RESTRICTED IMMUNOGLOBULIN VARIABLE REGION GENE USAGE BY NORMAL Ly-1 (CD5$^+$) B CELLS THAT RECOGNIZE PHOSPHATIDYL CHOLINE

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A substantial proportion of both murine and human CD5 B cell malignancies express a limited set of Ig variable region (V) gene segments that encode autoreactive antibodies (1, 2). In mice, the normal CD5 (Ly-1) subset of B cells is associated with the production of autoreactive antibody (3, 4), as are CD5 (Leu-1/T1) B cells from normal humans (5, 6). Cell transfer experiments indicate that the antibodies produced by normal CD5 B cells may also be encoded by a restricted Ig gene repertoire (7, 8). However, such a restricted use of Ig genes has not been demonstrated directly in normal, unmanipulated animals.

The most widely studied antibody associated with Ly-1 B cells is IgM, which, in the presence of complement, is hemolytic for bromelain-treated mouse erythrocytes (BrMRBC)[1] (4, 9, 10). Herzenberg and her colleagues originally established this association (11, 12). This group also showed that in normal mice, Ly-1 B cells occur at highest frequency in peritoneal cells (PerC) (3). By use of fluorochrome-loaded synthetic liposomes, we showed that antibody against the common membrane phospholipid, phosphatidyl choline (PtC), was produced by a substantial proportion of B cells present in PerC of normal mice. The PtC-binding cells were part of the Ly-1$^+$ B cell population, and after FACS they were found to include all the cells that could be stimulated by LPS to secrete antibody capable of complement-mediated lysis of BrMRBC (4).

We had found IgM with this same specificity produced by cells of the CH series of Ly-1$^+$ B cell lymphomas (13). Of 27 lymphomas tested, 6 produced PtC-specific antibodies that also reacted with BrMRBC (10). The six antibodies expressed a restricted set of idiotypes and were all encoded by one of three particular combinations of unmutated V_H, J_H, V_κ, and J_κ germline genes (2).

We wanted to know if the same restriction of Ig gene utilization applied to normally occurring PtC-specific Ly-1 B cells. One of the Ig gene combinations found

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[1] *Abbreviations used in this paper:* BrMRBC, bromelain-treated mouse erythrocytes; KLH, keyhole limpet hemocyanin; PC, phosphoryl choline; PtC, phosphatidyl choline; PerC, peritoneal cells.

in the CH series had been described previously by Reininger et al. (9) in hybridomas generated from normal spleen cells and selected for the ability to lyse BrMRBC. The idiotype associated with a second combination had been detected on Ly-1 B cells in the spleen of normal mice (14). Since PtC-specific antibodies produced by peritoneal B cells of B10.*H-2*^{a}*H-4*^{b}*p*/Wts (2^a4^b) mice are firmly established as belonging to the Ly-1 B cell population (4), we generated a panel of hybridomas from PerC of these mice. We found that of the 86 IgM-secreting hybridomas generated, 21 produced antibody that recognizes PtC in the membrane of synthetic liposomes. Of 18 of this subset tested, 17 bound to BrMRBC. The results presented in this paper demonstrate that the high frequency of antibodies from normal Ly-1 B cells specific for PtC derives primarily from the usage of two particular V gene pairs. These antibodies express the same idiotypes as the CH27 or CH34 lymphomas and, by Southern restriction fragment analysis, appear to be encoded by the same V gene pairs. The identification of multiple, clonally distinct hybridomas, derived from a single mouse, suggests that the high frequency of PtC-specific cells is not the result of the expansion of a single clone of Ly-1 B cells.

Materials and Methods

Mice and Tumors. B10.*H-2*^{a}*H-4*^{b}*p*/Wts (2^a4^b) mice were bred and maintained in our pathogen-free mouse colony. This strain had been developed from selected F_2 progeny of B10.A × B10.129 (21M) mice (15). The CH series of B cell lymphomas is of 2^a4^b origin, and was induced as described (16).

Immunofluorescence Analysis. For immunofluorescence analysis of reactivity against BrMRBC, BrMRBC were prepared as described by Bishop and Haughton (14). 50 μl of BrMRBC suspension containing 10^6 cells was mixed with 100 μl of hybridoma supernatant. This mixture was incubated for 20 min on ice then washed twice. Binding was detected using biotin-conjugated goat anti-mouse IgM (Fisher Biotech, Orangeburg, NY) followed by streptavidin-phycoerythrin and analysis on an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL) using 488 nm excitation from an argon-ion laser.

BrMRBC Lysis. 2 × 10^6 BrMRBC were mixed with 50 μl of tissue culture supernatant containing at least 600 ng IgM/ml and 50 μl of a 1:4 dilution of guinea pig complement in 96-well microassay plates. The mixture was incubated at 37°C for 90 min and the plate then centrifuged to pellet remaining intact RBCs. In all cases lysis was either complete or did not appear different from complement and irrelevant antibody controls. Positive controls were included in all assays.

Generation of the NC Series Hybridomas. 6–8-mo-old mice were killed by cervical dislocation. PerC were obtained from individual mice by injecting 5–10 ml RPMI 1640, supplemented with 10% FCS, 2 mM L-glutamine, 50 μM 2-ME, and gentamicin (B cell medium), into the peritoneal cavity. Medium was gently agitated then withdrawn aseptically with a Pasteur pipette. Before fusion (except for mice NC2, NC3 and NC7), cells were washed once and adjusted to 10^6/ml with B cell medium containing 50 μg/ml LPS W (Difco Laboratories, Detroit, MI). 2 ml volumes were incubated in 24-well tissue culture plates at 37°C in an atmosphere of 5% CO_2 for 24 h. PerC from mice NC2, NC3, and NC7 were cultured similarly for 4 d in B cell medium without LPS. After culture, cells were harvested using a rubber policeman, and viable cells were counted in trypan blue.

Hybridomas were produced by fusing cultured PerC with P3-X63-AG8.653 (8.653) using a modification of the procedure of Galfre et al. (17). We used a ratio of 1 PerC cell to 10 8.653 myeloma cells. We also reduced all reaction and culture volumes to ~1/100 that normally used for spleen cell fusions to reflect the lower number of PerC recoverable per mouse (10^6 to 10^7). As a result of this scaling down, 10–40 wells containing ~10^5 PerC per well were set up per individual mouse fusion.

In all fusions except NC12 and NC19, <30% of wells contained growing cells in the selective medium, and hence were assumed to be populated by single clones (18). Clones were isolated from fusion NC12 and NC19 by single cell deposition using the EPICS V cell sorter in combination with the Coulter Autoclone device. Clones that secreted IgM were detected using an isotype-specific ELISA. The wells of 96-well immunoassay plates (Nunclon Delte SI; Vanguard International, Neptune, NJ) were coated with goat anti-mouse Ig (CooperBiomedical, Malvern, PA). The plates were developed using alkaline phosphatase-conjugated goat anti-mouse Ig reagents specific for μ, κ or λ chains (Southern Biotechnology Associates, Walkersville, MD) and *p*-nitrophenyl phosphate as substrate (Sigma Chemical Co., St. Louis, MO). The developed plates were analyzed in an automated ELISA reader (Biotek Instruments, Winooski, VT). When used to quantitate Ig, standard curves were constructed using Ig isotype standards (ICN Immunobiochemicals, Lisle, IL).

PtC-Liposome Binding Assay. Liposomes, composed of 45 mole % synthetic distearoylphosphatidyl choline, 5 mole % distearoylphosphatidyl glycerol, and 50 mole % cholesterol that entrapped 100 mM sulforhodamine-B (SRB), were a gift of Dan McLaurin, Becton Dickinson Research Center (Research Triangle Park, NC). IgM-mediated liposome binding to polyacrylamide beads was performed as previously described (10). We used 96-well V-bottomed plates (Costar, Cambridge, MA) for this assay, which allows for semiquantitative, visual assessment of liposome binding.

DNA Isolation and Southern Analysis. High molecular weight DNA was isolated essentially as described (19) using 1 ml extraction volumes per 10^8 hybridoma cells. 10 μg of DNA from each cell type was digested with either 100 U Eco RI (New England Biolabs, Beverly, MA) or 160 U Hind III (IBI, New Haven, CT). Digested samples were electrophoresed, transferred to nitrocellulose filters, and hybridized with probes to detect rearrangements of the Ig loci as described (20). The probe used to detect IgH gene rearrangements was a 700-bp Xba I/Eco RI fragment from Ch4A142.7 (21), which hybridizes to the intervening sequence 3′ to J_{H4}, kindly provided by Dr. F. Blattner (University of Wisconsin, Madison, WI). κ gene rearrangements were detected with a 900-bp Hind III/Xba I fragment from pEC_κ (22) (a gift of Dr. C. Coleclough, Basel Institute for Immunology, Basel, Switzerland) which hybridizes to the intervening sequence 3′ to $J_{\kappa 5}$. DNA probes were labeled with α-[^{32}P]dCTP (Dupont/New England Nuclear, Boston, MA) using random primed DNA labeling (Pharmacia Oligo-labeling kit, Pharmacia Fine Chemicals, Piscataway, NJ) to a specific activity of 1–2 $\times$ 10^8 cpm/μg.

Antiidiotype Antisera. Antiidiotype antisera were prepared in Lewis rats by immunization with appropriate affinity-purified IgM (idiotype) conjugated to keyhole limpet hemocyanin (KLH). The rats received an initial injection of 100 μg of idiotype on KLH in CFA subcutaneously and a second injection of 100 μg of idiotype on KLH in IFA intraperitoneally 2 wk later. The rats were bled 12 d later to obtain serum. The antiserum was adsorbed over IgM, κ columns to remove constant region activity. Specificity of each antiserum was demonstrated by immunofluorescence staining of appropriate lymphoma cells and by ELISA (see Table I).

Monoclonal Anti-CH12 Idiotype. An 8-wk-old (21M $\times$ 2^a4^b)F_1 mouse was immunized with 100 μg of affinity-purified CH12 IgM conjugated onto KLH emulsified in CFA. The mouse received one boost 2 wk later with the same dose of idiotype on KLH in IFA and a second boost after an additional 12 d with 100 μg idiotype on KLH in PBS. 3 d later the spleen was removed and fused with the 8.653 myeloma (see above). Hybrids were selected for reactivity with the affinity-purified IgM from CH12 but not with affinity-purified IgM from CH27 or CH34. This reagent also specifically stains CH12 cells in an immunofluorescence assay. Biotinylated Ig (IgG2b) from one subclone, designated HyCH12Id.5E3, was used in these studies.

Analysis of Idiotype Expression. Idiotype expression on IgM secreted by the NC hybridomas was analyzed by ELISA. Goat anti-mouse IgM coated onto plastic plates was used to entrap the hybrid IgM. Rat anti-Id antisera were then added and developed with biotinylated monoclonal mouse anti-rat κ (MAR18.5) and avidin-alkaline phosphatase. Identification of the CH12 idiotype was performed with biotinylated 5E3 and avidin-alkaline phosphatase.

Results

Screening the NC Series for Hybridomas that Secrete Antibody Specific for PtC. We have generated 86 stable IgM-secreting hybridoma clones, which we call the NC series, from the fusion of PerC of 2^a4^b mice and the Ig nonsecreting myeloma, P3-X63-Ag8.653. These hybridomas were derived from fusions of PerC from 15 individual mice. 85% of the hybridomas produce κ light chains and the balance λ. To identify those hybridomas secreting IgM with specificity for PtC we screened hybridoma culture supernatants containing a minimum of 0.5 μg/ml of IgM against PtC-containing synthetic liposomes. A striking finding was the extraordinarily high frequency (21 of 86) of NC series hybridomas that secrete IgM specific for PtC (Table I). We have focused our initial analysis of the NC series hybridomas on the group with this specificity since we had established previously that they would be derived from B cells totally included in the Ly-1 population (4).

PtC-specific NC Hybridoma IgM Recognize BrMRBC. The IgM antibodies of 18 of the 21 NC hybridomas that recognize PtC liposomes were tested for their ability to recognize BrMRBC. Two assays were used; immunofluorescence and hemolysis. As shown in Table I, 17 of 18 could be shown to bind to BrMRBC by immunofluorescence. Only 5 of the 15 tested, however, were lytic for BrMRBC in the presence of added guinea pig complement. Lytic ability was not a function of the antibody concentration of the tissue culture supernatant (data not shown).

The PtC-binding NC Hybridomas Use a Restricted Set of Ig Heavy Chain/Light Chain Pairs. We used Southern blot analysis to look for restriction fragments that corresponded in size with the known productive Ig gene rearrangements of the CH series PtC-binding prototypes, CH12, CH27, and CH34 (2). Rearrangements at IgH were revealed by a probe that hybridizes to the intervening sequence 3′ to J_H4. Fig. 1 *A* shows that seven hybridomas (NC1-A7, NC19-F11, NC12-H8, NC19-E12, NC19-E6, NC5-A11, and NC12-G12) share a rearranged 3.3-kb Eco RI fragment at the J_H locus. The 3.3-kb fragment is the same size as that from CH27 that contains the productive rearrangement of its heavy chain locus (2). Eight other hybridomas (NC19-F5, NC17-D7, NC19-F2, NC19-F7, NC17-D8, NC19-F1, NC8-E1, and NC15,D3) share a 4.8-kb Eco RI fragment corresponding to a fragment generated from the productively rearranged chromosome of CH34 (2). The 4.8-kb fragment in NC17-D7 is very faint in the gel shown in Fig. 1, although repeated analyses of this hybridoma confirmed the presence of this fragment. None of the hybridomas appear to have a restriction fragment corresponding to the productive rearrangement of CH12. One of the 16 PtC-binding hybridomas tested (NC12-H1) did not display any fragment corresponding to CH12, CH27, or CH34. Fig. 1 *A* also shows that there is no example of a pair of hybridomas derived from the same mouse sharing any other heavy chain rearrangement except the 6.4-kb fragment derived from the 8.653 fusion partner. Four different mice (NC1, NC5, NC12, and NC19) yielded hybridomas with the 3.3-kb fragment (CH27-like) and four (NC8, NC15, NC17, and NC19) gave hybridomas with a 4.8-kb fragment (CH34-like). The single mouse NC19 yielded at least three hybridomas demonstrated to be of the CH27 type and four of the CH34 type (some have not yet been analyzed). NC12 provided two hybridomas with a 3.3-kb fragment and NC17 provided two with a 4.8-kb fragment. All hybridomas from a single mouse displayed a unique second J_H fragment, indicating that no two were derived from the same clone of B cells.

TABLE I

BrMRBC Reactivity, Ig Genes, and Idiotypes of PtC-Liposome Binding NC Series Hybridomas

	BrMRBC				Id‖		
	Immuno-fluorescence*	Lytic‡	V_H§	V_L§	12	27	34
[CH27]	+	+	27	27	–	+	–
NC1-A7	+	–	27	27	–	+	–
NC19-F11¶	ND	ND	27	27	ND	ND	ND
NC12-H8	–	–	27	27	–	+	–
NC19-E12	+	–	27	27	–	+	–
NC19-E6	+	–	27	27	–	+	–
NC5-A11	+	+	27	27	–	+	–
NC12-G12¶	ND	ND	27	27	ND	ND	ND
NC19-B8G2	+	ND	ND	ND	–	+	–
NC6-C12	+	+	ND	27	–	+	–
NC19-F5	+	+	34	34	–	–	+
NC17-D7	+	–	34	34	–	–	+
NC15-D3	+	–	34	34	–	–	+
NC8-E1	+	–	34	?	–	–	+
NC19-F1	+	–	34	34	–	–	+
NC17-D8	+	+	34	34	–	–	+
NC19-F7	+	–	34	34	–	–	+
NC19-F2	+	+	34	34	–	–	+
NC19-E11	+	ND	ND	ND	–	–	–
NC19-E10	+	–	ND	ND	–	–	+
[CH34]	+	+	34	34	–	–	+
NC12-G10	ND	ND	ND	ND	–	–	–
NC12-H1	+	ND	?	?	–	–	–
[CH12]	+	+	12	12	+	–	–

Brackets denote prototype PtC-binding CH lymphomas for V_H and V_L.

* Reactivity with BrMRBC determined by immunofluorescence.

‡ Reactivity with BrMRBC determined by hemolysis in the presence of guinea pig complement.

§ The assignment to CH lymphoma V_H or V_L is based on shared restriction fragment size with these prototypes in Southern-blot analysis. V_H "27", 3.3-kb; and V_H "34", 4.8-kb Eco RI fragments, respectively. V_L "27", 13.0-kb; and V_L "34", 2.5-kb Hind III fragments, respectively. ?, no fragment shared with either CH12, CH27, or CH34. ND, not done.

‖ Idiotype expression tested on affinity-selected IgM by ELISA. CH12 idiotype was detected with a mouse monoclonal anti-CH12Id; the CH27 and CH34 idiotypes were detected with specific rat antisera.

¶ Cell line has been lost.

We also analyzed the rearrangements at the κ locus (Fig. 1 *B*). The seven hybridomas that share an IgH rearrangement with CH27 also share a rearranged 13.0-kb Hind III fragment at the J_κ locus with that from CH27. Seven of the eight hybridomas (the exception being NC8-E1), which have a J_H rearrangement in common with CH34, are shown to share the 2.5-kb J_κ fragment corresponding to that of CH34. Other shared restriction fragments are in germline configuration (identical fragment length of 2.8 kb for liver DNA), or are derived from the fusion partner, 8.653 (10.2 kb). A summary of the restriction fragment analysis is given in Table I.

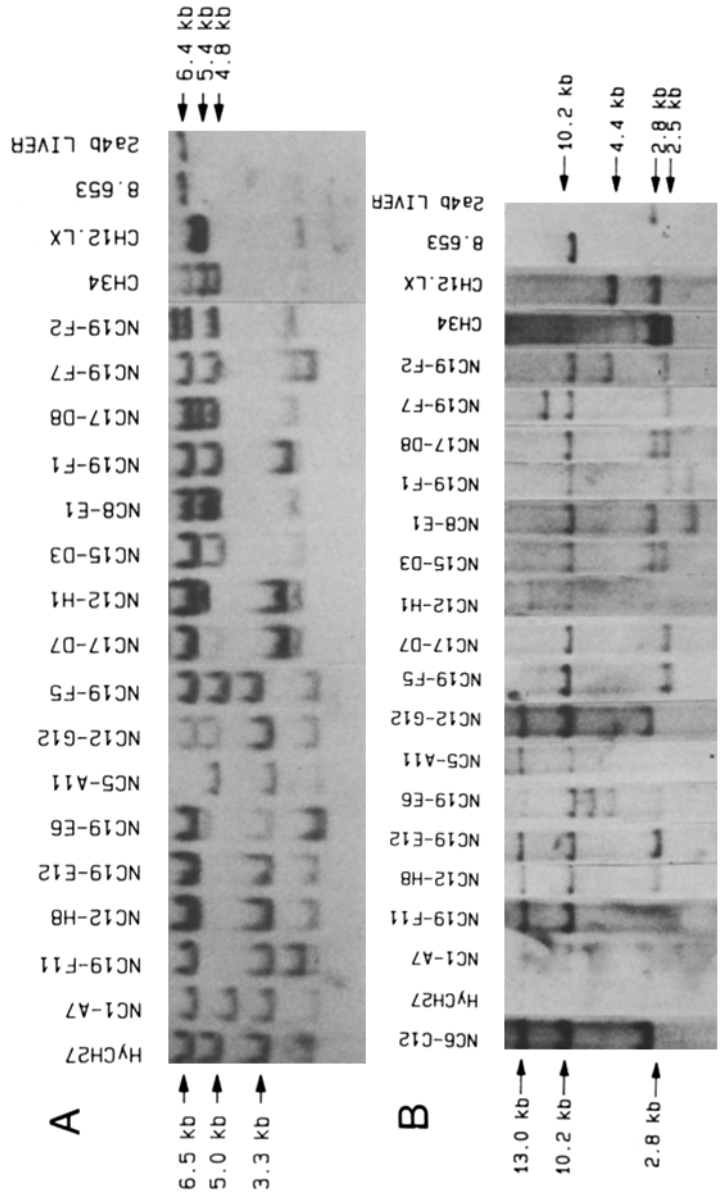

FIGURE 1. Rearrangements at IgH and κ in PtC-binding NC hybridomas are shared with PtC-binding CH lymphomas. (*A*) Eco RI restriction fragments generated by rearrangements at the IgH locus detected using a probe that hybridizes 3′ to J_H4. The low molecular weight bands of identical size are apparently λ DNA contamination in these gels since other gels did not show this band. (*B*) Analysis of the κ locus performed by hybridization of a probe to intervening sequence 3′ to $J_κ5$ with Hind III–restricted DNA. DNA from CH lymphomas was extracted from CH12.LX, an in vitro–adapted line, HyCH27, a hybrid of CH27 and 8.653, and CH34 was extracted from a tumor grown in vivo.

Idiotype Expression Correlates with Predicted V Gene Usage. To determine whether the restriction fragments shared by the CH27 or CH34 lymphomas and the PtC-specific NC hybridomas were indeed derived from the productive rearrangements, we analyzed the idiotype expressed by 19 of the 21 liposome-binding hybrids. As shown in Table I, in all cases where we could predict from Southern analysis a heavy or light chain gene usage, there was appropriate idiotype expression on the secreted IgM. Idiotype analysis of a group of 47 NC hybridomas whose secreted IgM does not react with PtC-liposomes showed that none of them expressed the CH12, 27, or 34 idiotype (data not shown).

Discussion

The Ly-1 B cell subset in normal animals has been implicated in the production of autoreactive antibody (12) in an environment where antigen levels would be expected to be tolerogenic for conventional B cells. As part of a continuing study of the antibodies produced by Ly-1 B cells, we made a library of hybridomas from the peritoneal B cells of normal adult 2^a4^b mice. We knew this tissue to be enriched for B cells producing anti-PtC antibody as revealed by the binding of fluorochrome-loaded synthetic liposomes, and that these cells were totally included within the Ly-1 B subset (4). We could thus be certain of the origin of hybridomas producing antibody of this specificity. This proved to be an advantage worthy of note, since we and others have observed that Ly-1 may cease to be expressed after fusion (our unpublished observations; 23).

Out of a total of 86 monoclonal IgM antibodies, 21 were specific for PtC liposomes and, as expected, 17 of 18 of these that were tested were found to bind to BrMRBC, confirming their derivation from Ly-1 B cells. However, only about one-third of them were hemolytic in the presence of complement. Since there was no systematic difference between hemolytic and nonhemolytic antibodies in terms of V gene usage or idiotype, this property probably reflects differences in post-transcriptional modification of $C\mu$.

Southern blot analysis showed that 14 of the 16 PtC-binding hybridomas examined produced restriction fragments that correspond with the productive rearrangements of both the heavy and light chains of either the CH27 or the CH34 lymphomas. One of the other two (NC8-E1) produced a restriction fragment corresponding to the CH34 heavy chain, but the size of the light chain fragment was not similar to that of CH34. In all cases tested, hybridomas displaying CH27-like gene rearrangements produced antibody bearing the CH27 idiotype and all hybridomas with CH34-like rearrangements (including NC8-E1) produced antibody with the CH34 idiotype. The one PtC-reactive hybridoma that did not share rearrangements with either CH27 or CH34 was the only one whose antibody displayed neither CH27 nor CH34 idiotype. None of the hybridoma antibodies reacted with the monoclonal anti-CH12 idiotype reagent, and of those tested that did not show PtC specificity (47 of 65), none reacted with anti-CH12, 27, or 34 idiotypes. The fact that the results of the idiotype analysis showed complete concordance with antigen specificity and Southern blot analysis strongly supports the conclusion that the shared fragments represent the productive V gene assemblies of the hybridomas and that they result from rearrangement and expression of the same V genes. Sequence analysis of some of these V_H genes confirms the CH27 or CH34 assignment based on the Southern and idiotype analyses (Pennell, C., personal communication).

The finding of a restricted repertoire among normal Ly-1 B cells raises questions regarding the ontogeny of these B cells. We have previously shown that early in life PtC-specific cells are rare, but that by 3–5 wk of age these cells reach the high levels seen in normal adult animals (4). Two mechanisms that could explain this generation of large numbers of Ly-1 B cells with the same antigen specificity are the expansion of a single clone of cells within each animal or the selective expansion of multiple clones of cells expressing particular antigen receptors. Each of the two characteristic pairings of V_H and V_κ genes appeared in PtC-binding hybridomas derived from different unimmunized mice. In addition, each of the two pairings was seen in three clonally distinct hybridomas from a single mouse and one of them was seen in two unrelated hybridomas from another, as demonstrated by the unique restriction fragments of the nonproductive allele. This implies that the high frequency of PtC-liposome binding Ly-1 B cells seen in the peritoneums of 2^a4^b mice (4) includes multiple clones of cells expressing a limited number of V gene assemblies. It further implies that no single clone develops an early selective advantage over the others, an implication that is in line with the scarcity of somatic mutations that have been found in Ig genes expressed by Ly-1 B cells (1, 2).

Summary

5–15% of lymphocytes in the peritoneums of normal adult B10.H-2^aH-4^bp/Wts (2^a4^b) mice are CD5$^+$ (Ly-1) B cells that recognize phosphatidyl choline (PtC), a phospholipid component of all mammalian cells. We produced a set of IgM-secreting hybridomas from the peritoneal cells of normal, adult 2^a4^b mice. We found that this set of hybridomas shows a similarly high frequency of antibodies specific for PtC (21 of 86) that also react with bromelain-treated mouse erythrocytes. Restriction fragment analysis of Ig gene rearrangements and analysis of expressed Ig idiotypes reveal that these cells use a restricted set of variable region genes to generate the PtC-specific antibodies. The Ig genes used by the PtC-specific hybridomas appear to be the same as those found in the PtC-specific Ly-1 B cell lymphomas, CH27 and CH34.

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