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_J Immunol_ 1988; 140:2436-2441;

http://www.jimmunol.org/content/140/7/2436
STRAIN-DEPENDENT EXPRESSION OF \( V_H \) GENE FAMILIES

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The tremendous diversity of the antibody specificity repertoire stems from the ability of each developing B cell to select one out of many possible variable, diversity, and joining gene segments by specific rearrangement of the DNA. The mechanism by which V region gene segments is selected is not known. Moreover, evidence for both random and nonrandom expression of \( V_H \) genes in mature B cells has been presented previously. In this report, the technique of in situ hybridization is used to accurately measure at the single cell level \( V_H \) gene family expression in LPS-induced cells from several strains. In this way, at least one-third of the B cells are stimulated and a large sampling of activated splenocytes from each strain analyzed. The use of in situ hybridization eliminates any potential biases resulting from transformation protocols. In addition, because all populations of cells are analyzed by both in situ hybridization and immunocytochemical staining with anti-IgM, the proportion of cells, blasts, and plasma cells in the population. It was concluded from these comparisons that the cells being detected by in situ hybridization under the conditions described are plasmablasts and plasma cells. Therefore, an accurate measure of the functional and expressed \( V_H \) gene repertoire could be made. The results clearly demonstrate strain-dependent variation in \( V_H \) gene family expression, particularly \( V_H \) 7183 and \( V_H \) J558 with up to three-fold differences observed. Thus, either there is considerable strain variation in the number of functional \( V_H \) gene family segments or the expression of \( V_H \) genes is not entirely random.

The \( V_H \) region of Ig which contributes to the Ag specificity of the molecule is encoded by three separate germ-line gene segments, \( V_H \), D, and J, which are the DNA by specific rearrangement of the DNA (1–5). The ability to select one out of many possible \( V_H \), D, and \( J_H \) gene segments by the B cell is the basis for the tremendous diversity of the antibody specificity repertoire (1–5). How the selection of V region gene segments is regulated has not been elucidated. Moreover, much of what we know about V region gene utilization is from studies of transformed cell lines (6–13) rather than normal B cells. Here, we report an analysis of \( V_H \) gene expression by normal cells from different strains using a technique sensitive enough to detect \( V_H \) gene transcripts at the single cell level.

Estimates of the number of \( V_H \) genes range from 120 (14, 15) to over 1000 (16), and these genes have been classified into nine families on the basis of homology (14, 15, 17). The \( V_H \) genes within a family are highly homologous with 80 to 100% sequence identity, whereas the degree of homology between members of different families is lower, approximately 50 to 70%. The families vary in size ranging from two members in the X24 family to 60 or more members in the J558 family, an estimate based on the number of hybridizing bands in genomic Southern blots. More recently, nucleic acid hybridization studies have indicated that the size of the J558 family may be significantly greater with at least 500 members (16). This discrepancy in the size of J558 may be due to the presence of many different, but related, \( V_H \) J558-containing fragments in a single Southern band (16). However, the actual number of functional genes is difficult to assess because the number of pseudogenes may be relatively high (2).

The size of each \( V_H \) gene family has been estimated in a number of inbred strains of mice by counting the number of hybridizing bands in genomic Southern blots (14, 15). It was concluded from these studies that the size of the \( V_H \) gene families from strain to strain is relatively constant (14, 15). However, functional studies comparing the proportion of B cells expressing each of the \( V_H \) gene families from strain to strain have led to conflicting results (18, 19).

To compare the functional \( V_H \) repertoire in a large proportion of B cells from various strains, the expression of \( V_H \) genes by individual, LPS-induced B cells was determined by in situ hybridization. In this way, over one-third of the B cells could be stimulated and hundreds of cells expressing Ig specific RNA could be analyzed for which particular \( V_H \) gene families were being expressed. In addition, the analysis of normal B cells eliminates any possible bias resulting from transformation protocols used previously. Furthermore, since each population of cells was analyzed by both in situ hybridization and immunocytochemical staining with anti-mouse, the proportion of cells detected by in situ hybridization could be directly compared with the percentage of B cells, B cell blasts, and plasma cells present in the population. From such a comparative analysis, the spectrum of B cell differentiation stages detected by in situ hybridization under the present conditions was deduced. The proportion of LPS-induced spleen cells expressing each of seven \( V_H \)
gene families was analyzed in several strains. The results indicate some strain dependent variation in the expression of Vn gene families, particularly Vn 7183 and Vn J558.

MATERIALS AND METHODS

Animals. Inbred BALB/c mice were purchased from Harlan Sprague-Dawley, Inc. C.AL2O mice obtained from National Institutes of Health were kindly provided by Dr. John Kung. C57BL/6, B10.D2/OSnJ, CBA/CaJ, and A/J mice were purchased from The Jackson Laboratory. Mice were used at 8 to 12 wk of age. C3H/HeJ, C57BL/6, B10.D2/OSnJ, CBA/CaJ, and A/J mice were used shortly after purchase. BALB/c and C. AL2O mice were maintained at UTHSCSA before LPS stimulation. All mice maintained at the University of Texas Health Science Center are routinely tested for pathogens, including mouse hepatitis, Sendai virus, Mycobacteria, pulmonitis, Salmonella, endotoxin, and ectoparasites. Both strains have tested negative for these pathogens.

Suspension cultures. Spleens were removed, dispensed into single cell suspensions, and plated into 24-well Costar dishes at 2 x 10^7/ml in DMEM containing 10% FCS (GIBCO Grand Island, NY), 10% NCTC 109 medium (Inland Laboratories, Austin, TX), 50 μg/ml gentamycin, 2 mM glutamine, 5 x 10^{-5} M 2-ME, 1 mM oxalacetaete, 3 x 10^{-5} M glucose, 0.2 U/ml insulin, and 0.1 mM nonessential amino acids (M.A. Bioproducts, Walkersville, MD). Cultures were incubated in 10% CO_2 for 5 to 6 days in the presence or absence of 10 to 40 μg/ml bacterial LPS (Escherichia coli-O111: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.

Immunocytochemical staining. Cytospin staining of lymphocytes was performed by a modified version of the method described by Hofman et al. (20) and described previously (21). Briefly, the cells to be stained were cytocentrifuged onto a microscope slide, allowed to air dry overnight, and subsequently rehydrated for 10 min with PBS. All incubations were carried out at room temperature in a humidified chamber and washed with PBS two times after each reagent incubation. After an initial 20-min incubation with 0.03% hydrogen peroxide to decrease the activity of endogenous tissue peroxidases, the cell button was incubated for 20 min with 50 μl of rabbit anti-mouse μ or rabbit anti-mouse Ig. Both antibody reagents were prepared in the laboratory and the specificity of the anti-α has been documented (22). When hybridomas and myelomas were tested against the remaining cell lines. However, no specific labeling was detected by in situ hybridization. Therefore, only cells exhibiting a high level of labeling were counted as positive when hybridized with the X24 probe. In subsequent experiments, all probes were tested against the remaining cell lines. Therefore, no labeling above background was observed (data not shown).

RESULTS

Demonstration of probe specificity. Because the degree of homology between members of different Vn gene families can be as high as 70%, it was important to determine stringency conditions for the in situ technique that would maintain specificity yet detect as many members of the appropriate family as possible. This was accomplished by testing each of the prototype Vn family probes on a number of myelomas and hybridomas expressing different Vn gene families. As shown in Figure 1 and Table I, conditions were established for specificity even when the degree of homology between families approached 70% (e.g., the S107 probe tested against J606, Fig. 1a and b). Not every cell is labeled when hybridomas and myelomas are used because continuous in vitro propagation of these lines usually results in some cells that are nonsecretors. This is corroborated by immunocytochemical staining with anti-mouse Ig. In most cases, specificity was nearly absolute. However, similar to the findings of Hartman and Rudikoff (8), the X24 probe cross-hybridized slightly to 7183. Consistent with this result, LPS-induced cells hybridized with X24 showed two distinct patterns of labeling: some cells contained large numbers of grains (>100) and some cells contained a small number of grains (10 to 15) above background (2 to 5). Therefore, only cells exhibiting a high level of labeling were counted as positive when hybridized with the X24 probe. In subsequent experiments, all probes were tested against the remaining cell lines. Therefore, no labeling above background was observed (data not shown).

The effect of varying the amount of probe added to each slide was also tested. The results indicated that increasing the amount of probe above 2 x 10^6 cpm/slide or approximately 2.5 ng, did not result in an increased frequency of cells labeled or alter the degree of specific labeling (data not shown).

Characterization of cells detected by in situ hybridization. To help characterize the cells being detected by in situ hybridization, all LPS-activated cell populations were initially analyzed by immunocytochemical staining with either anti-mouse μ or anti-mouse Ig to determine the proportion of Ig-producing cells. The vast majority of LPS-induced cells appeared to be producing IgM because the percentage of cells staining with anti-μ vs anti-λ usually did not vary by more than 10%. Inasmuch as the morphology of cells is well maintained with this tech-
The combination of morphology and intensity of staining could be used to assess the frequency of B cells, B cell blasts, and plasma cells. These frequencies were then compared with the frequency of cells detected by in situ hybridization with the Cµ probe. The results, shown in Table II, indicate that the proportion of cells detected by in situ hybridization under these conditions was greater than the proportion of plasma cells assessed by immunocytochemical staining. However, the fraction of cells positive by in situ hybridization with Cµ was never equivalent to the total proportion of cells that stained positive for Ig production [B cells + B cell blasts (lymphoblasts and plasmablasts) + plasma cells] nor was it as high as the combined frequency of plasma cells and blasts. Thus, only a portion of the blasts appear to be detectable by in situ hybridization by using these conditions. The results suggest that the cells scored positive by in situ hybridization include plasma cells and plasmablasts, but not lymphoblasts.

The data from in situ hybridization analyses in these

**TABLE I**

*Specificity of V\text{H} gene family probes*  

<table>
<thead>
<tr>
<th>V\text{H} Probe</th>
<th>TF2-76 (7183)</th>
<th>25-9 (952)</th>
<th>139C1.3 (36-60)</th>
<th>28-120 (X24)</th>
<th>S31.L1 (S107)</th>
<th>J606 (J606)</th>
<th>J558 (J558)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7183</td>
<td>&gt;120</td>
<td>8 ± 1</td>
<td>7 ± 1</td>
<td>&gt;120</td>
<td>7 ± 1</td>
<td>9 ± 1</td>
<td></td>
</tr>
<tr>
<td>952</td>
<td>&gt;120</td>
<td>7 ± 1</td>
<td>9 ± 1</td>
<td>&gt;120</td>
<td>6 ± 1</td>
<td>&gt;120</td>
<td></td>
</tr>
<tr>
<td>36-60</td>
<td>9 ± 1</td>
<td>&gt;120</td>
<td>2 ± 1</td>
<td>&gt;120</td>
<td>7 ± 1</td>
<td>&gt;120</td>
<td></td>
</tr>
<tr>
<td>X24</td>
<td>13 ± 1</td>
<td>43 ± 4</td>
<td>1 ± 1</td>
<td>30 ± 4</td>
<td>6 ± 1</td>
<td>&gt;120</td>
<td></td>
</tr>
<tr>
<td>S107</td>
<td>16 ± 1</td>
<td>2 ± 1</td>
<td>&gt;120</td>
<td>30 ± 4</td>
<td>6 ± 1</td>
<td>&gt;120</td>
<td></td>
</tr>
<tr>
<td>J558</td>
<td>6 ± 1</td>
<td>3 ± 1</td>
<td>1 ± 1</td>
<td>6 ± 1</td>
<td>&gt;120</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each V\text{H} gene family probe was tested against cell lines expressing known V\text{H} families. The cell lines used as negative controls for each probe were chosen on the basis of highest homology with the probe as reported by Brodeur and Riblet (14).

* Results represent the mean grain count ± SEM of 8 to 16 randomly chosen cells.
**V<sub>H</sub> GENE EXPRESSION**

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Comparison of proportion of LPS-induced cells producing IgM with proportion of cells containing detectable μ-specific RNA

<table>
<thead>
<tr>
<th>Expt. (Strain)</th>
<th>LPS</th>
<th>Percent of Total Cells of Indicated Morphology Found Positive by Immunocytochemical Staining with Anti-IgM</th>
<th>Percent of mRNA-Positive Cells by In Situ Hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total % positive</td>
<td>B cells</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>89.0</td>
<td>31.3</td>
</tr>
<tr>
<td>(B10.D2)</td>
<td>-</td>
<td>63.1</td>
<td>19.1</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>83.9</td>
<td>16.8</td>
</tr>
<tr>
<td>(CBA)</td>
<td>-</td>
<td>55.3</td>
<td>29.1</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>77.6</td>
<td>26.9</td>
</tr>
<tr>
<td>(A/J)</td>
<td>-</td>
<td>57.3</td>
<td>37.2</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>79.0</td>
<td>32.0</td>
</tr>
<tr>
<td>(C57BL/6)</td>
<td>-</td>
<td>56.3</td>
<td>32.6</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>83.4</td>
<td>29.8</td>
</tr>
<tr>
<td>(BALB/c)</td>
<td>-</td>
<td>51.2</td>
<td>35.5</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>75.7</td>
<td>30.8</td>
</tr>
<tr>
<td>(C.AL20)</td>
<td>-</td>
<td>49.8</td>
<td>40.3</td>
</tr>
</tbody>
</table>

* Spleen cells from the indicated strains were cultured in the presence and absence of LPS for 5 to 6 days. After incubation, cell cultures were harvested and cytocentrifuged onto slides for analysis by both immunocytochemical staining with anti-mouse IgM and in situ hybridization with 35S-labeled Cp probe.

**TABLE III**

LPS-induced V<sub>H</sub> gene expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>7183&lt;sup&gt;a&lt;/sup&gt;</th>
<th>gb2</th>
<th>36-60</th>
<th>X24</th>
<th>SI07</th>
<th>J606</th>
<th>J558</th>
<th>H2</th>
<th>IgH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>12.1±1.5</td>
<td>19.6±1.9</td>
<td>10.0±1.7</td>
<td>4.7±2.0</td>
<td>5.9±2.0</td>
<td>7.6±0.4</td>
<td>34.0±2.7</td>
<td>d</td>
<td>a</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>14.1±1.0</td>
<td>29.8±2.1</td>
<td>4.7±0.8</td>
<td>5.4±0.5</td>
<td>9.2±1.3</td>
<td>9.4±1.3</td>
<td>53.4±2.9</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>C.AL20</td>
<td>35.9±0.2</td>
<td>13.4±1.5</td>
<td>8.2±0.9</td>
<td>3.3±0.5</td>
<td>6.8±0.9</td>
<td>5.1±0.4</td>
<td>32.9±0.9</td>
<td>d</td>
<td>e&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B10.D2</td>
<td>10.7±1.4</td>
<td>3.9±0.4</td>
<td>3.5±1.0</td>
<td>7.7±0.5</td>
<td>9.5±0.2</td>
<td>49.5±2.2</td>
<td>30.3±0.7</td>
<td>k</td>
<td>j</td>
</tr>
<tr>
<td>CBA</td>
<td>10.7±1.0</td>
<td>17.9±2.8</td>
<td>4.4±0.3</td>
<td>0.9±0.5</td>
<td>8.8±0.8</td>
<td>5.3±1.0</td>
<td>43.6±2.9</td>
<td>a</td>
<td>e</td>
</tr>
<tr>
<td>A/J</td>
<td>19.3±0.4</td>
<td>12.1±0.9</td>
<td>6.5±0.6</td>
<td>1.6±0.2</td>
<td>4.4±0.7</td>
<td>6.5±1.1</td>
<td>43.6±2.9</td>
<td>a</td>
<td>e</td>
</tr>
</tbody>
</table>

<sup>a</sup> Spleen cells from the indicated strains were cultured in the presence and absence of LPS for 5 to 6 days. After incubation, cell cultures were harvested and cytocentrifuged onto slides for analysis by both immunocytochemical staining with anti-mouse IgM and in situ hybridization with 35S-labeled Cp probe.

<sup>b</sup> Results represent the mean ± SEM obtained from three or four separate experiments in which LPS-induced splenocytes were analyzed by in situ hybridization. Approximately 500 to 1000 cells were counted per slide.

<sup>c</sup> Numbers in parentheses indicate the published complexity of the V<sub>H</sub> gene families (14).

<sup>d</sup> The Igh<sub>V</sub> locus of C.AL20 is most likely ε (35).

The purpose of this study was to determine accurately the extent to which individual V<sub>H</sub> gene families in differ-
ent strains are expressed in normal B cell populations. With in situ hybridization, it is possible to analyze a large sample and obtain data at the single cell level. Spleen cells from a number of different strains were stimulated with the mitogen LPS. LPS was chosen so that a large proportion of the B cells (up to one-third) would be induced. Considering this level of induction, the majority of B cells stimulated would presumably represent the functional, primary B cell repertoire. LPS-stimulated splenocytes were cytocentrifuged onto slides and tested for VH gene expression by using seven different VH gene family probes. The percentage of LPS-induced splenocytes expressing each of the VH gene families is presented for a number of different strains.

The in situ hybridization technique described was not sufficiently sensitive to detect μ-specific RNA in un cultured adult splenocytes cytocentrifuged directly onto slides with the exception of a rare cell of plasma cell appearance (data not shown). However, when adult splenocytes were cultured in the presence of LPS, as many as 30 to 50% of the cells contained sufficient RNA to become labeled when hybridized with the Cμ probe. Less than 5% of cells cultured in the absence of LPS were labeled with Cμ. By comparing the morphology and proportion of cells that stained positive with anti-Ig by immunocytochemical staining with the proportion of cells that exhibited specific hybridization with a radiolabeled Cμ, it was concluded that the in situ hybridization technique was detecting plasma cells and a portion of blast cells, presumably plasmablasts. Given the sensitivity of the technique under the present conditions, it is highly unlikely that germ-line VH transcripts or sterile transcripts have been shown to be present at lower levels than functional Ig transcripts (29-34).

A comparison of VH gene family expression by LPS-induced splenocytes among a number of different strains provided evidence for strain-related utilization of certain families. The most striking differences were observed with the VH families 7183 and J558 where the difference was highly significant. For example, in C.AL20 mice, about 36% of LPS-induced splenocytes express VH 7183. Therefore, VH 7183 is utilized three times more than in most of the other strains tested. The A/J strain also utilizes VH 7183 at a relatively high rate with about 20% of LPS-induced splenocytes expressing 7183. Thus, VH 7183 is used more frequently in these strains than would be predicted based on family size. Similarly, the expression of VH, J558 in LPS-induced cells among the various strains ranged from about 30% in CBA to about 53% in C57BL/6. Significant differences among the strains were also found in the expression of VH, 36–60. Moreover, although an analysis of variance for all strains expressing a given family showed no significant difference with the remaining families, the range in the level of expression of these families did vary about two-fold or more, e.g., J552 9.2% to 19.6%.

Interestingly, the higher level of expression of VH, 7183 is found with both C.AL20 and A/J which appear to have the same IghV allotype (14, 35). Moreover, if one compares the proportion of cells expressing VH, J558 among BALB/c, C57BL/6, and B10.D2, the higher rate of expression again maps to the Igh locus. However, a number of recombinant strains would have to be analyzed to assess the relative contribution of IgM in the expression of VH genes. Our data also suggest that a given MHC type does not prevent the expression of a particular VH gene family, although it is likely that background genes such as MHC play a role in regulating repertoire expression. The use of in situ hybridization with appropriate congenic strains would be useful in determining the relative contributions of various genetic loci.

The strain-dependent expression of VH genes observed suggests that either the size of the VH gene families is substantially different among the inbred strains or that the expression of VH genes is not entirely random. The number of members in a given VH gene family was estimated by counting the number of hybridizing restriction fragments on Southern blots (14, 15). In comparing 18 different strains by this method, Brodeur and Riblet (14) concluded that there was extensive polymorphism of VH gene families among the various strains but that the size of the family was relatively conserved with about 30% variation in the number of hybridizing bands in some families. There are potential difficulties in counting genes by Southern blot hybridization that could result in both underestimates and overestimates. In addition, the number of pseudogenes may differ among the strains. However, if the strain variation in the functional size of the VH gene families is indeed minimal, then the expression of certain VH gene families may not be random. Whether LPS induction, itself, may introduce some bias that background genes such as MHC play a role in regulating repertoire expression. The use of in situ hybridization with appropriate congenic strains would be useful in determining the relative contributions of various genetic loci.
VH GENE EXPRESSION

Our results, when analyzing the C57BL/6 strain, are not substantially different from those of Dildrop et al. (13). Wu and Paige (18) found considerable differences between the expression of certain VH families when comparing BALB/c with C57BL/6 raising the possibility for a nonrandom expression of VH genes. In their studies strain dependent differences in the ratio of expression of VH J558 to VH 7183 were found when an RNA colony blot assay was used. This assay is limited to B cells that can form colonies in response to mitogens (LPS, SRBC) under semisolid agar culture conditions. Although our conclusion of strain-dependent differences in VH gene expression is in agreement with that of Wu and Paige (18), we do not find that the BALB/c strain expresses VH 7183 more frequently than VH J558. Another study, performed by Schulze and Kelsoe (19), compared the expression of VH J558, VH X24, and VH QS52 by LPS-induced B cell colonies growing on filter paper disks. They found no statistically significant differences between C57BL/6 and BALB/c in the expression of any of these families and conclude a random expression of VH gene families according to family complexity. Unfortunately, one of the most important families for distinguishing strain-dependent differences, VH J7183, was not done in this study. The reason for the discrepancies in the above-mentioned studies may be due to differences in assay systems, including different cloning efficiencies, and it is quite possible that different B cell subsets are read out by each of the protocols.

The results of this study in which hundreds of LPS-induced splenocytes derived from a number of different strains of inbred mice were analyzed indicate that the expression of VH gene families can vary significantly from strain to strain. Whether this finding is the result of strain variation in the number of functional VH gene segments or the result of a nonrandom expression of VH genes needs to be assessed.

Acknowledgments. We are grateful to Drs. Brodeur, Hood, Riblet, and Riley for providing probes; Dr. Drew Pardoll for sharing his protocol for in situ hybridization; Drs. Pollock, Scharff, and Schulze for hybridomas; and to Dr. Riblet for helpful discussions. We thank Elizabeth Morris for expert technical assistance and Mary Devados for helping to prepare the manuscript.

REFERENCES