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Brief Definitive Report

Rearrangement and Selection of V_H11 in the Ly-1 B Cell Lineage

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Summary

One of the predominant V_H genes utilized to encode the anti-BrMRBC specificity is a member of the small V_H11 family rearranged to J_H1. Using the polymerase chain reaction (PCR) we have determined that the frequency of B cells with a V_H11 rearrangement is 10–20 times higher in Ly-1 B than in Ly-1$^-$ "conventional" B cells regardless of location (spleen or peritoneal cavity). Conventional B cells rearrange this gene at comparable levels in pre-B cells and in mature B cells utilizing all J_H gene segments. In contrast, the increased levels of V_H11 rearrangement in Ly-1 B are restricted to J_H1 (and some J_H2) and therefore appear to be the result of selection. Furthermore, most peritoneal Ly-1 B cells with V_H11 rearrangements fall in a fraction stained by anti-BrMRBC antibody, likely bearing multivalent natural (likely self) antigen constitutively bound to their surface Ig receptors. Thus, we suggest that autoantigens are largely responsible for the accumulation of autoantibody specificities in the Ly-1 B cell lineage with time, whereas they do not exert this effect in the conventional B cells.

The question of whether there is restriction or overutilization of particular Ig V genes in distinct B cell subpopulations is unresolved and the mechanism(s) to account for such a phenomenon remains controversial. Recently, much attention has been focused on a B cell subset present in both mouse and human known as CD5$^+$ B cells (1–4). In mouse, CD5$^+$ B cells are preferentially generated from progenitors early in ontogeny (5) and have the ability to self-renew (6), maintaining their population through life as a distinct B cell lineage, termed Ly-1 B. In a previous paper we demonstrated that an autoantibody that binds to a cryptic determinant on mouse erythrocytes revealed by treatment with the proteolytic enzyme bromelain (anti-BrMRBC) was derived almost exclusively from Ly-1 B cells, and further, that this specificity was predominantly encoded by a novel V_H gene (a member of the small V_H11 family; reference 7) in association with a single member of the $V_\kappa9$ family (8). In this report we use PCR amplification of DNA isolated from cell sorter–purified pre-B and B cell populations to show that Ly-1 B cells overutilize V_H11, whereas other (Ly-1$^-$ or "conventional") B cells do not (although they do rearrange it). Furthermore, we observe that most of the V_H11 rearrangement found in peritoneal Ly-1 B cells is strikingly restricted to a cell fraction that is labeled by antibody of the same specificity (anti-BrMRBC) via "sandwich" binding. We interpret these results as evidence that V_H11 overutilization in Ly-1 B is largely due to combining site–driven selection, probably by autoantigen.

Materials and Methods

Immunofluorescence and Cell Sorting. Single cell suspensions prepared from either bone marrow, spleen, or peritoneal cavity (PerC) of BALB/c mice (bred and maintained in our animal facility) were stained simultaneously with the following reagents, then analyzed or sorted as described previously (8) using a dual-laser FACStar^{PLUS} (Becton Dickinson & Co., Mountain View, CA): bone marrow, PE anti-IgM (clone 331.12), and allophycocyanin (APC) anti-B220 (clone RA3-6B2); spleen and PerC, fluorescein (FL) anti-IgM, biotin (BI) anti-IgD (clone 10-4.2) revealed by PE-avidin and APC-anti-Ly-1 (clone 53-7). For staining by anti-BrMRBC, IgM^b anti-BrMRBC (clone 10E8; reference 8) revealed by FL-anti-IgM^b (clone AF6-78), PE-anti-IgM^a (clone RS-3.1) and APC-anti-Ly-1 were used. These reagents have been described previously (5, 6, 8).

Analysis of Secreted Anti-BrMRBC from Sorted Populations. 10E8$^+$ and 10E8$^-$ fractions of peritoneal Ly-1 B cells from 3-mo-old BALB/c mice were isolated by sorting and then cultured at 10^6 cells/ml for 3 d in culture medium containing 10 μg/ml LPS. Supernatant was assayed for total IgM^a (BALB/c Igh-6a allotype) by an ELISA sandwich assay (using rat anti-IgM and mouse anti-IgM^a). IgM^a anti-BrMRBC activity was determined by staining of bromelain-treated mouse erythrocytes as described previously (8).

DNA Preparation, PCR, and Analysis. DNA was isolated from 1–2 $\times$ 10^5 sorted cells by lysis and proteinase K digestion in 20 μl low gelling temperature agarose (1%) followed by solidification on ice and dialysis (3$\times$) versus 500 μl TE buffer (9). DNA was restricted with EcoRI while in agarose, then dialyzed (3$\times$) as above versus ddH_2O, and finally, diluted 1:5 with ddH_2O. One-fifth of a sample was then analyzed by PCR for V_H11 rearrangement

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using either a V_H11 leader oligo (ATGGAGTGGGAACTGAGCTTA) to a J_H1 oligo (GGTCCCTGCGCCCCAGACA) or the V_H11 oligo to a J_H4 oligo (TGACCCCAGTAGTCCATAGC). A 3′ of J_H4 oligo (CTAGAGAGGTCTGGTGGAGCC) to 5′ of $C\mu$ intron oligo (CTAAATACATTTTAGAAGTCGATAAACTTAAG) was used to standardize for variation in DNA loading. In addition to DNA template, reactions contained PCR buffer (50 mM KCl, 20 mM Tris, pH 8.4, 2.5 mM $MgCl_2$, 0.1 mg/ml nuclease-free BSA), 50 μM each of four dNTPs, 100 pM of each oligo, and 2 U of Taq polymerase (10). Amplified DNA was electrophoresed in 1.5% agarose, blotted, and then probed with either a J-less V_H11 or a normalizing fragment (made by amplification of genomic DNA using the normalizing oligos). After washing at high stringency (0.2× SSC, 0.1% SDS; 65°C), filters were autoradiographed at −80°C with intensifying screens, then bands were quantitated using a two-dimensional proportional detector (Ambis Radioanalytic Imaging Systems, San Diego, CA).

Cell type	Frequency of cells (%)	V_H11-J_H1 (%)	+/- SE
BM Pre-B	8	0.7	0.1
" B	10	0.8	0.3
Spl conv. B	50	0.9	0.1
" Ly-1 B	2	**9.0**	1.3
PerC conv. B	5	1.0	0.1
" Ly-1 B	26	**10.0**	1.1
Spl T cells	30	0.2	0.1

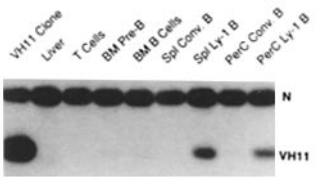

Figure 1. Frequency of V_H11 rearrangement in different B lineage subpopulations of BALB/c mice. DNA for PCR was prepared from 1–2 × 10^5 sorted cells as described in the text. Bone marrow pre-B and B cells were sorted as $B220^+/IgM^-$ and $B220^+/IgM^+$, respectively. Spleen and peritoneal conventional B cells and Ly-1 B cells were sorted as $IgM^+/IgD^{++}/Ly\text{-}1^-$ and $IgM^{++}/IgD^{+/-}/Ly\text{-}1^+$, respectively, by four-color fluorescence (including propidium iodide to eliminate dead cells). T cells were sorted from spleen as $IgM^-/Ly\text{-}1^+$ cells. Bone marrow B lineage populations, splenic T cells, and conventional B cells were all >98% pure as judged by reanalysis. Purities of splenic and peritoneal Ly-1 B cells were 75 and 92%, respectively, predominantly contaminated by $Ly\text{-}1^-/IgM^{+++}/IgD^{+/-}$ cells, not by conventional B cells. DNA was isolated from BALB/c liver according to standard procedures. Isolation of the V_H11 rearranged DNA was described previously (8). PCR conditions are described in Materials and Methods. Amplification used a 60-s denaturation time (95°C), a 30-s annealing time (57°C), and a 30-s polymerization time (72°C) for 23 cycles. Percentage of cells rearranged (at a single allele) was determined by measuring the ratio of (V_H11 cpm)/(normalizing fragment cpm) for each sample, then assuming that the ratio for cloned V_H11 DNA was equal to 200% (both alleles rearranged to V_H11). SE is standard error of five determinations. Exposure time for the blot was 4 h. V_H11 bands in bone marrow B lineage and conventional B cells are detectable in overnight exposures, whereas bands are not seen with liver or T cell DNA. Background is very low (corresponding to 0.2% or less), since we anneal (and wash) at relatively high temperature, amplify for a limited number of cycles, detect with specific probes, and require that the DNA migrates an appropriate distance.

Results and Discussion

Our earlier analysis of hybridomas made with Ly-1 B and $Ly\text{-}1^-$ B cells sorted from spleen demonstrated that anti-BrMRBC specific cells were at least 50-fold enriched in Ly-1 B, and further, that the majority utilized a V_H gene from the novel family V_H11 repeatedly rearranged to J_H1 (8). However, the frequency of V_H11 rearrangement in Ly-1 B and its potential restriction to Ly-1 B cells remained to be determined. We therefore sought to determine the extent of V_H11 expression in normal populations of B lineage cells by using PCR. Amplification (shown in Fig. 1) using oligos specific for the V_H11 leader and J_H1 (diagramed in Fig. 2 *B*) simultaneous with a normalizing fragment (from 3′ of J_H4 to 5′ of $C\mu$; "N" in Fig. 2 *B*) revealed differences in the frequency of V_H11 rearrangement in distinct populations of B lineage cells. Two points are notable: (*a*) this rearrangement is detectable in pre-B cells and B cells in bone marrow and in their progeny (conventional B cells) in spleen, demonstrating that V_H11 can be rearranged in such cells; and (*b*) there is a significant (10-fold) enrichment above this level of the V_H11-J_H1 rearrangement in Ly-1 B cells of spleen and PerC. Analysis of conventional B cells ($IgM^+/IgD^{++}/Ly\text{-}1^-$) in PerC reveals a low level comparable with that in spleen, demon-

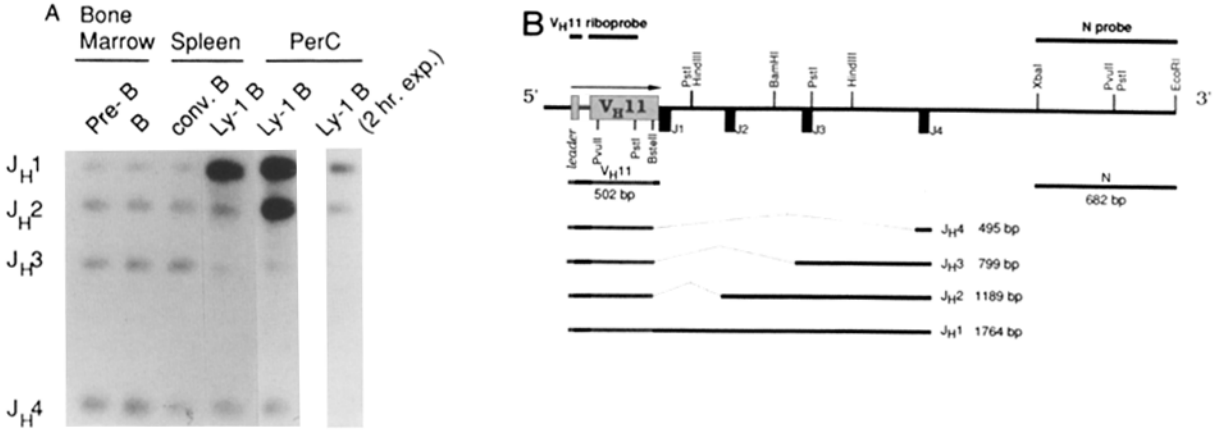

Figure 2. (*A*) V_H11-J_H4 PCR shows that most of the increased V_H11 rearrangement in Ly-1 B cells is restricted to J_H1 (with some J_H2). DNA was prepared as described in the legend for Fig. 1. Amplification was as described in Fig. 1 except for a polymerization time of 90 s and 27 cycles. Normalizing oligos were not included. Exposure time was 2 d, except as noted in the figure. (*B*) Map of the region being amplified in this paper and probes employed.

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strating that an increased level of V_H11 is not a function of location but rather of a particular B cell subpopulation, Ly-1 B.

Unselected rearrangement of V_H11 would probably show diverse J_H utilization, whereas selection for the BrMRBC specificity would show preferential use of J_H1. To determine whether the overutilization of V_H11 in Ly-1 B cells is due to selection, we have therefore used PCR with oligos specific for the V_H11 leader and J_H4 since this generates four resolvable fragments representing rearrangement to J_H1 through J_H4. Such analysis (Fig. 2) reveals that V_H11 rearrangements utilize all J_Hs in bone marrow B lineage cells and in mature conventional splenic B cells. Furthermore, the relative utilization of each J_H appears similar for these three cell types. As predicted, Ly-1 B cells in spleen and PerC show striking overutilization of J_H1. Curiously, we also detect significant overutilization of J_H2 (particularly in PerC Ly-1 B), not yet identified in V_H11 hybridomas with the anti-BrMRBC specificity. This may mean that a fraction of the V_H11 anti-BrMRBC specificity shows a rearrangement to J_H2 or else may reflect an as yet unrecognized specificity utilizing V_H11-J_H2 that is also selected for in the Ly-1 B population.

We (8) and others (11) have postulated previously that enrichment of particular specificities in the Ly-1 B cell population is likely due to antigen-driven selection because of the repeated usage of V_H11/J_H1 together with a single member of the $V_\kappa9$ family in clonally unrelated anti-BrMRBC hybridomas. In support of this concept, we have found that all of our anti-BrMRBC mAbs show direct binding to a fraction of Ly-1 B cells in PerC (10–20%, Fig. 3) that is responsible for all LPS-inducible anti-BrMRBC secretion (Table 1). This "sandwich" binding is specific since it occurs with anti-BrMRBC antibodies, but not with IgM antibodies of other specificities (data not shown). We have analyzed Ly-1 B cells separated on the basis of anti-BrMRBC (clone 10E8) binding for V_H11-J_H1 rearrangement. Over half of the $10E8^+$ fraction show the prototypic anti-BrMRBC rearrangement (V_H11-J_H1), whereas the $10E8^-$ fraction has severely depleted levels of this rearrangement compared with total PerC Ly-1 B (Figure 3, Table 1). In addition, the $10E8^+$ fraction largely overlaps the phosphatidylcholine-liposome binding fraction of PerC Ly-1 B cells (data not shown) previously reported

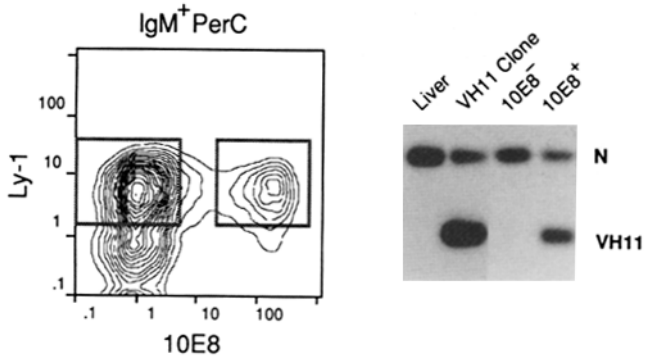

Figure 3. Cells washed out of peritoneum were stained as described in Materials and Methods. Gated IgM^+ cells are displayed in this figure and the indicated fractions were isolated by cell sorting (24% $10E8^-$ and 3% $10E8^+$ of total PerC). DNA was prepared as described and amplified by PCR using V_H11 leader and J_H1 oligos together with the normalizing oligos to standardize for variation in loading. PCR conditions as in Fig. 1.

as delineating the cells specific for BrMRBC (12). These data taken together lead us to conclude that this binding is through the Ig combining site. One possible explanation is that these cells bear multivalent antigen on their surface bound by membrane Ig. Thus incubation with an "anti-BrMRBC" antibody would result in its being bound to free determinants on the multivalent antigen. Although more difficult to quantitate due to their low frequency, it appears that at least 5–10% of the Ly-1 B cells in spleen are also stained by 10E8 (data not shown), consistent with the increased frequency of V_H11-J_H1 rearrangement shown above.

We suggest that during their generation, Ly-1 B cells specific for the determinant encoded by $V_H11/V_\kappa9$ are constantly being recruited by a natural antigen. This explains our observation that the percentage of $10E8^+$ Ly-1 B cells (among total Ly-1 B) increases sixfold between 2 wk and 3 mo of age (data not shown). Ly-1 B cells are generated from unrearranged precursors only in the first few weeks of life, then exist as a surface Ig^+ population (5, 6). If we assume that recruitment into this long-lived pool requires antigen receptor

Table 1. *Anti-BrMRBC Autoantibody Secretion and V_H11 Rearrangement Is Enriched in the $10E8^+$ PerC Ly-1 B Fraction*

B cells	Fraction of total cells	IgM secretion	Anti-BrMRBC activity	V_H11-J_H1 rearrangement
	%	$\mu g/ml$		% ± SE
Spl Conv. B	50	14.0	0.7	0.9 ± 0.1
PerC Ly-1 B total	26	29.5	8.0	10.0 ± 1.1
Ly-1 B $10E8^-$	24	26.5	0.6	2.0 ± 0.1
Ly-1 B $10E8^+$	3	10.0	8.9	76.0 ± 4.9

IgM levels were measured by ELISA, anti-BrMRBC activity was determined by a fluorescence staining assay (values reported are mean fluorescence intensity) and rearrangement was determined by PCR. Values reported are for 3-mo-old animals. SE is standard error of 5 determinations for conventional B and Ly-1 B and 10 determinations for 10E8 sorted fractions.

occupancy, then the adult Ly-1 B repertoire would be the result of selection by self and environmental antigens. Although the question remains as to why such selection does not occur in the conventional B cell population, we might hypothesize that this is due to differences in activation requirements between the two subsets. The maintenance of V genes in the germline capable of encoding certain autospecificities suggests that their expression by Ly-1 B has functional importance. Determining this function, the role of Ly-1 B cells in the immune system remains a fertile area for future investigation.

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References

1. Hayakawa, K., and R.R. Hardy. 1988. Normal, autoimmune, and malignant CD5$^+$ B cells: the Ly-1 B lineage? *Annu. Rev. Immunol.* 6:197.
2. Herzenberg, L.A., A.M. Stall, P.A. Lalor, C. Sidman, W.A. Moore, D.R. Parks, and L.A. Herzenberg. 1986. The Ly-1 B cell lineage. *Immunol. Rev.* 93:81.
3. Rajewsky, K., I. Forster, and A. Cumano. 1987. Evolutionary and somatic selection of the antibody repertoire in the mouse. *Science (Wash. DC).* 238:1088.
4. Kipps, T.J. 1989. The CD5 B Cell. *Adv. Immunol.* 47:117.
5. Hayakawa, K., R.R. Hardy, L.A. Herzenberg, and L.A. Herzenberg. 1985. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J. Exp. Med.* 161:1554.
6. Hayakawa, K., R.R. Hardy, A.M. Stall, L.A. Herzenberg, and L.A. Herzenberg. 1986. Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage. *Eur. J. Immunol.* 16:1313.
7. Reininger, L., A. Kaushik, S. Izui, and J.C. Jaton. 1988. A member of a new V_H gene family encodes antibromelinized mouse red blood cell autoantibodies. *Eur. J. Immunol.* 18:1521.
8. Hardy, R.R., C.E. Carmack, S.A. Shinton, R.J. Riblet, and K. Hayakawa. 1989. A single V_H gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the V_H11 family. *J. Immunol.* 142:3643.
9. Williams, G.T. 1987. High-efficiency preparation of DNA from limiting quantities of eukaryotic cells for hybridization analysis. *Gene (Amst.).* 53:121.
10. Innis, M.A., D.H. Gelfand, J.J. Sninsky and T.J. White, editors. 1990. PCR Protocols. A Guide to Methods and Applications. Academic Press, New York.
11. Pennell, C.A., T.J. Mercolino, T.A. Grdina, L.W. Arnold, G. Haughton, and S.H. Clarke. 1989. Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. *Eur. J. Immunol.* 19:1289.
12. Mercolino, T.J., L.W. Arnold, L.A. Hawkins, and G. Haughton. 1988. Normal mouse peritoneum contains a large population of Ly-1$^+$ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. *J. Exp. Med.* 168:687.