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V_H Gene Expression and its Regulation on Several Different B Cell Population by using *in situ* Hybridization technique

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B 세포의 V_H 유전자가 어떠한 기작으로 선택되어지는 지는 현재 명확히 밝혀져 있지 않다. 본 연구에서는 transformation 등의 방법에 의한 편향된 분석결과를 피하고자 *in situ* hybridization 기법을 이용하여 정상적인 single 세포가 발현한 V_H 유전자를 분석하였다.

V_H 유전자간에 나타나는 DNA 배열의 유사성 때문에 *in situ* 기법에서 가장 중요한 것은 probe 농도와 세척 stringency의 결정이다. LPS-stimulated된 spleen B 세포에 대해서 Cμ와 V_HJ558 ^{35}S-RNA probe는 2~4×106cpm/slide의 농도에서 낮은 background와 적정수의 positive 세포를 관찰할 수 있었으며 세척 조건으로서는 54℃에서 40~50%의 formamide를 사용할때 최적이라는 것을 Cμ, V_HS107, 그리고 V_HJ558 probe를 이용한 실험에서 결정하였다.

위의 조건하에서 spleen B 세포가 발현한 V_H 유전자를 분석하여 본 결과 각각의 V_H gene family 발현 빈도는 각각의 family 크기에 비례하여 결정된다는 것을 알 수 있었다. 이러한 결과들은 여러 다른 발달단계에 있는 bone marrow B 세포에 대해서도 동일한 결과를 보여 주어 어떤 특수 V_H gene family의 발현이 B 세포의 발달단계에 따라 특이하게 변화하는 것은 아니라는 것을 나타내 보여 주었다. 그러므로 V_H 유전자의 이용은 B 세포가 differentiation하는 것과는 무관하게 무작위 적으로 선택되어 진다는 것을 밝혔다.

Key Words : B cell, V_H gene expression, *in situ* hybridization, bone marrow B cells, RNA probes.

Antibody molecule is made up of two distinct types of polypeptide chain, the light(L) chain and heavy (H) chain. These polypeptide chains are linked together by covalent and non-covalent forces to give a four chain structure composed of pairs of identical heavy and light chains(Cooper *et al.*, 1974). Each heavy chain consists of two distinct regions. The carboxy terminal region or latter three fourths of the chain has a relatively constant amino acid sequence and is called C_H(Costant ; Heavy chain), whereas the amino terminal one fourth of the chain shows much sequence variability and is called V_H(Variable ; Heavy chain). Similarly, in the light chain, the corresponding regions are V_L and C_L for the variable and constant regions respectively. The heavy chain variable region is encoded by three separate germ line gene segments ; the variable region gene segments(V_H), diversity region gene segments(D), and junctional region gene segments(J_H) (Tonegawa, 1983) 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 eariest stage of B cell differentiation, D to J_H rearrangements generally occur first and on both chromosomes followed by V_H to D-J_H rearrangement(Tonegawa, 1983, Alt *et al.*, 1984, Sugiyama *et al.*, 1983, Brodeur *et al.*, 1984[a]). However, the exact mechamism of the rearrangement process and how it regulated remain unclear.

Several studies have been done to examine the number of V_H gene segments and their structures. Brodeur *et. al.*(Brodeur *et al.*, 1984[a], Brodeur *et al.*, 1984[b]) did genomic restriction enzyme fragment analysis by using many different V_H probes and defined the families of related V_H gene segments and they were called by the name of representitive probes used : V_HJ558, V_HJ606, V_H36-60, V_H3609, V_H11, VGAM3.8, V_H7183, V_HQ52, V_HS107, and V_HX24.

The extremely heterogeneous variable regions of antibody molecule are responsible for binding to antigen. In terms of that, it is not difficult to expect the structural diversity of antibody variable regions and apparently may be required to accomodate the enormous number of foreign antigens. Therefore, one of the fundamental questions at present time is how such a wide variety of antibody binding sites or variable regions can be generated and regulated. In this study, to compare the diversity of the functional heavy chain variable region genes in a large proportion of B cells from adult spleen and bone marrow cells, the expression of V_H genes 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 are routinely tested for pathogens, including mouse hepatitis, Sendai, *Mycoplasma pulmonis*, *Salmonella*, endoparasites, and ectoparasites. Mice have tested 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 2×106/mℓ 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 analysis by immunocytochemical staining and *in situ* hybridization.

Panning technique

Two panning methods were utilized : direct and indirect. For direct panning(used 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 70min at 4℃, nonadherent cells were removed, placed on a second

anti-MGG(Mouse gammaglobulin) coated plate, and incubated for 70min at 4℃. In the nonadherent cell population, there were no detectable sIg^+ cells by immunocytochemical staining.

For indirect panning(used with 14.8 monoclonal antibody that detects B220), 30×10^6 target cells were coated with 200μℓ of a 1 : 3 dilution of concentrated 14.8 culture suspernatant for 20min at 4℃. The cells were washed with cold PBS and suspended in 5mℓ 1% BSA/HBSS. Cells incubated with anti-B220 were then added to petri dishes that had been coated with the mouse anti-rat kappa light chain monoclonal antibody(Mar 18.5). The plates were incubated at 4℃ for 70min, swirling the plate once midway through the incubation period to redistribute cells. To recover the bound cells, the entire surface of each plate was vigorously flushed with 1% BSA/HBSS using a pasteur pipette. The recovered cells were counted in a hemacytometer.

The generation of radioactive transcripts

For the preparation of radiolabeled RNA transcripts, we followed the protocol of BRL(Bethesda Research Laboratories, Inc. Gaithersburg, MD) (Johnsion *et al.*, 1984, Buter *et al.*, 1982). 13μM of ^{35}S-UTP(Dupont New England Nuclear, Boston, MA) in volume of 5~7μℓ was incubated with 4μℓ of 5x transcription buffer(200μM Tris pH 7.5, 30 μM $MgCl_2$, 10mM spermidine, 50mM NaCl), 2μℓ 100mM DTT, 0.8μℓ RNAsin(Promega Biotec, Madison, WI), 1μℓ each of 10mM ATP, CTP, GTP, and 0.8μℓ of 100mM UTP, and 1μℓ of 0.5~1.0mg/mℓ linearized template. The total reaction volume was 20μℓ. The reaction was started by adding 25 units of T3 or T7 RNA polymerase. After 90min incubation at 39~41℃, addition of RNAse free DNAse(RQ1, Promega) to a final concentration of 5μg/mℓ followed by incubation at 37℃ for 15min was done to remove DNA template from the reaction mixture. All reactions were terminated by phenol/chloroform extraction which was routinely done two times. After ethanol precipitation, the transcripts generated were resuspended in 20μℓ of TE buffer(10mM Tris pH 7.5, 0.5mM EDTA).

Probes

The Cμ and V_H gene family probes were kindly provided by Drs. Hood, Brodeur, Riblet, and Riley and subcloned into pT7/T3~18 plasmid designed for transcription of either strand of DNA inserted into the multiple cloning site by using either T_7 or T_3 polymerase(8). The probes used were pV 36p[21](36~60), pV14 RI(J606), pVJ558(J558), pVS107(S107), pVQ52(Q52), pVSAPC-15(7183), and pV24(X24) and have been described elsewhere(Table 1) (Dildrop *et al.*, 1984, Riley *et al.*, 1986, Perlmutter *et al.*, 1985[a]).

Table 1. Probes used for *in situ* experiments

V_H Family	Probes	Insert (bp)	Specificities[a]
J558	V_HJ558	370	α1, 3-DEX, ARS, NP
Q52	QUPC52	350	α1, 6-DEX, OX
36-60	TH2-36	140	ARS
X-24	X-24	500	β1, 6-Galactan
7183	SAPC15	1,100	FLU HA
J606	J606	5,000	Inulin, SACHO
S107	S107	445	PC

[a] DEX, dextran ; ARS, arsonate ; OX, phenyloxazolone ; FLU HA, influenza hemagglutinin ; SACHO, streptococcal A carbohydrate ; PC, phosphorylcholine.

In situ hybridization

The *in situ* hybridization technique of Harper *et al.*

(Harper *et al.*, 1986) and Berger(Berger *et al.*, 1986) was used as modified by Sideras *et al.*(Sideras *et al.*, 1988). Slight modification of this procedure were carried out for the specific analysis of V_H gene expression. Briefly, cells were cytocentrifuged onto precleaned slides and fixed in freshly made 4% paraformaldehyde for 1min. Slides were transferred directly to 70 % ethanol and stored at 4℃ until used. Slides were then removed from 70% ethanol and prepared for hybridization by incubating successively in 2X SSC twice for 1min, 0.1M triethanolamine, pH 8.0, containing 0.25% acetic anhydride(10min), 2X SSC(twice for 1min), 0.1M Tris, pH 7.0, 0.1M glycine(30min), 2 X SSC(1min), 70% ethanol(1min), 80% ethanol(1 min), and 95% ethanol(1min). The slides were then allowed to air dry. A hybridization mixture(10μℓ) was pipetted directly onto the cell button of each slide ; it contained 5μℓ of deionized formamide(EM Science, Cherry Hill, NJ), 1μℓ 20X SSC/100mM DTT, 1μℓ 10 mg/mℓ *E. coli* tRNA, 1μℓ denatured salmon sperm DNA at 10mg/mℓ, 0.4μℓ of nuclease-free BSA at 50mg/mℓ and 0.6μℓ of ^{35}S-labeled V_H gene family probes or the Cμ probe(2~4×10⁶cpm/slide). Cover slips previously siliconized and baked at 280°F for 16hrs 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℃.

After incubation, the slides were washed by successively incubating in the following solutions ; 2X SSC containing 40% formamide for 3min at 54℃, 2X SSC containing 40% formamide at 54℃ for 5min, 2X SSC containing 40% formamide at 54℃ with shaking(1h), 2X SSC(twice for 1min), 2X SSC containing 100μg/mℓ RNAse A(Sigma Chemical Co., St. Louis, MO) and 1μg/mℓ RNAse T1(Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37(30min, twice), 2X SSC containing 40% formamide at 54℃(3min), 2X SSC containing 40% formamide at 54℃(5min), and 2X SSC containing 40% formamide at 54℃ with shaking (1h). The slides were dipped in 2X SSC, 70% ethanol, 80% ethanol, and 95% ethanol and allowed to dry. Finally, the slides were dipped in NTB2 emulsion(Eastman Kodak Co., Rochester, NY) for autoradiography, developed after 5~6d, and subsequently stained with hematoxylin and eosin.

RESULTS

Optimization of *in situ* hybridization conditions

The effect of probe concentration on signal and background was evaluated by using increasing concentrations of the probes. The probes tested were Cμ which has 100% homology with the cellular message and J558 which is the most heterogeneous V_H gene family(Brodeur *et al.*, 1984[a], Brodeur *et al.*, 1984[b]). The results indicate that increasing the probe above 2~4× 10⁶cpm/slide or approximately 2.5~5ng/slide, did not result in an increased frequency of cells labeled or alter the specificity of labeling(Table 2). However, more than 6×106cpm/slide resulted in increased levels of background, although it dod not alter the frequency of positive cells detected. Thus, it was concluded that addition of 2~4×10⁶cpm of RNA probe per slide was sufficient for saturation of the hybridization reaction.

The optimal washing conditions after hybridization was determined by varying the formamide concentration in the washing buffer(Table 3). The results indicated that lower washing stringency at 30% formamide in 2X SSC increased the frequency of V_H expressing cells accompanied by an increase in background grain numbers. Therefore, 40~50% formamide concen-tration in the washing buffer was found to be optimum(Fig. 1).

Table 2. Determination of optimal probe concentration for *in situ* hybridization on LPS-stimulated adult spleen cells[a]

Amount of probe (10^6cpm/slide)	μ probe		J558	
	Background	% of total cells detected	Background	% of total cells detected
1	4.3± 0.5[b]	43.4	4.6± 0.6	15.6
2	5.4± 0.6	40.7	4.3± 0.4	15.8
4	4.6± 0.6	38.8	5.7± 1.2	16.6
6	5.3± 0.4	45.6	10.0± 0.4	16.2
10	9.9± 0.5	45.1	14.8± 0.9	16.1

[a] Number of grains per positive cell is more than 120

[b] The mean grain number± SEM of 8~16 randomly chosen areas approximatelythe size of cells

Table 3. Effect of washing conditions on grain number, background, and frequency of cells detected using LPS-stimulated adult spleen cells

Probe	% formamide in washing buffer	grain number		% of total cells labeled
		Intracellular[a]	Extracellular[b]	
μ	50	96.5± 0.4	5.3± 0.6	45.6
	40	103.3± 3.5	11.7± 1.1	53.4
	30	102.4± 2.9	14.5± 1.2	47.7
S107	50	70.7± 9.1	5.0± 0.6	3.3
	40	69.0± 4.9	5.6± 0.5	3.6
	30	79.7± 0.4	13.1± 0.6	5.4
J558	50	41.4± 3.9	4.8± 0.5	16.5
	40		9.1± 0.4	17.1
	30		11.4± 0.9	23.4
(+)Cμ strand	50		2.6± 0.5	0

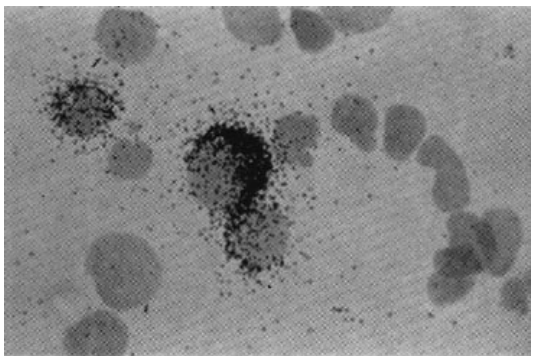
[a] The mean grain number± SEM of 8~16 randomly chosen positive cells

[b] The mean grain number± SEM of 8~16 randomly chosen areas approximately the size of cell

V_H gene family expression in LPS stimulated adult B lymphocytes

The repertoire of functional B cells in adult mice that can be determined by the analysis of the produced mRNA of V_H genes was evaluated by using *in situ* hybridization. The expressed V_H genes in LPS stimulated adult spleen B cells are presented as the percent of cells containing detectable Cμ specific RNA that

Fig. 1. Autoradiograph of LPS-stimulated adult spleen cells by *in situ* hybridization techniques.



Cells were hybridized with V_H J558 probe. Slides were exposed for 5~7 days at 4℃.

are expressing each of seven important V_H gene families(Table 4). All cell populations were routinely analyzed by immunocytochemical staining with anti-mouse gammaglobulin or anti-mouse Cμ(Fig. 2) and compared with the proportion of cells labeled with the control Cμ probe. Under the conditions used, it was come out that the number of cells detected by *in situ* hybridization were very closed to the number of plasma cells and plasmablasts, which stained more intensely than the remainder of B cell blast(data not shown). LPS-stimulated adult spleen B cells expressed V_H J558 predominantly as we can expect by the size of V_H gene family. The V_H J558 is the family containing the most numbers. Also, the level of expression of each of the other families analyzed approximated the complexity or size of that family. Therefore, the characteristic of adult B cells in spleen, in terms of V_H gene family expression, is apparently random utilization of V_H gene segment.

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

It has been proposed that the antigenic environment, e. g. self tolerance, anti-idiotypic regulation, influences the B cell repertoire through the antibody receptors on the B cell surface(Kinkade *et al.*, 1982, Metcalf *et al.*, 1979). Thus, it is thought that B cells specific for self antigens are deleted from the expressed repertoire. Therefore, pre-receptor B cells(pre B cells) that have not yet been exposed to the antigenic environment may have a distinct V_H gene repertoire from that of sIg^+ B cells. To study this possibility, adult bone marrow cells were separated into three different differentiation stages based on cell surface markers : 1) sIg^+, 2) sIg^- $B220^+$ and 3) sIg^- $B220^-$ cells. This

Table 4. V_H gene familly expression by LPS-stimulated adult spleen B lymphocytes[1]

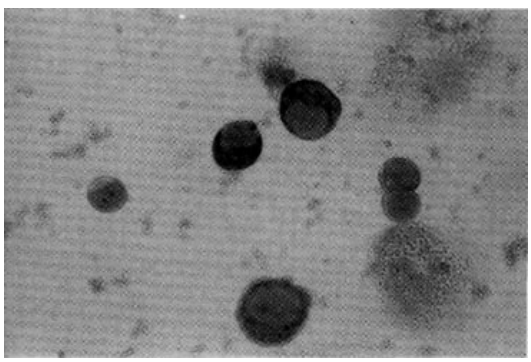
V_H gene familly	% of μ RNA containing cells expressing each of the following V_H gene families	relative complexity of V_H gene familly(%)
J558(60)[2]	37.1±4.13[3]	55.6
7182(12)	15.9±0.8	11.1
Q52(15)	19.6±1.9	13.9
36-60(5)	10.0±1.7	4.6
J606(10)	7.6±0.4	9.3
S107(4)	4.4±0.4	3.9
X-24(2)	3.5±1.2	1.9

[1] Spleen cells from BALB/c mice were cultured in the presence of LPS for 5~6 days. After incubation, cell cultures were harvested and cytocentrifuged onto slides for analysis by in situ hybridizaation using ^{35}S-labelled V_H gene familly probes and ^{35}S-labelled Cμ.

[2] Number in parenthesis represent the published complexity of V_H gene famililies by Brodeur(Brodeur *et al.*, 1984[a]).

[3] Results reprsents the mean±SEM of 8(spleen) complete experiments with different mice.

Fig. 2. Immunocytochemical staining of LPS-stimulated adult spleen cells.



Cells were stained with $F_{(ab)'2}$ rabbit anti-mouse Ig, biotin labeled $F_{(ab)'2}$ goat anti-rabbit immunoglobulin and mixture of streptoavidin and biotin labeled horse raddish peroxidase.

was done by the panning technique. Efficient cell separation was achieved by immunocytochemical staining, in that each isolated subpopulation showed less than 0.5% contamination by the other subpopulations. Separated B cells were stimulated with LPS for 5~6 days for analysis of the expressed V_H gene family using *in situ* hybridization. During the culture period, sIg^- B cells differentiated to LPS-responsive mature B cells. In the LPS stimulated sIg^+ population, more than 60% of the total cells were detected by *in situ* hybridization with the Cμ probe. In the LPS stimulated sIg^- $B220^+$ and sIg^- $B220^-$ cell population, the proportion of Cμ positive cells detected was 14~17% and 10~12% respectively. Presumably these findings reflect the frequency of LPS-responsive B cells during the stimulation period.

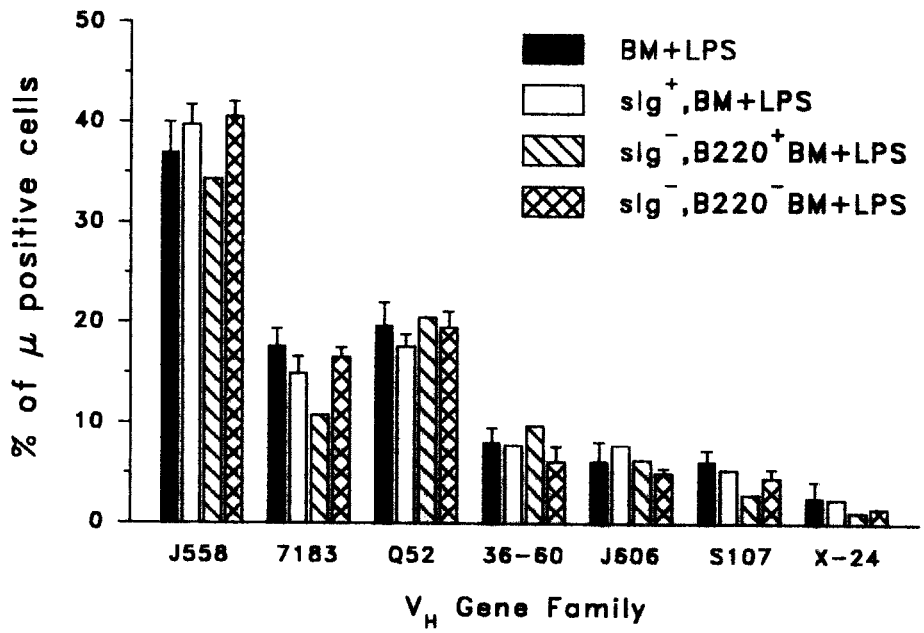
B lineage cell subsets separated according to differentiation stage were also analyzed for LPS-induced V_H gene family expression. The results indicated no apparent difference in V_H gene family profiles among adult subsets sIg^+, $B220^+$ sIg^-, and $B220^-$ sIg^-(Fig. 3). Therefore, at this level of analysis, no evidence was found for distinct repertoires in the pre-receptor vs post receptor pool of B cells.

These experiments also relate to potential differences in repertoire depending upon the maturation stage of the B cells. Thus, Malynn *et. al.*(Malynn *et al.*, 19 87) reported evidence that adult bone marrow B cells expressed higher levels of D-proximal V_H 7183 family compared with adult spleen B cells, when they analyzed and compared the amount of V_H gene family mRNA extracted from bone marrow cells and spleen cells. The authors proposed that D-proximal V_H gene family usage may be a characteristic of less mature B cells, because the bone marrow cells contain higher numbers of immature B cells than that of spleen B cells. However, our results indicated no differences between adult spleen and bone marrow populations(Table 4, Fig. 3). Moreover, the results shown in Fig. 3 confirm and extend our findings since both LPS-induced sIg^+ and sIg^- B lineage cells derived from adult bone marrow also showed a adult spleen-like V_H gene family expression pattern correlating with V_H gene family size. Thus, even after enriching for immature B lineage cells from adult bone marrow cells, there was no evidences to indicate preferential D-proximal V_H gene usage or an increased degree of preferential D-proximal V_H gene usage, respectively.

DISCUSSION

In this study, we examined the optimum conditions for *in situ* hybridization using ^{35}S labeled RNA probes. Among the technical variables examined were probe concentration and washing stringency. The results suggested that concentrations of labeled probe

Fig. 3. The expression of V_H gene families with LPS stimulation in various stages of adult bone marrow cells.



Adult bone marrow cells were panned on goat anti-mouse immunoglobulin coated plates for isolation of sIg^+ B cells. Nonadherent cells were coated with anti-B220(14.8) monoclonal antibody and layered on mouse anti-rat light chain monoclonal antibody(Mar 18.5) coated plated for the isolation of sIg^-, B220 B cells. After 5~7 days stimulation with LPS, cell cultures were harvested for the analysis of V_H gene family expression by *in situ* hybridization.

exceeding 6×10^6cpm/slide resulted in somewhat higher backgrounds without resulting in increased frequencies of positively labeled cells(Table 2). In terms of washing conditions, low stringency condition increased both the background level and the level of nonspecific binding(Table 3). We also tested the length of hybridization time. Extended hybridization times (overnight) were not shown to appreciably affect the degree of labeling(data not shown). The specificity of probes was also indicated by the fact that the total proportion of Cμ hybridizing cells approximated the total proportion of cells expressing V_H genes. This latter finding suggests that the majority of V_H gene families have been identified in agreement with previous studies(Dildrop *et al.*, 1985, Perry *et al.*, 1981). In some cases, the total proportion of cells expressing V_H genes was slightly greater than 100%. The most likely explanation is a small fraction of plasma cells are producing isotypes other than IgM and would not be scored by Cμ hybridization.

By the *in situ* hybridization technique, we can determine the expression of each functional V_H gene fa-

mily in the individual normal B cells without limitation of analyzable cell population. It made us possible to eliminate any possible bias resulting from transformation protocols used previously(Yancopoulos *et al.*, 1984, Alt *et al.*, 1981) and minimized limitations associated with sampling size. Spleen cells from adult mice were stimulated with the mitogen LPS. Up to one third of splenocytes would be stimulated by this treatment(Melchers, 1977). Considering the level of stimulation, the majority of B cells induced would presumably represent the functional, primary B cell repertoire. Generally, when adult splenocytes were cultured in the presence of LPS, as many as 30 to 50% of the cells contained sufficient amount of RNA to become labeled when hybridized with the Cμ probe. However, the normal *in situ* hybridization technique used in the experiments described in this study was not sufficiently sensitive to detect Cμ-specific or V_H gene specific RNA in uncultured splenocytes cytocentrifuged directly onto slides. Moreover, it was highly unlikely that germline V_H transcripts or sterile transcripts were detected ; such nonfunctional transcripts have been shown to be present at lower levels than functional immunoglobulin transcripts.

To analyze the functional B cell repertoire of the adult B cells in spleen were stimulated with the mitogen LPS. It was assumed that LPS acts as a polyclonal activator and would not selectively stimulate a subpopulation of B cells unique in terms of V_H gene expression. The result indicate that the LPS induced repertoire of adult splenocytes appears random for the V_H gene family utilization and is not significantly different from that of the bone marrow B cells(Table 4, Fig. 3). The predominant families expressed in adult splenocytes are V_H J558 the largest V_H gene family. These results of our study can be interpreted that the degree of V_H gene family expression in the population of adult splenocytes very much approximated to the complexity of V_H gene family and is not dependent upon the location of V_H gene family in chromosome as the results of Permutters analyzed by the transformed B cells(Perlmutter *et al.*, 1985[b]). Additionally, it would be interesting to determine if normal adult B cells may express different way the new V_H gene family analyzed very recently, especially VGAG 3~8 family that has been appeared as the most D-proximal V_H gene family(Christoph *et al.*, 1989). However, it would be not difficult to suspect the low expression levels of VGAM 3~8 gene family in LPS-induced adult splenocytes by the small size of this family, if our results that showed the complexity, not the positionin in chromosome, dependent V_H gene utilization in B cells of adult also can be extended to this new family.

Processes such as tolerance, anti-idiotypic regulation, and maternal antibodies could influence the emerging the B cell repertoire through the antibody receptors on the B cell surface(Kinkade *et al.*, 1982, Metcalf *et al.*, 1979). Therefore, it was of interest to compare V_H gene family expression on mature sIg^+ B cells with B cells that develop in culture from sIg^- or B220 precursor B cells. In these experiments, no apparent evidence was obtained for differences in V_H gene family expression when the starting populations were pre-receptor vs mature B cells(Fig. 3). If antigenic microenvironment plays a major role in shaping the B cell repertoire and self tolerance or idiotypic network results in elimination or expansion of B cells with altered V_H gene family repertoires, then differences in V_H region expression between B cells derived from sIg^- vs sIg population should be observed. However it is possible that the negative results could re-

lated to the sensitivity of analysis and that individual V_H genes would have to be analyzed. Another important aspect of these experiments is the analysis of B cell repertoire depending upon the differentiation stage of B cell. In the analysis of total RNA, Malynn *et. al.* (Malynn *et al.*, 1987) showed some evidence of an increase in V_H7183 specific RNA in bone marrow compared with spleen. The authors suggested that the restricted V_H gene usage of 7183 could be associated with differentiation stage of the B cell. 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. 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 indicated that LPS-induced B cells derived from both the sIg^- and sIg^+ subsets of adult bone marrow expressed complexity dependent V_H gene family utilization patterns. This suggests that the different maturational stages do not influence for the formation of functional V_H gene repertoire in the B cell lineage. However, it should be noted that our experiments were measuring the expressed V_H gene family in plasmablasts or plasma cells derived from separated sIg^+ and sIg^- B cells after in vitro stimulation rather than directly testing mature and immature cells. Nevertheless, the initial starting population was highly enriched for immature B cells in the sIg^- population and no evidence for increased usage of V_H 7183 was observed. The results of this study in which hundreds of LPS-induced adult B splenocytes were analyzed indicated that the expression of V_H gene families is random and dependent upon the the size of V_H gene families. Moreover, this pattern of random utilization of V_H gene family also appeared in the B cells of bone marrow and was not changed during maturation of the functional B cell lineage. Whether there is any specific contributory roles of cellular selective regulatory mechanisms in different lymphatic organs or special B lineage cells, like CD5 B cells, needs to be assessed further.

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V_H Gene Expression and its Regulation on Several Different B Cell Population by using *in situ* Hybridization technique

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The mechanism by which V_H region gene segments is selected in B lymphocyte is not known. Moreover, evidence for both random and nonrandom expression of V_H genes in matured B cells has been presented previously. In this report, the technique of *in situ* hybridization allowed us to analyze expressed V_H gene families in normal B lymphocyte at the single cell level. The analysis of normal B cells in this study eliminated any posssible bias resulting from transformation protocols used previously and minimized limitation associated with sampling size. Therefore, an accurate measure of the functional and expressed V_H gene repertoire in B lymphocyte could be made.

One of the most important controls for the optimization of *in situ* hybridization is to establish probe concentration and washing stringency due to the degree of nucleotide sequence similarlity between different families which in some cases can be as high as 70%. When the radioactive Cμ and V_HJ558 RNA probes are tested on LPS-stimulated adult spleen cells, 2~4×106cpm/slide shows low background and reasonable frequency of specific positive cells. For the washing condition, 40~50% formamide at 54℃ is found to be optimum for the Cμ, V_HS107 and V_HJ558 probes. The analyzed results clearly demonstrate that the level of each different V_H gene family expression is dependent upon the complexity or size of that family. These findings are also extended to the level of V_H gene family expression in separated bone marrow B cells depend upon the various stage of differentiation and conclude no preferential utilization of specific V_H gene family. Thus, the utilization of VH gene segments in B lymphocyte of adult BALB/c mice is random and is not regulated or changed during the differentiation of B cells.