LAM 402

Development of the Antibody Repertoire as Revealed by Single-Cell PCR of FACS-Sorted B-Cell Subsets[a]

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We distinguish three murine B-cell lineages[1-4]: conventional B cells (B-2 cells), which develop late and are continually replenished throughout life from progenitors in adult bone marrow; B-1a cells (Ly-1 or CD5 B cells), which develop early and maintain their numbers by self-replenishment; and B-1b cells (Ly-1 B "sister" cells), which share many of the properties of Ly-1 B cells, including self-replenishment and feedback regulation of development, but which can also readily develop from progenitors in adult bone marrow. Several studies indicate that the expressed antibody repertoire of mature B-1a and conventional B cells differ with respect to V_H gene usage and antigen specificity. There is no information on B-1b cells. Earlier methods of repertoire analysis, which include generation of immortalized cell lines (hybridomas and transformations), *in situ* hybridization with V_H probes, and the amplification of cDNA from polyclonal cells, are not necessarily representative of the populations studied. For example, transformation and hybridoma production are very inefficient and may select cells in favored states of activation, and bulk cDNA libraries favor cells with the most message.

We have developed a method for making cDNA from individual, unstimulated, FACS-sorted murine and human B cells that permits the recovery and amplification of transcripts from up to 90% of the cells and will allow the analysis of multiple genes from a single cell. Cells are sorted at one cell per tube, lysed in hypotonic medium containing RNAse inhibitors, and cDNA is prepared by using random hexanucleotide primers. No purification of the RNA or cDNA is needed. The murine cDNA is amplified with primers homologous to the 5′ portion of $C\mu$ and to a conserved consensus region in framework I of V_H. We have recovered transcripts from hybridomas and sorted B cells that represent the 14 murine V_H families. This result, along with our high recovery rate, demonstrates that the procedure is void of bias for particular rearrangements.

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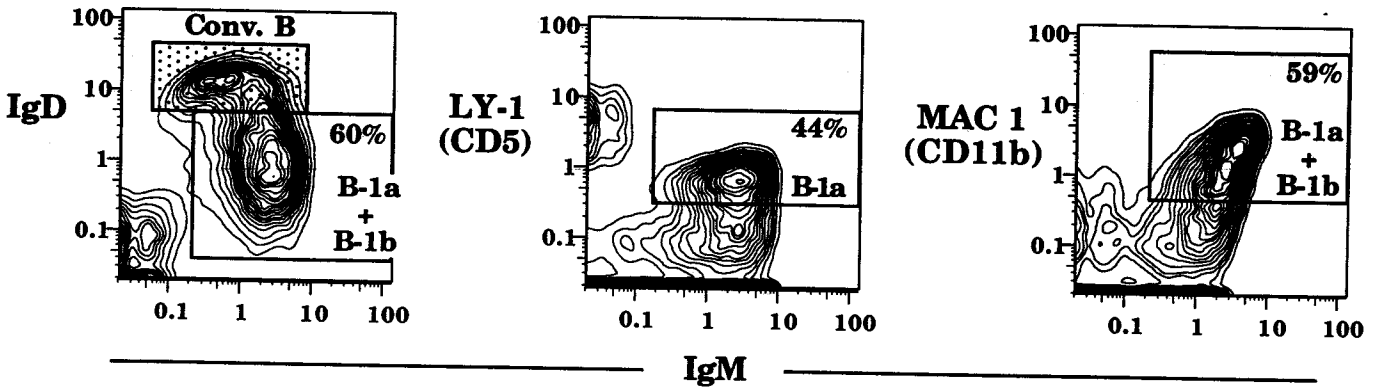

FIGURE 1. FACS analysis of peritoneal B-cell populations. Conventional B cells are identified by a broad, positive IgM and tight, bright IgD FACS profile. They are negative for Ly-1 and Mac 1. All B-1 cells are IgM bright and low to moderate for IgD. B-1 cells are also MAC 1 positive in the peritoneum. B-1 cells are divided into B-1a cells, which are $CD5^+$, and B-1b cells, which are $CD5^-$.

The method allows us to analyze V gene expression from any population of B cells that we can define phenotypically by FACS. We begin with B-1a, B-1b, and conventional B cells from the adult Balb/c peritoneum. The phenotype is shown in FIGURE 1. Conventional B cells are IgM^{lo}, IgD^{hi}, and $CD5^-$. Both B-1a and B-1b cells are IgM^{hi}, IgD^{lo}; however, B-1a cells are $CD5^+$, and B-1b cells are $CD5^-$. Each population is bulk sorted first, based on size, viability, and expression of IgM, IgD, and Ly-1. After reanalysis, single cells are sorted into the lysis solution and snap-frozen for future analysis. The data described below is from 170 transcripts distributed among the three populations.

The three B-cell populations show some similarities in V_H gene usage with respect to the V_H families. In all cases, the J558 family is used most frequently, and the Q52 family is used the second most frequently. Interestingly, the V_H 10 and 3660 families are overrepresented among B-1b cells. In order to evaluate the use of individual V_H gene segments, we developed a database of known and putative germline genes. The most striking finding from this analysis is that a single Q52 V_H element is used in 22% of all B-1a cells, 13% of all B-1b cells, but only 3% of B-2 cells. Some of these cells are among the phosphatidylcholine liposome-binding cells (anti-bromelein-treated mouse red blood cells). Cells with this specificity also commonly use V_H11 and V_H12, as previously shown by others.[5–8]

Does the repertoire of B-1a cells found in the adult animal reflect any features of fetal/neonatal B-cell development? It has been well established that fetal and neonatal B cells rarely have N-region insertions at the VD and DJ junctions, whereas most such junctions recovered from adults have N-regions.[9–13] Contrary to a previous study that evaluated B-1a hybridomas,[12,14] we find that B-1a cells isolated from the adult peritoneum have a significantly larger proportion of V_H transcripts with no (or few) N bases at both the V-D and D-J junctions than either B-1b or conventional B cells. In fact 25% of the B-1a cell transcripts, but only 5% of the conventional B-cell transcripts, lack N-region insertions. This result is consistent with the model that many of the B-1a cells present in the adult, which contain no or few N-region nucleotides, arose very early in ontogeny when TdT activity is low[15,16] and survived via self-replenishment into adulthood. However, TdT expression, as monitored by the incidence of N-region insertions, clearly does not define B-1a cells, since most V_H transcripts contain N-regions, consistent with B-1a cell VDJ rearrangement occurring through the first six weeks of life.[2]

We anticipate that the approach presented here will be extremely powerful in analyzing the developmental and selective events that produce the expressed antibody repertoire.

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REFERENCES

1. STALL, A. M., S. ADAMS, L. A. HERZENBERG & A. B. KANTOR. 1992. Characteristics and development of the murine B-1b (Ly-1 B sister) cell population. Ann. N.Y. Acad. Sci. **651:** 33.
2. KANTOR, A. B. & L. A. HERZENBERG. 1993. Origin of murine B cell lineages. Annu. Rev. Immunol. **11:** 501.

3. KANTOR, A. B., A. M. STALL, S. ADAMS, L. A. HERZENBERG & L. A. HERZENBERG. 1992. Differential development of progenitor activity for three B-cell lineages. Proc. Natl. Acad. Sci. USA **89:** 3320.
4. KANTOR, A. B., A. M. STALL, S. ADAMS, K. WATANABE & L. A. HERZENBERG. 1994. De novo development and self-replenishment of B cells. Intl. Immunol. **7:** 55–68.
5. HARDY, R. R., C. E. CARMACK, S. A. SHINTON, R. J. RIBLET & K. HAYAKAWA. 1989. A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. J. Immunol. **142:** 3643.
6. MERCOLINO, T. J., L. W. ARNOLD, L. A. HAWKINS & 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.
7. MERCOLINO, T. J., A. L. LOCKE, A. AFSHARI, D. SASSER, W. W. TRAVIS, L. W. ARNOLD & G. HAUGHTON. 1989. Restricted immunoglobulin variable region gene usage by normal Ly-1 (CD5$^+$) B cells that recognize phosphatidyl choline. J. Exp. Med. **169:** 1869.
8. PENNELL, C. A., T. J. MERCOLINO, T. A. GRDINA, L. W. ARNOLD, G. HAUGHTON & S. H. CLARKE. 1989. Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. Eur. J. Immunol. **19:** 1289.
9. BANGS, L. A., I. E. SANZ & J. M. TEALE. 1991. Comparison of D, JH, and junctional diversity in the fetal, adult, and aged B cell repertoires. J. Immunol. **146:** 1996.
10. CARLSSON, L. & D. HOLMBERG. 1990. Genetic basis of the neonatal antibody repertoire: Germline V-gene expression and limited N-region diversity. Int. Immunol. **2:** 639.
11. FEENEY, A. J. 1990. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. J. Exp. Med. **172:** 1377.
12. GU, H., I. FORSTER & K. RAJEWSKY. 1990. Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: Implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. EMBO J. **9:** 2133.
13. MEEK, K. 1990. Analysis of junctional diversity during B lymphocyte development. Science **250:** 820.
14. GU, H., I. FORSTER & K. RAJEWSKY. 1992. Study of murine B-cell development through analysis of immunoglobulin variable region genes. Ann. N.Y. Acad. Sci. **651:** 304.
15. DESIDERIO, S. V., G. D. YANCOPOULOS, M. PASKIND, E. THOMAS, M. A. BOSS, N. LANDAU, F. W. ALT & D. BALTIMORE. 1984. Insertion of N-regions into heavy chain genes is correlated with the expression of terminal deoxytransferase in B cells. Nature **311:** 752.
16. GREGOIRE, K. E., I. GOLDSCHNEIDER, R. W. BARTON & F. J. BOLLUM. 1979. Ontogeny of terminal deoxynucleotide transferase-positive cells in lymphohemopoietic tissues of rat and mouse. J. Immunol. **123:** 1347.