and cytocentrifuged onto slides for analysis by in situ hybridization using <sup>35</sup>S-labeled V<sub>H</sub> gene family probes and <sup>35</sup>S-labeled C<sub>μ</sub>.

\* Numbers in parenthesis represent the published complexity of V<sub>H</sub> gene families (11).

<sup>†</sup> Results represent the mean  $\pm$  SEM of four (bone marrow) or eight (spleen) complete experiments with different mice.

TABLE IV  
LPS-induced  $V_H$  Gene Family Expression in B cells from Various Stages in Ontogeny

Age	Percent of $\mu$ -RNA containing cells expressing		
	J558	7183	Q52
Fetal liver (18-19 day)	21.8 $\pm$ 1.4	25.8 $\pm$ 3.1	30.5 $\pm$ 1.7
1-d spleen	19.0-20.1	22.5-24.3	33.2-33.3
4-d spleen	24.4-24.6	16.6-18.8	28.3-30.3
7-d spleen	35.4-37.5	14.1-18.5	20.0-21.7
Adult spleen* (8-12 wk)	37.1 $\pm$ 4.1	15.9 $\pm$ 0.8	19.6 $\pm$ 1.9

Fetal liver or neonatal spleen cells from BALB/c mice were cultured in the presence or absence of LPS for 5-6 d. After incubation, cell cultures were harvested and cytocentrifuged onto slides for analysis by in situ hybridization using  $^{35}\text{S}$ -labeled  $V_H$  gene family probes and  $^{35}\text{S}$ -labeled  $C\mu$ . Data for fetal liver represent the mean  $\pm$  SEM for five separate experiments using pool of two to four fetal livers per experiment. Data for neonatal spleen represent the range obtained from two separate experiments using a pool of 3-10 neonatal spleens/experiment.

\* Adult data are taken from Table III for ease of comparison.

V. In the absence of antigen the responses as measured by immunocytochemical staining ranged from 7.8 to 14.9% of the antigen response for the fetal liver cultures and 10.3-17.5% for the adult spleen cultures. Fig. 4 shows a comparison of the level of expression of each  $V_H$  gene family when fetal B cells are stimulated with DNP vs. LPS. The LPS responses were generated with unfractionated fetal liver cells. The most dramatic differences between the DNP- and LPS-induced responses are the increased proportion of cells expressing  $V_H$  36-60 and the somewhat decreased proportion of cells expressing J558 and Q52. Fig. 5 shows a comparison of DNP-induced  $V_H$  gene family expression using DNP-enriched fetal vs. DNP-enriched adult B cells. The results indicate that a higher proportion of DNP-induced fetal B cells express  $V_H$  36-60,  $V_H$  7183, and  $V_H$  Q52 and a lower proportion express  $V_H$  J558 gene families compared with DNP-induced adult B cells.

### Discussion

The purpose of this study was to compare the functional B cell repertoires from fetal and adult mice. It has been shown recently that the vast majority of AMuLV-transformed fetal pre-B cells and fetal pre-B cell hybridomas (18, 19) preferentially rearrange  $V_H$  genes of the 7183 family with the  $V_H\delta 1X$  member used most frequently

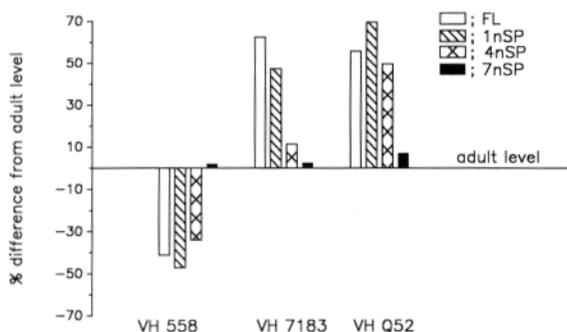


FIGURE 3.  $V_H$  gene family expression at various stages in ontogeny. The results presented in Table IV are graphed as percent difference from adult levels.

TABLE V  
Level of Stimulation of DNP-enriched B Cells

Population	Percent rosettes	Percent plasma/plasmablasts after stimulation determined by immunochemical staining	Percent $\mu$ RNA-containing cells
Fetal liver	<0.5%	4.2 $\pm$ 0.8	3.9 $\pm$ 0.4
Adult spleen (8-12 wk)	0.9 $\pm$ .18	8.3 $\pm$ 2.7	8.4 $\pm$ 1.2

DNP-ABC were isolated from fetal liver or adult spleen using the rosetting protocol described in Materials and Methods. The DNP-ABC were then cocultured with KLH-specific T cells in the presence or absence of DNP-KLH. Cultures were incubated for 7 d, harvested, and cytocentrifuged onto slides for analysis by both immunocytochemical staining with anti-MGG and in situ hybridization with  $^{35}\text{S}$ -labeled C $\mu$  probe. The results represent the mean  $\pm$  SEM for three to five separate experiments.

(18). The functional significance of this finding has been unclear since most of the rearrangements analyzed were nonproductive. Also,  $V_H\beta 1X$  is rarely found in an expressed antibody (20, 21), except that observed in a transgenic mouse model (42). Moreover, Reth et al. (43) had shown that AMuLV transformants derived from adult bone marrow of an NIH/Swiss outbred mouse preferentially rearranged members of the  $V_H Q52$  family, confusing further the importance of preferential rearrangement of  $V_H$  gene families early in development (20, 21). Therefore, it was important to determine if the nonrandom rearrangement pattern observed with fetal pre-B cell lines extended through to the emerging functional B cell repertoire of the fetus.

In this study, the functional B cell repertoire of the fetus and adult were probed in two ways. In the first set of experiments, fetal liver cells or adult splenocytes were stimulated with the mitogen LPS so that a large proportion of the B cells would be stimulated. It was assumed that LPS acts as a bona fide polyclonal activator and would not selectively stimulate a subpopulation of B cells unique in terms of  $V_H$

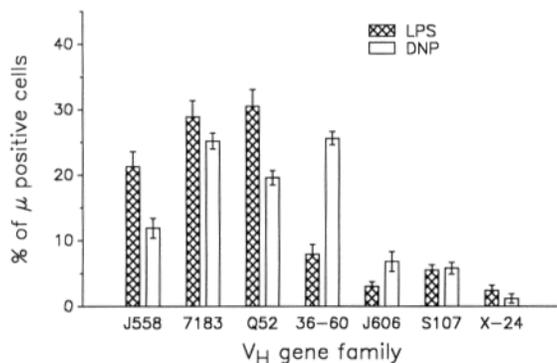


FIGURE 4.  $V_H$  gene family expression in fetal B cells stimulated with mitogen (LPS) vs. antigen (DNP-KLH). DNP-ABC were isolated from 18-20-d fetal liver using the rosetting protocol described in Materials and Methods. The DNP-ABC were then cocultured with KLH-specific T cells in the presence or absence of DNP-KLH. Cultures were incubated for 7 d, harvested, and cytocentrifuged onto slides for analysis by in situ hybridization with  $^{35}\text{S}$ -labeled C $\mu$  and  $^{35}\text{S}$ -labeled  $V_H$  gene family probes. Results are expressed as the percent of  $\mu$ RNA containing cells expressing each of the  $V_H$  gene families. LPS-induced  $V_H$  gene expression of the unfractionated fetal B cells is also shown as described in Fig. 2. The results represent the mean  $\pm$  SEM of three to five separate experiments.

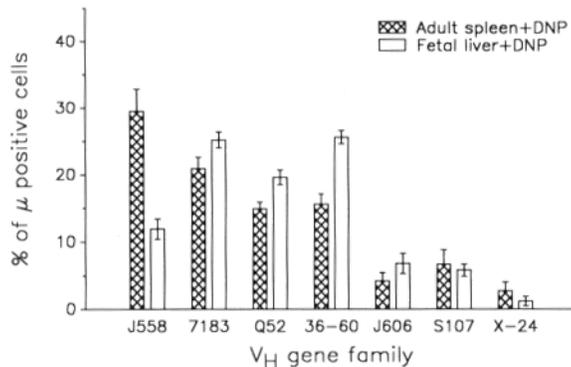


FIGURE 5. V<sub>H</sub> gene family expression after DNP stimulation in adult B cells. DNP-ABC isolated from adult spleen were analyzed as in Fig. 4. The results represent the mean  $\pm$  SEM of five separate experiments. For ease of comparison, the results shown in Fig. 4 for fetal DNP-ABC are also included.

gene expression. Instead LPS stimulation should reflect the available, functional repertoire, an assumption strengthened by our preliminary findings of V<sub>H</sub> gene family usage in unstimulated B cells. After LPS stimulation the cells were harvested and analyzed by both immunocytochemical staining with anti-MGG, and in situ hybridization with the C $\mu$  probe. In this way it was determined that, under the conditions used, the cells detected by in situ hybridization were most likely plasma cells and plasmablasts (Table II, reference 32). Therefore, a large sampling of LPS-stimulated, untransformed B cells could be analyzed for V<sub>H</sub> gene family expression at the single cell level.

The results indicate that the LPS-induced BALB/c fetal repertoire appears non-random in terms of V<sub>H</sub> gene family expression and is significantly different from that of the adult. The predominant families expressed in the LPS-induced fetal repertoire are V<sub>H</sub> Q52 and V<sub>H</sub> 7183 with each being represented at about two times the expected level based on the number of family members (11). V<sub>H</sub> J558 is expressed at nearly half the expected level based on family size. In contrast, V<sub>H</sub> J558 is clearly the predominant family in the adult repertoire. The prevalent expression of the most D proximal V<sub>H</sub> gene families, 7183 and Q52, in LPS-induced fetal B cells suggests that the preferential rearrangement in transformed pre-B cells of members of V<sub>H</sub> 7183 (18, 19) and Q52 (41, 45) is physiologically relevant. Presumably such a position dependent rearrangement mechanism (18-21, 46) plays a significant role in shaping the early repertoire. However, a substantial proportion of the fetal B cells after LPS stimulation also expressed more 5' families including V<sub>H</sub> J558. This is in contrast to the conspicuous lack of rearrangements to V<sub>H</sub> J558 members in transformed fetal pre-B cells (18, 19, 44). Consequently, other regulatory factors must be operative early in development and influence the expressed repertoire.

The results of Wu and Paige (47) showed essentially no differences in V<sub>H</sub> gene family expression between adult vs. fetal B cells when using an RNA colony blot assay. The reason for this discrepancy is unclear. It is possible that the different assay systems detect distinct B cell populations, particularly since colonies derived from sIg<sup>-</sup> precursors are analyzed in the RNA blot assay (47). However, it would seem that the ability to analyze less mature B cell precursors would favor the detection of B cells that preferentially rearranged D proximal V<sub>H</sub> gene families.

A comparison of LPS induced V<sub>H</sub> gene expression in B cells derived from adult

bone marrow vs. adult spleen yielded no detectable differences. This is in contrast to the results of Malynn et al. (21) who studied total RNA from these tissues and found some evidence of an increase in  $V_H$  7183-specific RNA in bone marrow compared with spleen. However, it is difficult to draw conclusions from total RNA obtained from heterogeneous populations of cells since a small proportion of cells containing high levels of specific RNA could account for the differences. In the studies reported here the fetal-like bias observed in  $V_H$  gene expression, including the increased expression of  $V_H$  7183, began to change at day 4 after birth and was essentially identical to the adult by day 7. Therefore, in terms of the functional B cell repertoire, a position-dependent bias in  $V_H$  gene family expression appears to be a characteristic of developmental age rather than a particular maturational stage in the B cell lineage. Whether or not the difference in  $V_H$  gene expression in the fetus and the adult can be explained on the basis of distinct B cell subsets, e.g., Ly1 B cells or early B cells exhibiting autoreactive, interconnecting antibodies (48-51), remains to be clarified.

The fetal and adult B cell repertoires were also compared in terms of  $V_H$  gene family expression after the stimulation of B cells by the antigen DNP. The results suggest that the anti-DNP response, in general, is very heterogeneous and that member(s) of all of the  $V_H$  gene families tested can potentially code for anti-DNP antibodies in both the fetus and adult. The heterogeneous nature of the anti-DNP response and the ability of anti-DNP antibodies to be encoded by more than one  $V_H$  gene family is consistent with other reports (35, 52-55).

The DNP antigen was one of the haptens used to establish that the B cell repertoire is acquired during ontogeny in a predictable, temporal order with DNP-responsive B cells representing the earliest detectable antigen responsive B cells (3, 4, 8). Consequently, it was of interest to determine whether the  $V_H$  gene families expressed by DNP-stimulated fetal B cells would reflect the rearrangement biases of pre-B cell lines (18, 19); biases which were also observed to a certain extent in the LPS-induced fetal repertoire as shown here. The results indicate that there may be some increased expression of  $V_H$  7183 and  $V_H$  Q52 and decreased expression of  $V_H$  J558 in the DNP-induced fetal repertoire as compared with the DNP-induced adult repertoire. Furthermore, an important observation was the major contribution of  $V_H$  36-60 expression to the DNP response. This was most pronounced in the fetal response and is consistent with our previous results indicating that predominant idiotypes 36 (52) and 460 (53-55), both associated with  $V_H$  36-60 (35, 55, 56), were produced in the DNP-specific fetal response (56). In addition, the 460 idiomotype is also a predominant idiomotype found in the adult DNP response (53-55). These findings reemphasize the potential of antigen to specifically select and expand a small subset of B cells. Moreover, the results indicate that if preferential  $V_H$  gene family rearrangement is involved in the developmental acquisition of the B cell specificity repertoire, other mechanisms must also be operative.

In summary, the functional fetal B cell repertoire upon stimulation results in a nonrandom expression of  $V_H$  genes in that there is a predominant expression of gene segments from  $V_H$  7183 and  $V_H$  Q52. Therefore, it appears that the preferential rearrangements to members of these more  $DJ_H$  proximal families in B cell precursors observed previously in mice (17, 18) and more recently in humans (45) is also seen in normal cells and is therefore functionally significant. The exact mech-

anisms involved in the nonrandom expression of  $V_H$  gene families in the fetus and the progressive change to a more random expression during development remain unclear. The contributory roles of distinct B cell subsets, distinct rearrangement mechanisms, evolutionary constraints, and cellular selective regulatory mechanisms need to be addressed.

### Summary

The functional B cell repertoire in BALB/c mice was assessed at various stages in ontogeny. This was done by analyzing  $V_H$  gene family expression using the sensitive technique of in situ hybridization. The B cell repertoire was probed with the mitogen, LPS, and the antigen DNP. DNP was chosen because B cells responsive to this hapten appear very early in ontogeny. The APCs that developed after stimulation with LPS or DNP were analyzed for  $V_H$  gene expression by in situ hybridization of individual cells using radiolabeled  $V_H$  gene family probes. The results indicated that  $V_H$  gene expression in fetal B cells after stimulation was distinct from adult B cells in that there was a biased expression of D proximal families. The results indicated that this bias was associated with developmental age and not a given differentiation stage in the B cell lineage. In addition, stimulation of fetal B cells with DNP resulted in a large increase in expression of member(s) of  $V_H$  36-60, suggesting that the early appearance of DNP-responsive B cells is not strictly correlated with preferential rearrangement of D proximal families,  $V_H$  7183 and  $V_H$  Q52. However, the results suggested that a large proportion of pre-B cells that preferentially rearrange D proximal families early in ontogeny become part of the functional developing repertoire.

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