Rearrangement and Selection of \( \text{VH11} \) in the Ly-1 B Cell Lineage

By Condie E. Carmack, Susan A. Shinton, Kyoko Hayakawa, and Richard R. Hardy

From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Summary

One of the predominant \( \text{VH} \) genes utilized to encode the anti-BrMRBC specificity is a member of the small \( \text{VH11} \) family rearranged to \( \text{JH1} \). Using the polymerase chain reaction (PCR) we have determined that the frequency of B cells with a \( \text{VH11} \) 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 \( \text{JH} \) gene segments. In contrast, the increased levels of \( \text{VH11} \) rearrangement in Ly-1 B are restricted to \( \text{JH1} \) (and some \( \text{JH2} \)) and therefore appear to be the result of selection. Furthermore, most peritoneal Ly-1 B cells with \( \text{VH11} \) 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.

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® (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-IgD, biotin (BI) anti-IgM, and APC anti-Ly-1 (clone 53-7). For staining by anti-BrMRBC, IgM<sup>+</sup> anti-BrMRBC (clone 10E8; reference 8) revealed by FL-anti-IgMb (clone AF6-78), PE-anti-IgM<sup>+</sup> (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<sup>+</sup> and 10E8<sup>-</sup> fractions of peritoneal Ly-1 B cells from 3-mo-old BALB/c mice were isolated by sorting and then cultured at 10<sup>6</sup> cells/ml for 3 d in culture medium containing 10 μg/ml LPS. Supernatant was assayed for total IgM<sup>+</sup> (BALB/c Igh-6a allotype) by an ELISA sandwich assay (using rat anti-IgM and mouse anti-IgM<sup>+</sup>). IgM<sup>+</sup> 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 × 10<sup>5</sup> sorted cells by lysis and proteinase K digestion in 20 μl low gelling temperature agarose (1%) followed by solidification on ice and dialysis (3×) versus 500 μl TE buffer (9). DNA was restricted with EcoRI while in agarose, then dialyzed (3×) as above versus ddH<sub>2</sub>O, and finally, diluted 1:5 with ddH<sub>2</sub>O. One-fifth of a sample was then analyzed by PCR for \( \text{VH11} \) rearrangement.
using either a V_{H11} leader oligo (ATGGAGTGGGAACTGAGCTTA) to a J_{H1} oligo (TGACCACCACTGGCTCCTAGGC) or the V_{H11} oligo to a J_{H4} oligo (CCTAGAGGGTCTGAGGACCC) to 5' of C_{µ} intron oligo (CGATCGTAATAACTCTAAG) 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 MgCl₂, 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 VH11 or a normalizing fragment (made by amplification of genomic DNA using the normalizing oligos). After washing at high stringency (0.2x 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).

**Results and Discussion**

Our earlier analysis of hybridomas made with Ly-1 B and Ly-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 VH gene from the novel family VH11 repeatedly rearranged to J_{H1} (8). However, the frequency of VH11 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 VH11 expression in normal populations of B lineage cells by using PCR. Amplification (shown in Fig. 1) using oligos specific for the VH11 leader and J_{H1} (diagramed in Fig. 2 B) simultaneous with a normalizing fragment (from 3' of J_{H4} to 5' of C_{µ}; "N" in Fig. 2 B) revealed differences in the frequency of VH11 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 VH11 can be rearranged in such cells; and (b) there is a significant (10-fold) enrichment above this level of the VH11-J_{H1} rearrangement in Ly-1 B cells of spleen and PerC. Analysis of conventional B cells (IgM+/IgD+/Ly-1-) in PerC reveals a low level comparable with that in spleen, demon-

| Cell type | Frequency of cells (%) | VH11-J_{H1} |
|-----------|------------------------|-------------|
| BM Pre-B  | 0.7                    | 0.1         |
| B         | 0.1                    | 0.3         |
| Spleen conv. B | 0.9                  | 0.1         |
| Ly-1 B    | 0.5                    | 0.1         |
| PerC conv. B | 1.0                  | 1.3         |
| Ly-1 B    | 2.0                    | 1.0         |
| Spl T cells | 0.2                  | 0.1         |
| PerC T cells | 0.1                 | 0.3         |

**Figure 1.** Frequency of VH11 rearrangement in different B lineage subpopulations of BALB/c mice. DNA for PCR was prepared from 1-2 x 10⁶ 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-1- and IgM+/IgD+/Ly-1+, respectively, by four-color fluorescence (including propidium iodide to eliminate dead cells). T cells were sorted from spleen as IgM-/Ly-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-1-/IgM+/IgD+/- cells, not by conventional B cells. DNA was isolated from BALB/c liver according to standard procedures. Isolation of the VH11 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 (VH11 cpm)/(normalizing fragment cpm) for each sample, then assuming that the ratio for cloned VH11 DNA was equal to 200% (both alleles rearranged to VH11). SE is standard error of five determinations. Exposure time for the blot was 4 h. VH11 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.

**Figure 2.** (A) VH11-J_{H4} PCR shows that most of the increased VH11 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.
Rearrangement of VH11 is not a function of location but rather of a particular B cell subpopulation, Ly-1 B. Unselected rearrangement of VH11 would probably show diverse JH utilization, whereas selection for the BrMRBC specificity would show preferential use of JH1. To determine whether the overutilization of VH11 in Ly-1 B cells is due to selection, we have therefore used PCR with oligos specific for the VH11 leader and JH4 since this generates four resolvable fragments representing rearrangement to JH1 through JH4. Such analysis (Fig. 2) reveals that VH11 rearrangements utilize all JHS in bone marrow B lineage cells and in mature conventional splenic B cells. Furthermore, the relative utilization of each JH appears similar for these three cell types. As predicted, Ly-1 B cells in spleen and PerC show striking overutilization of JH1. Curiously, we also detect significant overutilization of JH2 (particularly in PerC Ly-1 B), not yet identified in VH11 hybridomas with the anti-BrMRBC specificity. This may mean that a fraction of the VH11 anti-BrMRBC specificity shows a rearrangement to JH2 or else may reflect an as yet unrecognized specificity utilizing VH11-JH2 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 VH11/JH1 together with a single member of the Vγ9 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 VH11-JH1 rearrangement. Over half of the 10E8+ fraction show the prototypic anti-BrMRBC rearrangement (VH11-JH1), 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 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 quantify 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 VH11-JH1 rearrangement shown above.

We suggest that during their generation, Ly-1 B cells specific for the determinant encoded by VH11/Vγ9 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

| B cells | Fraction of total cells | IgM secretion | Anti-BrMRBC activity | VH11-JH1 rearrangement |
|---------|------------------------|---------------|----------------------|-----------------------|
|         | %                      | µg/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

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. Cuman. 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. Reinginer, L., A. Kaushik, S. Izui, and J.C. Jaton. 1988. A member of a new \( V_{H} \) gene family encodes antibrinolysinized 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 \( V_{H}11 \) 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.

374 V_{H}11 Gene Expression