Selection of Antigen-specific, Idiotype-positive B Cells in Transgenic Mice Expressing a Rearranged M167-\(\mu\) Heavy Chain Gene

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Summary

Flow cytometric analysis of antigen-specific, idiotype-positive (id+) B cell development in transgenic mice expressing a rearranged M167-\(\mu\) gene shows that large numbers of phosphocholine (PC)-specific, M167-id+ B cells develop in the spleen and bone marrow of these mice. Random rearrangement of endogenous \(V_x\) genes, in the absence of a subsequent receptor-driven selection, should give rise to equal numbers of T15- and M167-id+ B cells. The observed 100-500-fold amplification of M167-id+ B cells expressing an endogenous encoded \(V_x24\) light chain in association with the M167 \(V_\mu\)-id transgene product appears to be an antigen driven, receptor-mediated process, since no amplification of non-PC-binding M167 \(V_\mu1/V_\mu2\), T15-id+ B cells occurs in these \(\kappa\)-only transgenic mice. The selection and amplification of antigen-specific, M167-id+ B cells requires surface expression of the \(\mu\) transgene product; thus, no enhancement of M167-id+ B cells occurs in the M167 \(\kappa\)-mem-transgenic mice, which cannot insert the \(\mu\) transgene product into the B cell membrane. Surprisingly, no selection of PC-specific B cells occurs in M167-K-transgenic mice although large numbers of B cells expressing a crossreactive M167-id are present in the spleen and bone marrow of these mice. The failure to develop detectable numbers of M167-id+, PC-specific B cells in M167-\(\kappa\)-transgenic mice may be due to a very low frequency of M167-\(\mu\)-region formation during endogenous rearrangement of \(V_\mu1\) to D-J\(\_\) segments. The somatic generation of the M167 version of a rearranged \(V_\mu\)-gene may occur in less than one of every 10⁶ bone marrow B cells, and a 500-fold amplification of this M167-Id+ B cell would not be detectable by flow cytometry even though the anti-PC antibody produced by these B cells is detectable in the serum of M167-\(\kappa\)-transgenic mice after immunization with PC.

B lymphocyte development in the mouse is a complex and dynamic process in which the adult bone marrow produces \(\sim 6 \times 10^7\) new B cells each day (1). However, very few of these B cells appear to enter the stable, long-lived peripheral B cell pool where the half-life of a B cell, as measured by bromodeoxyuridine (BrdUrd) incorporation, is 3 mo or longer (2, 3). It is of interest to understand how the few B cells that enter this long-lived pool are chosen from the millions of B cells produced each day.

Analysis of the adult peripheral B cell \(V_\mu\) repertoire (4-9) suggests that it is randomly generated in as much as it reflects the complexity of the \(V_\mu\) gene families expressed in the mouse genome (8, 10); however, several studies (11-17) have also suggested that the peripheral B cell pool is selected and does not simply reflect the repertoire that emerges from the bone marrow. Yancopoulos et al. (11) found that the preferential utilization of J\(\mu\) proximal \(V_\mu\) genes in Abelson virus-transformed pre-B cells, which persists in the bone marrow of adult BALB/c mice (9, 12), is not mirrored in the B cell repertoire of the adult spleen. Freitas et al. (9) have also found that local environmental factors can lead to increased representation of the \(V_\kappa\)-J558 family in lymph nodes, while the \(V_\mu\)-X24 family is overexpressed in Peyer's patches. The selection of individual idiotypes or H/L chain combina-
tions into restricted B cell subsets may also occur during B cell ontogeny (15–17). We have shown (13, 14) that T15 idiotype-positive (id+) B cells are functionally restricted to the Lyb-5+ B cell subset in normal mice, and that all phosphocholine (PC)-specific B cells appear to be negatively selected via clonal deletion in M167 μ/κ anti-PC transgenic mice coexpressing the xid gene (15). Other laboratories have suggested that T15-id+ B cells are highly selected into the peritoneal CD5+ B cell subset (16, 17). The T15 (V₅₁/V₅₂₂) clone of B cells is the most frequently occurring individual B cell in the mouse (18). This overexpression of the T15 clone may be due to antigenic (14) or antiidiotypic (19) selection and/or biotin-conjugated antibody. Cells were then washed twice with HBSS, the cells were analyzed on an EPICS 753 cytofluorograph for dual fluorescence (Coulter Electronics, Hialeah, FL). Forward and right-angle light scatter and green (FITC) and yellow (PE) fluorescence were detected. Dead cells were excluded from light scatter, and green fluorescence analysis was based on propidium iodide uptake. Compensation for spectral overlap of FITC and PE was determined using single-labeled samples. PC inhibition of anti-id binding to spleen cells from M167 μ/κ 207-4 transgenic mice was performed by staining cells as described above but in the presence of 5 × 10⁻³ M PC (Sigma Chemical Co., St. Louis, MO).

Antigen-binding Cells (ABC). The number of spleen cells in TG+ and TG- mice capable of binding PC was determined in a rosette assay (29). SRBC were conjugated with diazophenylphosphocholine, as previously described (14), and adjusted to 2% (vol/vol). Spleen cells were diluted to 10⁶/ml, and 200 μl of spleen cells and 100 μl of PC-SRBC were placed together in a 12 × 75 tube, spun at 800 rpm for 10 min in the cold, resuspended, and placed on a hemocytometer. The number of ABC was determined by counting the number of white cells having four or more PC SRBC bound.

Isolation of M167-id+ B Cells. PC-specific, M167-id+ splenic B cells were isolated from μ-transgenic mice by staining them with FITC-conjugated anti-Id⁺ and biotin-conjugated anti-M167-id (28-5-15) plus PE-conjugated streptavidin. The double-positive

| Abbreviations used in this paper: ABC, antigen-binding cells; id, idiotype; ODN, oligodeoxynucleotide; PC, phosphocholine; RT, reverse transcriptase; TG, transgene. | 1190 Expression of Antigen-specific, Idiotype-positive B Cells |
RNA Isolation and PCR Reactions. RNA from 2 x 10^6 μ^V/M167-id* spleen cells or M167-id* (V,1/V24) hybridoma cells from 207-4 transgenic mice were isolated using the guanidine thiocyanate-CiCl centrifugation method (30). CsCl was obtained from Bethesda Research Laboratories (Gaithersburg, MD) and guanidine thiocyanate from Fluka Biochemicals (Ronkonkoma, NY). For CDNA synthesis from RNA, 1.0 μg of total RNA was added to PCR reaction buffer (Perkin-Elmer Corp., Norwalk, CT) with final concentrations of: 5 mM MgCl_2, 50 mM KC1, and 10 mM Tris-HCl, pH 8.3. 1 mM dATP, dCTP, dGTP, and dTTP was added to the mixture, as well as 1.0 μM 3' primer specific for Cc and 1 U of RNase inhibitor (Perkin-Elmer Corp.). The reaction mixture was heated to 65°C for 10 min, then placed on ice. M-MLV reverse transcriptase (Bethesda Research Laboratories) was added to a final concentration of 200 U/reaction and transcribed allowed to proceed for 30 min at 37°C. After cDNA synthesis, the reactions were incubated at 99°C for 5 min and placed on ice. A PCR reaction was set up according to the instructions (GeneAmp RNA PCR kit; Perkin-Elmer Corp.). The PCR reactions contained cDNA, 2 mM MgCl_2, 50 mM KC1, 10 mM Tris-HCl, pH 8.3, 2.5 U Taq Polymerase, 1.0 mM DTT, 0.2 mM dNTPs, and 0.2 μM 5' and 3' primers. A thermocycler (Perkin-Elmer Corp.) was used for the amplifications. The reactions were allowed to proceed for 30 cycles consisting of 90 s at 94°C, 90 s at 55°C, and 150 s at 72°C. A soak cycle at the end of the 30 cycles at 72°C for 6 min terminated the reaction.

Analysis of PCR Products. 20 μl of the 100 μl amplified products was separated on a 3% agarose gel containing 2.25% Nu-sieve GTG agarose (FMC Bioproducts, Rockland, ME) and 0.75% regular agarose (Bethesda Research Laboratories), then stained with ethidium bromide and photographed. Only products of the size predicted by the locations of the primers within the μM167 and MOPC-21 sequences were present. μM167 PCR products were sequenced using the Sequenase 2.0 sequencing kit (U.S. Biochemicals Corp., Cleveland, OH) with the following modifications: 80 μl of 100 μl PCR product was extracted once with an equal volume of phenol/chloroform (1:1), then primers were removed by passage through a Sepharose C6B spin column (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The final volume from the spin column was 80 μl. NP-40 was added to the sequencing reaction to a final concentration of 0.4%, 7 μl of PCR product and 20 pmol of Cc primer were used. The reaction components were denatured by boiling for 3 min, then snap-cooled in powdered dry ice. The dGTP labeling mix was diluted 1:20, the sequencing enzyme 1:8. The labeling reaction was incubated at room temperature for 2 min, and the termination reaction proceeded for 4 min at 37°C.

Oligodeoxynucleotide Primers. Unmodified oligodeoxynucleotides (ODNs) were prepared by the Nucleic Acid Synthesis Laboratory (Program Resources, Inc./DynCorp, NCI-FCRDC, Frederick, MD) using B-phosphoramidite chemicals on an automated DNA synthesizer (8750; Biosearch, Millipore, Milford, MA). The ODNs were purified on denaturing polyacrylamide gels, electro-eluted, and ethanol precipitated. Concentrations were established by densitometry (A_260 of 1 = 20 μg).

A V, 167-specific 5' primer, MOPC-21-specific 5' primer, and Cc 3' primer were synthesized using the published sequences (32–35) obtained through computer database searches. Primers were selected from these sequences through the use of the PCR Primer Selection Program (Henry M. Jackson Foundation for the Advancement of Military Medicine, Rockville, MD): V,M167 5' primer; 5'-GCT-GGTCGTG-CTTC-ATTCGTC-3'; V,M-21 5' primer; 5'-AAG-GAC-GCA-ATC-TCC-CAAGA-3'; 3' Cc primer: 5'-GCC-ATT-TCG-ATG-ATC-3'.

Transfection of V, Genes into a M167-Cell Line. The J558L-M167μ cell line was obtained from Dr. Carol Sibley (University of Washington, Seattle, WA). This cell line, which was produced by transfection of the J558L cell line with the p167μ plasmid constructed by Storb et al. (21), produces an IgM antibody that bears the V,1-id but does not bind PC (see Table 2). This cell line was then transfected by electroporation (capacitance of 960 μF at 180 V) with 10 μg of DNA from either the pSV2-Neo-S407-κ plasmid (provided by Dr. Phil Tucker, University of Texas, Dallas, TX) or the p67κ-plasmid (21) (provided by Dr. Ursula Storb, University of Chicago, Chicago, IL) plus SV2-Neo, and the resulting lines were selected in the presence of Geneticin (microbiological potency of 400 μg/ml) (Gibco Laboratories, Grand Island, NY). The antibodies produced by these cell lines were tested for the presence of T15-id and M167-id using the antiidiotype antibodies described above, and they were also tested in ELISA for their ability to bind PC-BSA (36).

Results

Idiotype Analysis of Spleen Cells from M167 μ/k Transgenic Mice. We have previously shown that phenotypically normal M167 μ/k transgenic mice express the IgM* (μ^* ) transgene-encoded anti-PC antibody on 97% of their splenic B cells (36). When the spleen cells of these μ/k TG+ mice were stained with FITC-anti-μ plus biotin-conjugated antiidiotype antibodies, as shown in Fig. 1, >95% of the B cells stained with the H-chain-specific anti-V,1-id (A), and also with the H + L combinatorial anti-M167-ids 28-5-15 (C) and 28-6-20 (D). None of the B cells from these M167 μ/k transgenic mice stained with anti-T15-id antibody (B), and <1% of spleen cells from TG- littermates stained with any of the anti-id antibodies (data not shown). Fig. 2 shows the staining of spleen cells from these same M167 μ/k transgenic mice with a combination of FITC-anti-μ plus anti-id in the absence (A–C) and presence (D–F) of 5 x 10^-3 M PC. As previously shown by Desaymard et al. (23), the binding of the T68.3 anti-V,1-id antibody to its idiotope is not blocked by PC (A vs. D). The binding of the crossreactive anti-M167-id 28-6-20 is also unaffected by the presence of PC (C vs. F), indicating that its idiotope is not in or near the binding site. On the other hand, the binding of the anti-M167-id antibody from clone 28-5-15 to the M167-id* spleen cells is inhibited by >90% in the presence of PC (B vs. E).

Idiotype Analysis of B Cells from M167 μ-only Transgenic Mice. The combined use of these anti-id antibodies allows us to follow the independent development of both H chain and H + L chain id− B cells and to quickly determine the frequency of those B cells that are also antigen specific. Using these anti-id reagents, we have analyzed B cell development in transgenic mice carrying either the M167 μ-only or κ-only transgenes. In the μ-only transgenic mice, the V,1-id will be expressed on all B cells expressing the μ^* transgene product, while the 28-6-20 and 28-5-15 combinatorial idiotopes will be expressed only on those B cells in which the μ^* transgene product has associated with an endogenous
germline M167 (V\textsubscript{p},24) light chain. Since there is only a single copy of the M167 V\textsubscript{p},24 L chain gene in the mouse genome (34), and this V\textsubscript{p} gene must rearrange to a J\textsubscript{p}5 gene segment to produce the M167 κ L chain (37), these M167 combinatorial idiotypes should occur in ~0.1% (1/800) of the μ\textsuperscript{+} B cells. This estimate is based on the assumption that the 100–200 endogenous κ genes (38) are expressed in a random fashion and randomly rearranged to the four functional J\textsubscript{p} genes during transgenic B cell development. This estimate represents the upper limit for M167 V\textsubscript{p},24:J\textsubscript{p}5 expression in as much as V\textsubscript{p} rearrangement is not completely random (39) and J\textsubscript{p}5 is under expressed in adult spleen (40).

Spleen cells from μ\textsubscript{κ}-243-4 transgenic mice were stained with FITC-conjugated goat anti-μ or anti-μ\textsuperscript{a}, and biotin-conjugated anti-V\textsubscript{p}1-id, anti-T15-id, or anti-M167-id followed by PE-streptavidin. Fig. 3 A shows that 14% of the spleen cells or 28% of the B cells in this mouse expressed the transgene-encoded H chain. The expression of the M167 μ transgene product is highly variable (25–80%) on B cells from 243-4 mice, and >50% of these μ\textsuperscript{+} B cells also express the endogenous μ\textsuperscript{κ} allotype (Kenny et al., manuscript in preparation). The data in Fig. 3 B show that none of the B cells in these M167 μ-only transgenic mice express the T15-id. Formation of a T15-id\textsuperscript{+} B cell would require that the V\textsubscript{p}1 transgene associate with an endogenous V\textsubscript{p}22 L chain, and this should occur at the same frequency (~0.1%) as the generation of M167-id\textsuperscript{+} B cells. By contrast, ~20% of the total B cells in this mouse express both the 28-5-15 binding site–specific and the crossreactive 28-6-20 M167-ids (Fig. 3, C and D). When one considers only the V\textsubscript{p}1\textsuperscript{+} B cells, these V\textsubscript{p}24-dependent M167-ids are expressed on 57% and 71% of the TG\textsuperscript{+} B cells, respectively. This is significantly higher (500-fold) than the 0.1% M167-id\textsuperscript{+} B cells expected from random expression of endogenous κ genes. Two distinct M167-id\textsuperscript{+} B cell populations are present, one with high levels of M167-id (5% of spleen cells) and one with low levels of M167-id (3–5%) (Fig. 3, C and D). Three color flow cytometric analyses will be needed to determine if the low M167-id expression is due to B cells expressing high levels of endogenous μ\textsuperscript{κ} allotype. The majority of M167-id bright cells are expressing only the μ\textsuperscript{κ} transgene product, but some coexpression of endogenous μ\textsuperscript{κ} also occurs in this population (data not shown).

Idiotyp Analysis of the Bone Marrow B Cells in M167 μ-only Transgenic Mice. The above data indicate that splenic B cells expressing the V\textsubscript{p}1 transgene product in association with a V\textsubscript{p}24 L chain have been selectively amplified at least 500-fold over the level expected from random association of H and L chains. We therefore analyzed bone marrow B cells for M167-id expression to determine whether this amplification of id\textsuperscript{+} B cells also occurs during early B cell ontogeny or mainly reflects selection and amplification of these B cells in the peripheral lymphoid tissues. In the five μ-only bone marrow samples analyzed, an average of 2.4 ± 0.3% of the μ\textsuperscript{+}V\textsubscript{p}1\textsuperscript{+} B cells also expressed the 28-5-15 binding site–specific M167-id. In these same five mice, 10.6 ± 0.7% of the splenic μ\textsuperscript{κ}V\textsubscript{p}1\textsuperscript{+} B cells carried the M167-id. Thus, there is approximately a fivefold increase in M167-id\textsuperscript{+} B cells in the spleen.
compared to the bone marrow of the same mouse, but the M167-id levels in the bone marrow are still 24-fold higher than expected if the H and L chains were associating randomly. However, it is possible that many of these M167-id+ bone marrow B cells could have reentered the bone marrow from the periphery. Forster et al. (2) have recently shown that the vast majority of δ+ bone marrow B cells do not appear to arise from the rapidly dividing pre-B cells, but appear to represent long-lived, nondividing B cells that circulate through the bone marrow. When the bone marrow of μ-only 243-4 mice was stained with FITC-anti-δ and biotin-anti-Vn1-id plus PE/streptavidin, ~50% of the Vn1+ B cells also expressed δ (data not shown). In transgenic mice, this may represent a minimal estimate of recirculating TG+ B cells, since the B cells that express the Vn1 transgene product in the absence of endogenous μ or δ may also be cycling back to the bone marrow. Thus, the elevated numbers of M167-id+ B cells in the bone marrow are probably due to recirculation of mature peripheral B cells back to the marrow.

Selective Amplification of B Cells Bearing the M167-id Is Dependent on Cell Surface Expression of the μ Transgene Product. Storb et al. (21) had observed that M167 κ-mRNA was elevated in all the M167 μ-only transgenic mouse lines they produced, whereas, mRNA for this L chain was not detected in M167 μΔmem transgenic mice where the transgene product could not be inserted into the B cell membrane. We have confirmed this observation by staining the spleen cells from M167 μΔmem 254-3 transgenic mice with FITC-anti-μ plus biotin-anti-M167-id. As shown in Fig. 4, none of the μ+ B cells from these mice stained with either the binding site-specific 28-5-15 or crossreactive 28-6-20 anti-M167-ids. The selective amplification of M167-id+ B cells in the 243-4 μ-only mice would therefore appear to be a receptor-mediated event and possibly antigen driven as suggested by Storb et al. (21).

M167-id+ B Cells in μ-only Transgenic Mice Are PC Specific. To determine if the M167-id+ B cells in the μ-only transgenic mice were indeed antigen specific, spleen cells from TG+ and TG− mice were stained with the binding
Site-specific anti-M167-id 28-5-15 in the presence and absence of 5 × 10⁻³ M PC and also rosetted with PC-conjugated SRBC. Greater than 95% of the μ⁺/M167-id⁺ B cells shown in Fig. 3 C were PC inhibitable (data not shown), and 5% of the μ-243-4 spleen cells also formed PC-specific ABC in the rosette assay (Table 1). On the other hand, the number of ABC in TG⁺ μΔmem 254-3 mice was no different than that seen in their TG⁻ littermates.

The spleen cells from two strains of M167 κ-transgenic mice were also analyzed for PC-specific ABC. As shown in Table 1, there was no difference in the number of ABC detected in TG⁺ and TG⁻ κ 234-4 mice, whereas TG⁺ κ-233-8 mice exhibited elevated numbers of ABC compared to their TG⁻ littermates. However, these ABC were not PC inhibitable and their exact specificity has not been determined. The data in Table 1 suggest that M167-id⁺, PC-specific B cells are highly expanded in μ-only but not in either μΔmem or κ-only M167 transgenic mice.
Table 1.  *PC-specific ABC in M167 Transgenic Mice*

| Mouse Transgene(s) present | Percent ABC  |
|---------------------------|--------------|
| 243-4  μ                  | 5.05         |
| 254-3  μΔmem              | 0.04         |
| 234-4  κ                   | 0.05         |
| 233-8  κ                   | 2.40         |
| 207-4  μκ                  | 46.0         |

*S* Spleen cells from various strains of M167 transgenic mice were adjusted to 10^6 per ml; 200 μl of spleen cells was rosetted with 100 μl of 2% PC-SRBC as previously described (15, 36). ABC were counted on a hemocytometer and the ABC data expressed as a percent of the total number of spleen cells. The μ/κ 207-4 mice were used as a positive control since >95% of their B cells have been shown to bind PC-SRBC (36). The PC specificity of ABC was tested by rosetting in the presence of 5 × 10^-3 M PC. Greater than 90% of the ABC in the 243-4-μ- transgenic mice were inhibited by PC, while none of the ABC in the 233-8-κ-transgenic mice were PC inhibitable.

Antibodies Produced by the Association of the M167 μ Chain and the κ22 L Chain Are T15-id + but exhibit Low Affinity for PC. At least three L chains (V8, V22, and V24) are known to associate with a V1-id gene product to form PC-specific antibodies (41). Yet only the V41/V24 H/L combination has been selectively amplified in the M167 μ-transgenic mice, while T15-id + (V41/V22) B cells, if present, are below the level of detection (Fig. 3). To determine whether or not the M167-μ transgene product would form a PC-specific antibody when associated with a κ22 L chain, we electroporated rearranged V22 and V24 L chain genes into a cell line containing the M167 μ gene. The antibodies produced by these cell lines were tested for both id expression and for their ability to bind PC-BSA-coated plates. As shown in Table 2, the antibody formed by association of the M167-μ H chain and the V22 L chain expresses the T15-ids detected by both the T139.2 monoclonal and rabbit polyclonal anti-T15 antibodies, but this antibody is at least 100 times less efficient at binding PC than the T15 - IgM antibody HPCM2, which was used as a control to generate the standard curves in the PC-specific ELISA. On the other hand, the M167-id + antibody formed by association of the M167-μ transgene and the V24 L chain was PC specific and bound PC-BSA to the same extent as the control. Yet antibodies formed by a V24 L chain plus either an M603 or a T15 H chain were M167-id + but not capable of binding PC (data not shown). These data suggest that the in vivo selection and amplification of M167-id + B cells in the μ-transgenic mice is an antigen-driven rather than an antiidiotype-driven event. B cells expressing the normally dominant T15-id + are not selectively amplified because the antibody product formed by the M167-μ transgene product and the endogenous V22 L chain has little or no affinity for PC.

Analysis of the Endogenous κ L Chain Expressed in the M167-id + B Cells of μ-transgenic Mice. To demonstrate that the endogenous L chain expressed in the M167-id +, PC-specific B cells of μ-transgenic mice was the product of an endogenous V24-J5 gene rearrangement, the μ/κ 207-4 transgenic mice were sorted by flow cytometry. RNA from these double-positive B cells was PCR amplified using a V16 primer (Fig. 5 A). PCRs were carried out after

Table 2.  *The IgM T15-id + Antibody Formed by M167-μ and V,22 Does Not Exhibit Good Binding to PC-BSA*

| Cell line†  | Transfected V genes  | κ5  | λ  | IgM† | V41-id | T139.2 | RdT15† | 28-5-15 | 28-4-3 | PC-BSA  |
|-------------|----------------------|-----|----|------|--------|--------|--------|--------|--------|--------|
| J558L + V167μ | +                    | −   | +  | +    | +      | −      | −      | −      | −      | −      |
| J558L + V167μ | +                    | +   | +  | +    | −      | +      | −      | −      | −      | −      |
| J558L + V22   | +                    | −   | −  | −    | −      | −      | +      | −      | −      | +      |

† The J558L-V167μ cell line produces a V41-id +, IgM-λ antibody that lacks the V22- and V24-dependent T15 and M167 idotypes and does not bind PC.
‡ When this cell line is transfected with either a V22 or V24 L chain gene, the resulting cell lines produce antibodies that express κ and the appropriate T15 or M167 idotypes, respectively.
§ The total amount of antibody bearing each of the above markers was determined in a capture ELISA in which plates coated with goat anti-μ were developed by addition of biotin-conjugated antibodies specific for the indicated isotype, allotype, or idiotype as described in Materials and Methods. The same biotin conjugates were used to develop PC-BSA-coated plates. Standard curves were generated in all assays using either the IgM T15-id + hybridoma HPCM2 or the M167-id + IgM hybridoma HPCM27 (24).
| Polyclonal rabbit anti-T15id antiserum (25).
All the V<sub>i</sub> genes expressed in PC-binding myelomas and hybridomas have been rearranged to the J<sub>5</sub>S joining segment (37). The leucine at position 96-L is a contact-determining residue for PC (42), and J<sub>5</sub>S is the only J chain coding for leucine at this position. To demonstrate that the M167-like k mRNA expressed in the μ<sup>+</sup>/M167-id<sup>+</sup> B cells represented a V<sub>24</sub> germ line gene rearranged to J<sub>5</sub>S, the mRNA from the μ<sup>+</sup>/M167-id<sup>+</sup> double-positive B cells was PCR amplified and the DNA product was sequenced from the 3' end using the C<sub>μ</sub>-PCR primer. RNA from a μ<sup>+</sup>/M167-id<sup>+</sup> hybridoma expressing the somatically mutated M167-k L chain was PCR amplified and sequenced as a positive control. The 171-bp sequence obtained was identical to the published M167<sub>i</sub> germ line sequence (34) over the last 103 bp of V<sub>i</sub> (i.e., starting with nucleotide 197, which is the last nucleotide of amino acid 61) (34), J<sub>5</sub>S, and 34 bp of C<sub>κ</sub> (data not shown). The DNA sequence obtained from the double-positive cells differed from that of the hybridoma sequence by two nucleotides (216 and 243) that are known to be somatically mutated in the M167-k L chain (34).

Idiotype Analysis of B Cells in M167 k-only Transgenic Mice. In M167 k-transgenic mice, the V<sub>24</sub> L chain should be expressed in all B cells, and endogenous k L chains should be suppressed (43). Thus, the combinatorial, binding site-specific 28-5-15 V<sub>κ</sub>1/V<sub>λ</sub>24-dependent id recognized by the 28-5-15 antibody will be generated only when the endogenous V<sub>κ</sub>1 gene is rearranged to the DFL16.1 D gene that has rearranged to a J<sub>κ</sub>1 gene. If the single genomic copy of the endogenous V<sub>κ</sub>1 gene (44) is randomly rearranged during B cell development in k-only transgenic mice as it is in normal adult mice (4-9), then one should find ~0.006% (1/16,000) of the B cells in M167 k-transgenic mice expressing the binding site-specific 28-5-15 idiotope. This estimate is based on the assumption that the mouse genome contains ~200 V<sub>κ</sub> genes, 20 D genes, and four J<sub>κ</sub> genes. On the other hand, the M167 crossreactive 28-6-20-id could be generated by the association of the V<sub>κ</sub>24 transgene product with a variety of endogenous V<sub>κ</sub> gene products.

All the data presented thus far indicate that the expression of the M167-μ transgene leads to a selective amplification of PC-specific B cells that coexpress the M167-endogenous L chain. However, the data in Table 1 suggest that PC-specific B cells are not selected in k-transgenic mice expressing the M167-k chain. Since Storb et al. (21) have demonstrated that mRNA from both the k transgene and the secretory form of an endogenous T15 family gene are expressed at high levels in many of their M167-k-transgenic mice, it was important to determine whether large numbers of B cells in these mice were also expressing these gene products as B cell surface receptors. To analyze id development in M167 k-only transgenic mice, spleen cells from 234-4 and 233-8 mice were stained with FITC-anti-μ and the biotin-conjugated anti-ids as described above. The flow cytometric data shown in Fig. 6 demonstrate that splenic B cells bearing V<sub>κ</sub>1-id, T15-id, or the M167 binding site-specific-id (28-5-15) were below the level of flow cytometric detection (A–C). However, as shown in Fig. 6 D, two-thirds of the B cells from TG<sup>+</sup> κ 234-4 mice expressed the crossreactive, 28-6-20 id on their sur-

Figure 5. PCR analysis of light chains expressed in mRNA from M167-id<sup>+</sup> B cells from μ-only transgenic mice. (A) PCR amplification using the M167, 5′ primer and C<sub>κ</sub> 3′ primer. The correct size of the PCR product is 319 bp. Even-numbered lanes were amplified after mRNA was converted to cDNA by RT, odd-numbered lanes are without RT. Lanes 1 and 2, mRNA from the MOPC-21 myeloma; lanes 3 and 4, from flow cytometry-sorted μ<sup>+</sup>/M167-id<sup>+</sup> (double-negative) cells; lanes 5 and 6, from the C47 M167-id<sup>+</sup> hybridoma line; lanes 7 and 8, from the J55L cell line transfected with the M167 k heavy chain; lanes 9 and 10, mRNA from double-positive (μ<sup>+</sup>/M167-id<sup>+</sup>) cells; and lanes 11 and 12, from a second M167-id<sup>+</sup> hybridoma, C46. (B) PCR amplification using the MOPC-21 5′ primer and C<sub>κ</sub> 3′ primer. The correct size of the PCR product is 466 bp. Even-numbered lanes, without RT; odd-numbered lanes, after RT. Lanes 1 and 2, amplification of mRNA from the double-negative cells; lanes 3 and 4, from MOPC-21; lanes 5 and 6, double-positive cells; and lanes 7 and 8, from the M167-id<sup>+</sup> hybridoma, C46.

RNA was converted to cDNA by reverse transcriptase (RT; even-numbered lanes), or without RT (odd-numbered lanes). RNA from the double-positive cells amplified a product of the correct size (319 bp) when the M167 5′ primer was used (lanes 9 and 10), proving that this population contained M167-like k mRNA. A faint PCR product was also present after PCR amplification of the double-negative mRNA (lanes 3 and 4), and RNA from both M167-id<sup>+</sup> hybridoma lines (lanes 5 and 6, 11 and 12) amplified the M167-specific product. RNA from the J55L κ-μM167 cell line and from MOPC-21 did not amplify the κ-167-specific product (lanes 7 and 8, 1 and 2, respectively). The bands seen in lane 2 are at a higher molecular weight than the κ-167 product.

To demonstrate that the μ<sup>+</sup>/M167-id<sup>+</sup> cells contained primarily M167-like k mRNA, a MOPC-21 5′ primer was also used in conjunction with the C<sub>κ</sub> 3′ primer to amplify mRNAs from this population of cells. In Fig. 5B, PCR products amplified from these primers are shown. No PCR product was seen in mRNA from the double-positive population (lanes 5 and 6, plus and minus RT, respectively) or in the M167-id<sup>+</sup> hybridoma mRNA (lanes 7 and 8). MOPC-21 mRNA amplified a PCR product of the correct size (466 bp) (lanes 3 and 4), and a band was also detectable in the PCR product of the double-negative cells on longer exposure or when more mRNA was used (data not shown).
face, while 23% of the splenic B cells from \( \kappa \)-233-8 mice expressed this idiotype (data not shown). The IgM + B cells in the bone marrow of these mice also exhibited high levels of the crossreactive 28-6-20 id; 86% of the total IgM + cells in the single 234-4 mouse analyzed, and 16 and 22% of the total IgM + cells in the two 233-8 TG - mice analyzed. TG - mice always exhibited <1% M167-id + B cells in their bone marrow. These data suggest that the \( \kappa \) transgene product is being expressed in association with endogenous \( \mu \) chains in a large number of the B cells from these M167 \( \kappa \) mice, but very few of these spleen cells express an endogenous M167-\( \mu \)H chain, which is required to produce a PC-specific antibody.

Two populations of 28-6-20 M167-id + B cells are present in the \( \kappa \)-only transgenic mice, one with high levels of id and one with low levels of id (Fig. 6 D). This difference in M167-id expression is not due to a difference in density of IgM expression on the B cells but might be due to differences in IgD expression, or it could result from the coexpression of endogenous L chains, which would lower the intensity of M167-id staining.

**Discussion**

In this paper, we have presented data suggesting that there is a preferential selection and expansion of M167-id +, PC-specific B cells in transgenic mice that express a rearranged M167-H chain gene, and that a similar amplification of M167-id +, PC-specific B cells does not occur in mice expressing a rearranged M167 \( \kappa \) transgene. In the M167-\( \mu \)-transgenic mice, 20–75% of the splenic B cells expressing the \( \mu \) transgene product also coexpress an endogenous V_{24}J_{5} L chain. The frequency of expression of this H/L chain pair is 100–500-fold higher than the 0.1% frequency expected from a random expression and association of this or any other endogenous \( \kappa \) L chain gene product with the M167-\( \mu \) transgene product. The selective expansion of M167-id +, PC-specific B cells in these \( \mu \)-transgenic mice appears to be the result of an antigen-driven, receptor-mediated process; hence, it is dependent on the expression of the transgene product on the surface of the B cell, and it only occurs when the transgene-encoded H chain pairs with a light chain that confers PC binding specificity. Thus, there is no selection of PC-specific B cells in the M167-\( \mu \Delta \)mem-transgenic mice, which cannot insert the transgene-encoded antibody into their B cell membranes (22; Fig. 4 and Table 1), and furthermore, there is no selection for T15-id + B cells even though the M167-\( \mu \) chain can associate with a V_{22}L chain to form T15-id + antibodies. The failure to selectively expand these T15-id + M167V_{\mu}/V_{22} B cells is probably due to the fact that they have little or no affinity for PC, as was demonstrated in gene transfection studies (Table 2). On the other hand, the PC-specific V_{\mu}1/V_{24}-expressing B cells are likely amplified in vivo via encounter with autologous or environmental PC in these \( \mu \)-transgenic mice.

The observations presented in this paper may provide important insights into how B cells in general are selected by antigen- or other receptor-mediated interactions from the short-lived pool of rapidly renewing bone marrow B cells.
into the long-lived stable B cell population that is present in peripheral lymphoid tissues. It would appear that the rapidly renewing bone marrow B cells, like the majority of thymocytes, are destined to die within a few days unless they encounter a ligand capable of signaling via their antigen-specific receptor. The B cells' encounter with ligand may have very different consequences depending on the developmental state of the B cell or the type of secondary signals concurrently generated by T cells or accessory cells. Thus, we have recently shown (16) that PC-specific B cells are clonally deleted in M167/κ and μ-only transgenic mice that coexpress the X-linked immunodeficiency gene, xid, whereas these same B cells are clonally expanded in normal mice. The clonal elimination of PC-specific B cells in xid mice may occur because the xid B cell is unable to respond to soluble T cell or accessory cell signals (45), or because it remains in a developmentally immature, toleralizable state much longer than a normal B cell (46). The PC-specific B cells that develop in the bone marrow of xid mice (15, 20) can be rescued from clonal deletion if they are provided with antigen and cognate T cell help (Kenny et al., manuscript in preparation).

The data presented in this paper support the idea that the PC-specific B cells that develop in normal mice are selectively expanded from the bone marrow in the long-lived peripheral B cell pool without specific immunization, and we hypothesize that this is because they can clonally expand when they encounter environmental or autologous PC in the presence of low levels of cytokines. Our observation that M167-id+ B cells are expanded 100-500-fold in μ-transgenic mice while T15-id+ B cells are not selectively expanded might also provide insight into the reason for T15-id dominance in normal mice (18, 47–51). As shown in our transfection studies, both the Vκ22 and Vκ24 L chains can associate with the M167-μ transgene product, and B cells expressing these endogenous genes should be generated at the same frequency in the bone marrow of the M167-μ-transgenic mice; however, the antibody formed by M167-Hk22L has little or no affinity for PC even though it expresses T15-id. According to our working hypothesis, B cells expressing this H/L chain pair would not be selected into the long-lived B cell pool because this selection is an antigen-dependent, receptor-mediated process. These data further suggest that an anti-id-induced network selection of T15-id+ B cells is not operating in the M167-μ-transgenic mice. In TG- and other normal mice, T15-id+ B cells may be expanded more than M167-id+ B cells because they have a higher affinity for PC (52, 53). However, as recently shown by Feeney and Thuerauf (53), the lower affinity M167-id+ B cells can dominate the immune response to PC in M167-κ-transgenic mice where the κ24 L chain should be expressed in every B cell and the κ22 L chain should be suppressed by allelic exclusion. Our flow cytometry data on M167-κ-transgenic mice demonstrate that M167-id+, PC-specific B cells are not expanded to the same degree as seen in the M167-μ-transgenic mice. In as much as the M167-id+ B cells generated in M167-μ-only and κ-only transgenic mice should have the same affinity, the lower number of PC-specific B cells in the κ-transgenic mice must be due to the infrequent generation of the rearranged endogenous M167-Vκ1 gene, whose H chain product binds PC when associated with the Vκ24 transgene product. The rearrangement of Vκ1 to DFL16.1/Jκ1 should occur in ~1/16,000 B cells. If this endogenous Vκ1 H chain always associated with the M167-κ-transgenic product to form a PC-specific B cell in the κ-transgenic mice and these B cells were expanded 100-500-fold, as they are in the M167-μ-transgenic mice, they should represent from 0.6 to 3% of the B cells in M167-κ-transgenic mice. Since this frequency of M167-id+ cells could easily be detected by flow cytometry, the probability of forming a M167-H chain must be much less frequent. Feeney et al. (52, 53) have demonstrated that most M167/M511 Vκ1 H chains have an alanine at position 96, which is needed to get good PC binding when associated with a Vκ24 L chain. The alanine at this position is generated by alternate slicing of the Vκ1 gene or by N-region diversification, and additional somatic changes also occur at the D-J junction in M167 H chains. By contrast, the T15 form of the Vκ1 gene, which lacks the alanine at position 96, is generated entirely from germ line nucleotide sequences. During early neonatal development, when N-region diversification appears to play little or no role in generating H chain diversity (54), the formation of T15-Vκ1 sequences may be greatly favored over M167-Vκ1 rearrangements. In the adult animal, the generation of M167-Vκ1 sequences will also be rare among all the variants of Vκ1-D-Jκ1 that can be generated by N-region diversification. In fact, Feeney (54) found no M167/M511 Vκ1 sequences among the 34 Vκ1 genes PCR amplified from an adult mouse spleen, and Decker et al. (55) have estimated that >10⁴ unique sequences can be generated from a single Vκ gene segment rearranged in conjunction with a single Jκ segment. It is therefore not surprising that large numbers of M167-id+ B cells are not generated from rearrangement of the endogenous Vκ1 gene in the κ-transgenic mice, although such cells exist in these mice and are activated after immunization (53). Large numbers of M167-id+, PC-specific B cells are produced in M167-μ-transgenic mice because N-region diversity does not occur during the generation of the endogenous κ repertoire, and one out of every four B cells that rearranges the germ line M167 Vκ24 gene segment will generate the Vκ24/Jκ5 L chain necessary for generating PC-specific B cells in conjunction with the M167-μ transgene product.

Even though PC-specific B cells are not generated in large numbers in the M167-κ-transgenic mice, 20–80% of B cells present in these mice express the crossreactive M167-id. In the 233-8 line of M167-κ-transgenic mice, there were in fact increased numbers of B cells that bound PC-conjugated SRBC, however, these ABC were not PC inhibitable. It is possible that these ABC have specificity for nitrophenylphosphocholine (NPPC) in as much as the Vκ24 L chain has been shown to associate with Vκ genes from the J558 and 7183 families to produce specific antibodies (56). To detect binding site-specific M167-id+ B cells by flow cytometry, there would have to be >0.1% id+ cells present in the spleens of the κ-transgenic mice. Neither the M167-id detected by hybridoma 28-5-15 nor Vκ1-id+ B cells were above the levels
seen in TG− controls. However, Storb et al. (21) were able to detect elevated levels of the secretory form of Vκ-167 mRNA in some of the M167-κ-transgenic mice using a S107 Vκ family-specific probe. Thus, VκT15 family B cells are amplified and activated in the κ-transgenic mice but not to a level detectable by flow cytometry.

In conclusion, our data suggest that the expression of the M167-μ H chain in the B cells of transgenic mice results in an antigen-specific skewing of the B cell repertoire. A disproportionate number of the B cells of such animals express the M167-id and bind to PC. By contrast, when the same transgene product cannot be inserted into the cell membrane, the B cell repertoire is unaffected; there are no M167-id+ cells detected by flow cytometry, and the PC response in the μΔmem TG− animals is dominated by T15-id+ B cells. The skewing of the repertoire in μ-only transgenic mice appears to result from an antigen-driven rather than an antidiotype-driven process. The M167-μ transgene product can form a T15-id+ antibody by associating with an endogenous Vκ22 L chain, but such an antibody does not bind to PC, and cells expressing such antibodies are not expanded in these mice. If the T15-id domination of the PC response in normal animals were based primarily upon id selection, we should have seen many PC-nonbinding T15-id+ B cells in the μ-only transgenic mice. Data from the M167 κ-only mice also support the conclusion that the repertoire selection is antigen driven. The Vκ24 L chain associates with the μ H chain of many Vκ genes to generate a M167-crossreactive id, but forms a PC-binding antibody only in the rare event that it associates with a particular alternatively spliced Vκ1-rearranged gene product that generates an alanine residue at position 96.

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