The Characteristics of V_H Gene Family Expression in Early B Cells

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Defining the mechanisms of B cell diversification which establish the immune repertoire is fundamental to understand how the immune response is regulated. In this report, B cell differentiation and diversification focused on the regulation of immunoglobulin V_H gene expression during ontogeny were analyzed by *in situ* hybridization technique. Fetal liver B cells in different gestational days from 16d to 20d showed the predominant expression of V_H7183 and V_HQ52 without transition of repertoire during the observed gestation days. The two subsets of fetal liver B cells separated according to different differentiation stages based on the presence of cell surface immunoglobulin also did not indicate apparent difference in expressed V_H gene family profiles. B cells in fetal spleen as an another hematopoietic lymphoid tissue in fetus also expressed similar V_H gene repertoire to that in fetal liver B cells. This distinct pattern of V_H gene expression in fetal B cells from that of adult B cells were not changed even after four weeks contact with adult bone marrow microenvironment supplied by the established adult bone marrow stromal cell layers. Thus, the restricted V_H gene repertoire of B cells in fetus which is distinct from that in adult appears to be associated more with the genetic potential of fetal B cell progenitors and less with environmental influences or differentiation stages or compartmentalization.

Key words : B cell, V_H gene, Bone marrow culture, *in situ* hybridization, Fetal B cells.

Introduction

The variable regions of Ig H and L chains each consist of a framework of relatively conserved amino acid sequences and three regions of highly variable amino acid sequences known as hypervariable regions or complementarity determining regions (CDR). The heavy chain variable region is encoded by three separate germ line gene segments; the variable region gene segments (V_H), diversity gene segments (D), and junctional region gene segments (J_H) (Tonegawa 1983). The light chain variable regions are encoded by just V_L and J_L segments. The germ line V_H and V_L gene segments each encode two of the CDRs; the third (CDR3) arises from the junctional region where the component gene segments are joined (Tonegawa, 19 83).

A key feature of the generation of a functional gene for heavy chain variable regions is the recombination of the three separated gene segments. The assembly of these gene segments is a highly ordered process. During the earliest stage of B cell differentiation, D to J_H rearrangements generally occur first and followed by V_H to DJ_H rearrangement (Alt et al., 1984).

Several studies have been done to examine the number of V_H gene segments and their structures. One of these studies used cloned V_H genes to determine the number of genomic restriction enzyme fragments (REF) by Southern blot (Brodeur and riblet 1984; Dildrop, 1984). By this analysis, each V_H gene probe binds to REFs containing related V_H gene sequences. Using many different V_H probes, these patterns define the families of related V_H gene segments, and they were called by the name of representative probes used; V_HJ558, V_H J606, V_H36-60, V_H3609, V_H11, VGAM 3.8, V_HQ52, V_HS107, and V_HX24.

The size of the families range from very small to very large. For example, the X24 family has only

two V_H gene members and the J558 family has at least 60 members (Brodeur and Riblet 1984, Brodeur et al., 1984). However, the size of the J558 family examined by other analyses led to an estimate as high as 1000 to 2000 (Livant et al., 1986; Schiff et al., 1985) implying that the number of total V_H gene segments in the mouse may be much larger. And the most recent order of V_H gene on chromosome determined by deletion analysis is 5'-V_HJ558 - V_H3609 - (VGAM3.8 - V_H36-60 - V_H X24 - V_HS107) - V_HQ52, V_H 7183-D-J_H-C_H-3'(Brodeur et al., 1988). V_H7183 and Q 52 are highly interspersed and located at the 3' end of the V_H gene loci.

In fish, at least several distinct V_H families have been identified, these are related to murine V_HS107 gene family with some divergence. It would not be surprising if genes related to the murine V_H family to be found with a germ-line representation in many vertebrates since selective pressure excreted by the invasion of pathogens and parasites has been constant during the evolution of the verterbrates and mammalians.

At present time, one of the fundamental questions is how the enormous number of antigen sites or variable region genes can be generated and regulated. In previous studies (Jeong, 1993; Jeong and Teale, 1988) a large proportion of fetal B cells that preferentially rearrange D proximal V_H gene families, V_H7183 and V_HQ52, became part of the functional developing reportoire. Clearly, it is different from the adult spleen B cells that utilize the largest V_H gene family, V_HJ558 the most frequent. In this report, to analyze the diversity of the functional heavy chain variable region genes in a B cells from fetus present at different developmental stages or environment, the expression of V_H genes focused on three important V_H gene families, V_H7183, V_HQ52 and V_HJ558, by individual, LPS induced B cells was determined by *in situ* hybridization.

Materials and Methods

Animals

Inbred BALB/c mice were purchased from Harlan Sprague-Dawley, Inc. and maintained at National Fisheries University of Pusan. All mice were routinely tested for pathogens, including mouse hepatitis, Sendai, *Mycoplasma pulmonis, Salmonella,* endoparasites, and ectoparasites. Mice have appeared negative for these pathogens.

Stimulation of lymphocyte cultures with LPS

Spleen of 6~8 wks old mouse was removed, dispersed into single cell suspensions, and plated into 24-well Costar (Cambridge, MA) dishes at 2×10^6ml in DME (Dulbecco's Modified Eagle) containing 10% FCS (Grand Island Biological, Grand Island, NY), 10 % NCTC medium (Inland Laboratories, Austin, TX), 50μg/ml gentamycin, 2mM glutamine, 5×10^{-5}M 2ME (2 mercaptoethanol), 1ml oxalacetate, 3×10^{-6}M glycine, 0.2U/ml insulin, and 0.1mM nonessential amino acids (M.A. Bioproducts, Walkersville, MD).

This medium was referred to as DME enriched. Cultures were incubated in 10% CO_2 for 5~6 d in the presence or absence of 10~40μg/ml bacterial LPS (*Escherichia coli* 0111 : B4 phenol/water extracted; List Biological Laboratories, Campbell, CA). Cultured cells were harvested, counted and cytocentrifuged onto slides for analyses by immunocytochemical staining and in situ hybridization.

Panning technique

Direct panning method was utilized for sIg^+ cell separation; the polystyrene petri plates (Fisher, 100× 15mm) were coated with 100μg of goat anti-mouse immunoglobulin in a volume of 5mls for 1hr at room temperature. After washing with HBSS (Hanks Balanced Salt Solution) twice, the plates were coated with 1% BSA/HBSS for 1hr at room temperature. After allowing the cells (30×10^6 per plate) to adhere for 70 min at 4℃, nonadherent cells were removed, placed

on a second anti-MGG (Mouse gammaglobulin) coated plate, and incubated for 70min at 4℃. To recover the bound cells, the entire surface of each plate was vigorously flushed with 1% BSA/HBSS using a pasteur pipette. In the nonadherent cell population, there were no detectable sIg^+ cells by immunocytochemical staining. The recovered cells were counted in a hemacytometer.

In situ hybridization

The in situ hybridization technique of Harper et al. (1986) and Berger (1986) was used as modified by Pardoll et al. (1987). Slight modifications of this procedure were carried out and have been described in previous reports with the origion of the used $C\mu$ and V_H gene family probes (Jeong, 1993).

Lymphocyte cultures with established stromal cell layers

The establishment of stromal cell layers derived from adult bone marrow was followed the methods of Dorshkind et al. (1986). For lymphocyte cultures, different source of progenitors were suspended at a concentration of 10^8 cells in 1ml of DMEM containing 0.1% BSA. The cell suspension was passed over Sephadex G-10 beads (Sigma) contained in a 5ml syringe according to the conditions described by Ly and Mishell (1974). The G-10 eluted cells were washed twice with BSS at 4℃ and seeded on established stromal layers in T25 flasks at a concentration of 2 to 3× 10^5 cells per 5.5ml. At intervals of 3 to 4 days, 75% of the culture medium, along with the nonadherent cells was removed from the seeded feeder layer flasks and replaced with fresh medium. After two changes of medium, the cells were harvested and refered to as cultured cells.

Results

1. V_H gene expression at various gestational ages in fetal B lymphocytes

It has been shown that more than 80% of transformed BALB/c fetal pre-B cells and B cells utilized the D proximal V_H7183 family (Alt et al., 1984). Moreover the early functional B cell repertoire examined by *in situ* hybridization showed somewhat similar tendency of bias with different degree in terms of V_H gene family utilization as was observed with fetal pre-B cell transformants (Jeong and Teale, 1988). Thus, it was of interest to determine if the use of fetal liver from an earlier age in gestation would result in a B cell population more skewed D-proximal V_H family expression. For this, V_H gene family expression was compared among LPS-stimulated B cells obtained from 16d, 18d, 19d and 20d fetal livers. The fetal B cells earlier than 16d were not responsive to LPS stimulation.

The results for expression of V_H gene families, J 558, 7183 and Q52 were normalized to C positive cell numbers represented about 20-40% of total cells. The results, shown in Fig. 1, provide no evidence for distinct B cell populations in different gestational fetal liver that results in different V_H repertoires or more skewed VH utilization. Again, even though there is the skewness of normal fetal liver B cell repertoire to D proximal V_H gene families, it is not severe like the transformant analyses.

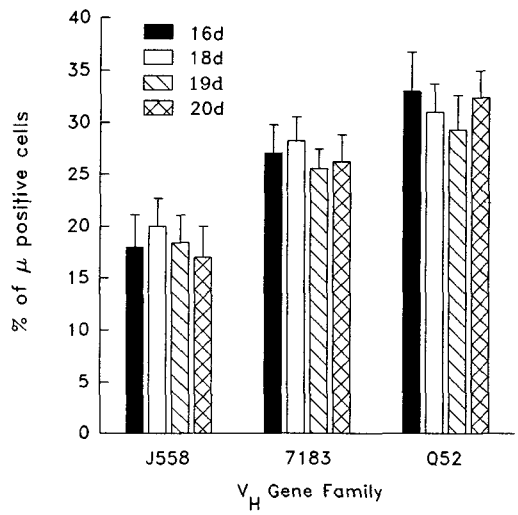

Fig. 1. V_H gene family expression in fetal liver B cells at different gestational days after LPS stimulation.

2. Determination of V_H gene family expression in fetal B lymphocytes of various stages of differentiation.

The antigenic environment, e.g. self tolerance, anti-idiotypic regulation, influences the B cell repertoire through the antibody receptors on the B cell surface (Teale and Mandel, 1980). Thus, it is thought that B cells specific for self antigens are deleted from the expressed repertoire. This regulation in fetal stages would be very critical for the establishment of complete immune system. To study this possibility, fetal liver cells at 18d gestation were panned on goat anti-mouse immunoglobulin coated plate for the separation of sIg^+ B cells. The separated adherent (sIg^+)/ nonadherent (sIg^+) cells were cultured in the presence of LPS for 5~8days. During the culture period sIg^- B cells differentiated to LPS-responsive mature B cells. After incubation, cell cultures were harvested and cytocentrifuged for the analysis of V_H gene family expression by *in situ* hybridization.

The results indicate no apparent difference in V_H gene family profiles between fetal sIg^+ and sIg^- B cell subsets (Table 1). Both sIg^+ and sIg^- B cells in fetal liver showed fetal-like VH gene family expression pattern with similar degrees of bias to the 3′ V_H gene families, V_H7183 and V_HQ52 as shown in Fig. 1 with unseparated fetal liver B cells.

Table 1. V_H gene family expression in different stages of fetal liver cells after LPS stimulation

Cells	V_H gene families		
	J558	7183	Q52
sIg^+ FL	17.2*	29.3	38.4
sIg^- FL	15.5	28.1	36.5

*; % of Cμ RNA expression cells

3. V_H gene family expression in B lymphocytes of fetal liver vs fetal spleen

To determine if the biased early functional B cell repertoire observed in fetal liver B cells can be found in other fetal lymphoid tissue, V_H gene family expression in fetal spleen B cells was analyzed. For this, 19 day fetal spleen cells were stimulated with LPS for 6~8days and cytocentrifuged for the *in situ* hybridization analysis. The results were compared with the previous data of fetal liver B cells. As shown in figure 2, fetal B cells derived from two different fetal lymphoid tissues expressed similar V_H gene repertoires. Both fetal spleen and fetal liver B cells showed a fetal-like V_H gene family expression pattern, that is, the predominat usage of V_H7183 and Q52 families. The less dominant expression of the largest family, J558, was also comparable to that of fetal liver cells. It indicates that two different lymphoid tissues of the fetus, liver and spleen, both contained similar B cell populations in terms of V_H gene family expression.

4. V_H gene family repertoire of developing B cells in fetal liver and fetal spleen after cultured in stromal cell layers.

It was shown in the previous data that LPS induced fetal B cells were charaterized by a biased utilization of two D-proximal V_H gene families, V_H 7183 and V_H Q52 (Figs. 1 and 2), compared with a random V_H gene utilization in adult B cells (Jeong and Teale, 1988). In order to determime the relative influence of environmental vs genetic factors on the distinct V_H gene family repertoires of different sources of B cells, the lymphocyte culture technique described by Whitlock and Witte (1982) as modified by Denis (1987) and Dorshikind (1983) was used. Sources of B cell progenitors from fetal liver, fetal spleen and adult bone marrow were seeded onto already established stromal cell layers derived from adult bone marrow. In this way, essentially the same microenviroment was provided for different progenitor sources.

The appeared cells after 5~6day culture were harvested and found that over 90% were B lineage cells determined by immunocytochemical staining for the presence of cytoplasmic immunoglobulin (data not shown).

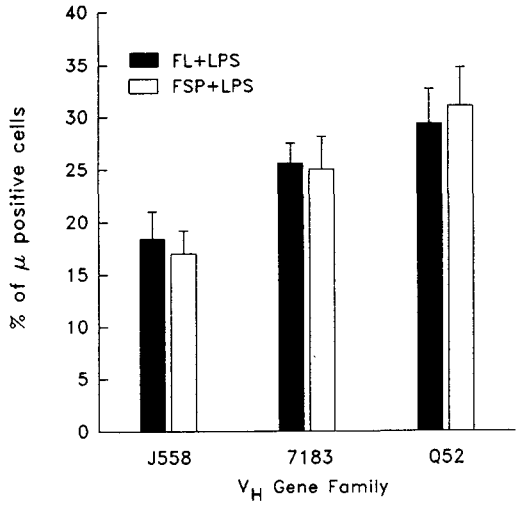

Fig. 2. V_H gene family expression in fetal liver and fetal spleen B cells after LPS stimulation.

Harvested cells were analyzed for V_H gene family expression after LPS stimulation. The data are shown in Fig. 3 and indicate that the B cells developing from fetal liver and fetal spleen maintain the fetal-like V_H gene repertoire with a higher frequency of V_H 7183 and Q 52 expression and a lower frequency of V_H J 558 expression compared with adult bone marrow cells. And both the B cells developing from adult and fetal progenitors resulted in an LPS-induced V_H gene family repertoire similar to uncultured adult and fetal B cells, respectively (Jeong, 1993).

Moreover, the cultured fetal liver cells for different periods, 6d, 11d, 21d and 28d, also resulted in a V_H gene repertoire similar to uncultured fetal liver B cells (Fig. 4). Thus, even longer contact period with adult stromal cell layer as a microenvironment does not give influence to the formation of V_H gene repertoire in fetal B cell progenitors.

Discussion

B cells in the fetus appear in many respects to be different from B cells in the adult in terms of average cell size, surface antigen expression, susceptibility to tolerization, and functional characteristics (Maureen, 1982). Moreover, many studies have demonstrated that fetal and neonatal B cells have restricted antigen specificities compared with those of the adult. For example, fetal and neonatal B cells are either unresponsive to particular antigens or are present in very low frequency (Denis and Klinman, 1983; Teale, 1985). In many cases, the idiotype profiles obtained

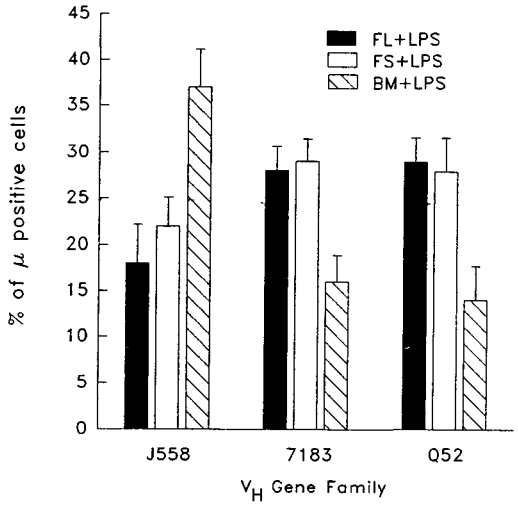

Fig. 3. V_H gene family expression after LPS stimulation of cultured fetal liver, fetal spleen and adult bone marrow cells for 1 week.

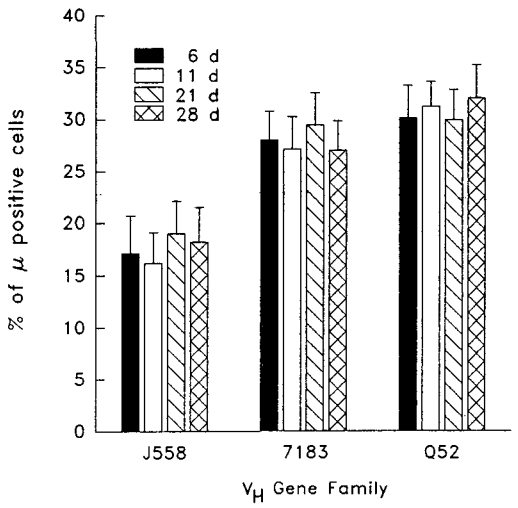

Fig. 4. LPS induced V_H gene family expression of cultured fetal liver cells for different periods on stromal cell layers.

with fetal and neonatal B cells are substantially different from the idiotypes produced by adult B cells (Klinman and Press, 1975; Fung and Kohler, 1980). Unresponsiveness or a restricted response to certain antigens could be the result of restricted V_H gene rearrangements as suggested by recent molecular studies of fetal pre-B cell lines (Permutter et al., 1985).

The main focus of our studies was to concentrate on the early functional repertoire, since most published studies have been concerned with rearrangement frequencies of transformed pre-B cells. Therefore, we developed the *in situ* hybridization technique which allowed us to analyze expressed V_H gene families in normal B lymphocytes at the single cell level (Jeong et al., 1988). The analysis of normal B cells in this study eliminated any possible bias resulting from transformation protocols used previously and minimized limitations associated with sampling size. However in this technique, three important V_H gene families, V_HJ558,V_H7189 and V_HQ52 were focused, because the smaller V_H gene families showed no clear differences or too rare positive cells to analyze (Jeong, 1993).

In the previous studies (Jeong, 1993; Jeong and Teale, 1988), the LPS stimulated cells derived from adult spleen cells of BALB/C mice showed the predominant expression of V_H J558 family, the largest V_H gene family. In contrast, the most D proximal V_H gene families, V_H Q52 and V_H 7183 were the predominant families expressed by LPS-stimulated fetal liver B cells. However, the degree of bias was considerably less than the rearrangement biases observed with fetal pre-B cell transformants in which the vast majority precursors had rearranged to V_H 7183 (Alt et al., 1984).

In the studies to determine whether the fetal liver B cells from an earlier age in gestation would result in more skewed toward D-proximal V_H gene family expression, no apparent differences in V_H gene expression among different gestational ages, 16d, 18d, 19d and 20d were found (Fig. 1). This suggests that the repertoire of fetal B cells for V_H gene utilization does not change from day 16 of gestation to birth.

Another important aspect of these experiments is the analysis of B cell repertoire depending upon the differentiation stage of B cell. Malynn et al. (1987) showed some evidence of an increased V_H 7183 in total RNA analysis of bone marrow cells compared with spleen cells. He used very heteregeneous cell populations, however, for the isolation of RNA. To analyze the B cell repertoire in different maturational stages, immature sIg^- B cells were isolated and analyzed for V_H gene family expression after they matured *in vitro* and were stimulated with LPS. The results showed that LPS-induced B cells derived from both the sIg^- and sIg^+ subsets of fetal liver expressed fetal-like V_H gene family utilization patterns (Table 1). This suggests that developmental age determines the formation of functional V_H gene repertoire rather than a maturational stage in the B cell lineage. It is matched with the results of Wu and Paige(1988) who also analyzed the V_H gene expression in CFU-B (colony forming B cells) by RNA colony blot assay.

Considerable experimental evidence indicates that different lymphoid may contain distinct subpopulations of lymphocytes (Scher, 1981). Also, it is known that the cells migrate from the fetal liver to the fetal spleen and bone marrow early in development (Teale, 1985). However, it is not known whether fetal hematopoietic tissues, fetal liver and spleen, contain essentially the same progenitor pools in terms of B cell repertoire. We found that B lymphocytes of fetus derived from fetal liver and spleen had a similar V_H gene repertoire with predominant expression of the D proximal V_H gene families, V_H 7183 and V_H Q52 (Fig. 2). It means that the compartmentalization of B cells does not result in different B cells in terms of V_H gene repertoire. It is also interesting to expand these experiments to other vertebrate or fish for the analysis of the distinct antibody repertoire established through the evolution of long time.

In order to address the possibilities of microenvironmental influences to the B cell progenitors during ontogeny, long term B cell culture systems were developed which represent modifications of protocols established by Whitlock and witte(1982) and Dennis

and witte(1987). In these cultures, an adherent stromal cell layer provides the same microenvironment for the development of and growth of progenitor B cells derived from different sources, fetal spleen, fetal liver and adult bone marrow. In our experiments, the cultured cells contained very high proportion of B cells and similar to previously published results (Denis, 1987).

When cultured cells were analyzed for LPS-induced VH gene family expression, it was found that adult bone marrow cells and fetal cells both expressed identical V_H gene family with the uncultured cells, respectivly (Fig. 3). Moreover, fetal liver cells grown on bone marrow stromal cell layers for different periods still expressed D proximal V_H gene families, V_H 7183 and V_H Q52 predominantly without skewness to any specific families (Fig. 4). Because the environment provided by stromal layers was presumably the same, the results have suggested that the B cell progenitors are distinct in fetus and adult. These progenitors are committed to express a preprogrammed expression of V_H gene repertoire and not influenced by different microenvironments.

Consequently, the restricted V_H gene repertoire of B cells in the fetus which is distinct from that of the adult appears to be associated with the genetic potential of B cell progenitors and less with environmental influences or compartmentalization or differentiation stages.

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어린 B 세포가 갖는 V_H 유전자 발현의 특성

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B 세포가 다양화 되어가는 기작을 규명한다는 것은 면역 반응의 조절이 생체 내에서 어떻게 이루어 지고 있는 가를 이해하는데 가장 기본이 되는 것이다. 본 연구는 기 확립한 *in situ* hybridization 기법을 이용하여 항체의 항원 결합 부위 유전자가 B 세포의 발달 과정중 어떻게 조절이 되고 있으며 이것은 B 세포의 다양화라는 측면과 어떻게 연관이 되어 있는 지를 분석하였다. Gestation 시기가 16일, 18일, 19일, 20일 되었을 때 간에 있는 B 세포는 V_H7183와 V_HQ52 두개의 V_H 유전자군을 가장 많이 이용하고 있었으며 이러한 경향은 gestation 기간 전체를 통하여 변화없이 일정하게 나타났다. 간에 있는 fetal B 세포를 differentiation 단계별로 구분하기 위하여 표면 항체를 갖고 있는 집단과, 갖고 있지 않은 두 집단으로 나눈 후 각 집단이 발현하는 V_H 유전자를 분석하였을 때 뚜렷한 차이를 나타냄이 없이 양쪽 집단 모두 fetus의 특징적 V_H 이용양식을 보여주었다. 또 다른 조혈 기능 임파 기관인 fetal spleen에 있는 B 세포 또한 fetal liver의 B 세포와 동일한 양상의 V_H 유전자 이용 양식을 보여 주어 각 임파 기관별 B 세포의 다양성 차이를 발견 할 수 없었다. 이와 같이 adult의 B 세포에 대비하여 독특한 V_H 유전자 이용 양상을 보이는 fetal B 세포의 전구 세포를 4주 이상 미리 형성시킨 adult 골수 세포와 직접 접촉시키면서 발달, 성숙시킨후 다시 나타난 B 세포를 분석하여도 여전히 fetal B 세포로서의 V_H 유전자 이용 양상을 보이는 것은 fetal B 세포의 전구 세포가 갖고 있는 유전적 잠재력에 의한 것이지 환경이나 B 세포의 differentiation 단계 또는 B 세포가 머무르고 있는 특수 임파 장기의 생리적 환경 등에 좌우되는 것이 아니라는 것이 확인되었다.