ROLE OF VARIABLE REGION GENE EXPRESSION AND ENVIRONMENTAL SELECTION IN DETERMINING THE ANTIPHOSPHORYLCHOLINE B CELL REPERTOIRE*

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Although the B cell specificity repertoire of adult individuals is extraordinarily diverse, inbred murine strains reproducibly display certain clonotypes at a relatively high frequency (1–9). Often such clonotypes dominate the in vivo and in vitro response to given antigens. The response to phosphorylcholine (PC)[1] of BALB/c mice is one such response, wherein the repertoire of responsive clonotypes includes over 100 distinct specificities (10, 11), yet one clonotype, that which is indistinguishable from the TEPC-15 (T15) myeloma protein, represents the majority of the response (5–11). The basis for such clonal dominance within an immune response is not yet fully understood. In the case of the T15 response in BALB/c mice, it has been found that B cells expressing this clonotype are present in high frequency in the spleen before immunization (7–9), which could, in part, account for its dominance within the response to PC. However, the mechanism responsible for the high frequency of expression of this clonotype and the low frequency of expression of the vast majority of other clonotypes has yet to be determined.

To assess the relative role of environmental selective processes vs. variable region gene expression in determining clonotype representations, we have assessed the PC-specific repertoire as it is expressed in bone marrow cells of the B cell lineage before their acquisition of surface immunoglobulin (sIg) receptors (prereceptor B cells) (12–16). Such cells should closely reflect the readout of expressed variable region genes since they are not affected by environmental influences that could act through positive or negative selection via the sIg antigen receptor. The results of this analysis indicate that B cells of the T15 clonotype dominate the PC-specific repertoire even at the prereceptor B cell level in BALB/c mice. Furthermore, by the analysis of prereceptor B cells in (CBA/N × BALB/c)F₁ male mice, it has been possible to demonstrate that the deficit in PC-responsive mature B cells characteristic of this murine strain (17–20), occurs after receptor acquisition, since the prereceptor B cells in the bone marrow of these mice express a normal frequency of PC-responsive and T15-expressing

cells. Finally, an analysis of the occurrence of T15$^+$ and T15$^-$ precursor B cells in bone marrow cells derived from individual leg bones of (CBA/N × BALB/c)F$_1$ male mice has demonstrated that T15 predominance is the result of the frequent recurrence of this variable region within the generative cell pool.

Materials and Methods

Mice. BALB/c, and (CBA/N × BALB/c)F$_1$ male and female mice were obtained from the breeding colony at Scripps Clinic and Research Foundation.

Antigens and Immunizations. The preparation of *Limulus polyphemis* hemocyanin (Hy) phosphorylcholine (PC)-bovine serum albumin and 3-(*p*-azophenylphosphorylcholine)-*N*-acetyl-L-tyrocylglcylglycine Boc hydrazide-Hy (PPC-TGG-Hy) has been described previously (7–9). 6–10-wk-old BALB/c mice were immunized intraperitoneally with 100 µg of Hy in complete Freund's adjuvant and boosted intraperitoneally with 100 µg Hy in saline 4 wk later. Mice were used 4–8 wk after boosting as recipients for cell transfer.

Splenic Fragment Cultures. Monoclonal anti-PC antibodies were produced in splenic fragment cultures as described previously (7–10). Briefly, 15–20 × 10^6 donor spleen cells, 3 × 10^7 bone marrow cells, or 3 × 10^7 bone marrow cells depleted of sIg$^+$ B cells (see below) were transferred intravenously into Hy-primed BALB/c recipients that had received 1,300 rad whole-body irradiation from a cesium source 1–4 h earlier. Fragment cultures were prepared from recipient spleens 16–20 h after cell transfer and were stimulated for 3 d with PPC-TGG-Hy at a hapten concentration of 10^{-6} M. Cultures were incubated at 37°C in an atmosphere of 93% O$_2$ and 7% CO$_2$. Medium was changed every 2–4 d and culture fluids were individually collected from days 9 to 28 and assayed for antibody activity.

Radioimmunoassay and Hapten Inhibition Studies. Culture supernates collected between days 9 and 28 of culture were assayed for PC-specific antibodies by solid-phase radioimmunoassay (RIA) by a method previously described (7–10). Hapten-inhibition studies were carried out by adding an appropriate volume of 10^{-3} M PC to the binding assay before the addition of the antibody (10).

Idiotype Assays. Assays for the presence of the T15 idiotype were carried out using either a hybridoma-produced monoclonal anti-T15 idiotype antibody (AB1-2), which was reported by Kearney et al. (21), or affinity-eluted rabbit anti-T15 antibody, which has been described previously (8–10). The method used for these assays has been described (7–10). Briefly, 50 µl of a 0.2 µg/ml solution of affinity-purified AB1-2 protein or rabbit anti-T15 antibody was adsorbed to wells of polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA). 25 µl of culture fluid containing monoclonal anti-PC antibodies was added and its relative ability to inhibit, stoichiometrically, the binding of 1–2 ng of ^{125}I-labeled T15 in 25 µl was assessed. Antibodies whose inhibition of binding was equivalent on a weight basis to the inhibition exhibited by the T15 myeloma protein (±20%) were considered positive for the T15 idiotype.

Idiotype Suppression. Neonatal BALB/c mice were injected at days 2 and 4 after birth with 0.2 ml i.p. of clarified ascities fluid obtained from pristane-primed mice bearing the AB1-2 hybridoma. These mice were used as donors of spleen and bone marrow cells at 2–3 mo of age.

Depletion of sIg$^+$ B Cells from Bone Marrow Preparations. sIg$^+$ B cells were removed from adult bone marrow cell preparations by a modification of the rosetting technique described by Zharhary and Klinman (14) and Walker et al. (22). In brief, 10^8–10^9 washed bone marrow cells were incubated on ice with a 5–10-fold excess of tanned, sheep erythrocytes that were glutaraldehyde fixed and coated with goat anti-mouse IgM + IgG heavy and light chain-specific antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Rosetted (sIg$^+$) bone marrow cells were pelleted by centrifugation at 4°C through Ficoll-Hypaque density gradients. The unrosetted cells at the interface were carefully removed and extensively washed. 30–50% of the input bone marrow cells were generally recovered in the interface band. These nonrosetted bone marrow cells, depleted of sIg$^+$ lymphocytes, have been termed prereceptor B cells. At least 90% of sIg$^+$ lymphocytes

were depleted by this method and the prereceptor B cell preparations contained <2% sIg$^+$ lymphocytes as assessed by direct fluorescence analysis using fluorescein-labeled anti-mouse (IgM + IgG + IgA) rabbit F(ab')$_2$ fragments (Zymed Laboratories, Burlingame, CA) using a Becton, Dickinson FACS IV (B-D FACS Systems, Sunnyvale, CA).

Results

Response of sIg$^-$ BALB/c Bone Marrow Cells to PC.   Table I summarizes the data obtained from an extensive analysis of the responsiveness to PC of cells obtained from the spleen and bone marrow of BALB/c mice. The frequency of splenic cells that gave rise to anti-PC antibody-producing clones in the fragment culture system is consistent with previous reports from this and other laboratories (7–10, 19, 20). If one assumes that 50% of injected spleen cells are B cells and assumes an efficiency for homing and stimulation in fragment cultures of 4%, as previous determined for responses to PC (9), then ~1/35,000 B cells responded in fragment culture to stimulation with PPC-TGG-Hy to yield a clone of anti-PC antibody-producing cells. Also consistent with previous reports using other antigens (14), the frequency of responsive bone marrow cells was ~40% that of spleen cells on a per injected cell basis. After removal of sIg$^+$ cells from the bone marrow population, the frequency of remaining cells responsive to PC was ~1/20 million injected cells, or 8.8% of the frequency of spleen cells. Since <2% of

TABLE I

Frequency and Idiotype of PC-responsive Cells in Various Lymphoid Cell Populations of Normal and Suppressed BALB/c mice

Source	Total No. of cells injected* (×10⁶)	Clones per 10⁶ injected cells‡	T15$^{+§}$	T15$^-$
			%	
Normal BALB/c mice[I]				
Spleen	690	0.57	62	38
Bone marrow	930	0.20	59	41
sIg$^-$ bone marrow	3,150	0.05	63	37
AB1-2-suppressed BALB/c mice[¶]				
Spleen	1,32ↄ	0.19	6	94
Bone marrow	540	0.12	14	86
sIg$^-$ bone marrow	240	0.04	50	50

* Each BALB/c recipient received 15–20 × 10⁶ spleen cells, 3 × 10⁷ bone marrow cells, or 3 × 10⁷ sIg$^-$ bone marrow cells pooled from 6–8 donor mice.

‡ Antibody-producing clones were detected by RIA of culture fluids collected from days 9–28 of culture.

§ Monoclonal antibodies bearing T15 idiotypic determinants were identified by the ability of test antibodies to inhibit the binding of ^{125}I-labeled T15 myeloma protein to microtiter wells coated with AB1-2 antibody. Monoclonal antibodies were judged positive if inhibition was equivalent on a weight basis to the inhibition obtained with unlabeled T15 (±20%) and negative if inhibition was <10% of inhibition of T15. Approximately 10% of antibodies in each group gave intermediate inhibition values and were not included in the above calculations. At least 20 monoclonal antibodies from each set, chosen at random, were analyzed except antibodies derived from sIg$^-$ cells obtained from AB1-2-suppressed mice, of which six antibodies were analyzed.

[I] Normal BALB/c donor mice were 2–4 mo old.

[¶] AB1-2-suppressed mice received 0.2 ml of clarified hybridoma ascites fluid at 2 and 4 d after birth and were used as cell donors at 2–3 mo of age.

the injected cells could have been sIg$^+$, it is apparent that the majority of responsive cells in the sIg-depleted population represented cells that had not yet acquired their immunoglobulin receptors. This conclusion was corroborated by an analysis of the isotypes of antibodies produced from stimulated sIg$^-$ cells. <10% of the monoclonal antibodies obtained included IgG subclasses. The production of only IgM and/or IgA antibodies is characteristic of immature B cells derived either from the bone marrow or fetal and neonatal cell populations (23).

Idiotypic Analysis of sIg$^-$ Bone Marrow Cell Responses to PC. The majority of BALB/c B cells responsive to PC have been shown previously to bear idiotypic determinants identical to those found on the T15 myeloma protein. Recently, a series of monoclonal hybridoma antibodies has been derived that recognize different determinants present on T15 molecules (11, 21). One of these, AB1-2, has proven extremely useful in standardizing assays for indicating the presence of markers that are diagnostic of members of the T15 family. As shown in Table I, the majority of anti-PC monoclonal antibodies derived from BALB/c spleen, bone marrow, or sIg$^-$ bone marrow cells displayed the T15 idiotypic marker. This finding for splenic B cells is consistent with previous observations (6–9). The findings presented here extend the observation of T15 dominance to the sIg$^-$ population of bone marrow cells. Indeed, the similar ratio of T15$^+$ to T15$^-$ clonal responses in all three donor cell populations indicates that the representation of idiotypes in the mature splenic B cell population may be a close reflection of the representation of idiotypes within the developing pre-B cell pool.

To verify the designation of idiotype-positive and -negative monoclonal antibodies, several of the antibodies were also tested by an idiotypic analysis using affinity-eluted rabbit anti-T15 antibodies. In all instances, this analysis confirmed the analysis using the AB1-2 antibody.

Response to PC of Cells Derived from Mice that Were Neonatally Suppressed with Anti-T15 Antibody. Previous studies from this and other laboratories (11, 21, 24, 25) have demontrated that responses of the T15 idiotype can be markedly suppressed when BALB/c mice are treated as neonates with large amounts of anti-T15 antibody. Recent studies from other laboratories (21) have demonstrated that this suppression can be obtained by treatment of neonatal mice with the AB1-2 monoclonal antibody. Additionally, these studies demonstrated that 2–3 mo after idiotypic suppression, B cell responses to PC can be observed but that these responses are deficient in antibodies of the T15 idiotype (21, 24). Table I presents the data obtained when cells were obtained from mice that were suppressed as neonates with the AB1-2 antibody. It can be seen that the overall frequency of response to PC of spleen cells and cells of intact bone marrow populations was substantially reduced in mice after treatment with the AB1-2 antibody. In both populations, most of this diminution in frequency can be attributed to a profound reduction in the frequency of PC-responsive B cells that produce antibodies bearing the T15 idiotype. Significantly, neither the overall frequency of cells responsive to PC nor the proportion of resultant antibodies that bore the T15 idiotype was substantially reduced in the sIg$^-$ bone marrow cell population of mice which were profoundly suppressed in their mature B cell populations by the antiidiotypic treatment. These findings are consistent with

the supposition that sIg^- bone marrow cells represent a population of cells that had not yet acquired their immunoglobulin receptors in situ and, therefore, would be impervious to environmental manipulation such as idiotypic suppression or, as previously demonstrated, in vivo tolerance induction (13). This finding also serves as confirmation of the assumption that the majority of responses from the sIg^- cell populations are derived from cells that have not as yet acquired their immunoglobulin receptors, as opposed to a few remaining sIg^+ cells in these preparations.

Responses to PC of sIg^- Cells Derived from (CBA/N × BALB/c)F_1 Mice. Previous studies from several laboratories (17–20, 26–28) have demonstrated a multifaceted sex-linked immunologic deficiency in CBA/N mice and F_1 male mice derived from a CBA/N parent. One of the deficiencies of these mice is an almost total absence, from the primary B cell repertoire, of cells that can respond to PC, even presented on highly immunogenic carriers (17–20). In (CBA/N × BALB/c)F_1 male mice, this deficiency includes a lack of all primary responses which bear the T15 idiotype. To assess the level at which this deficiency occurs, we examined the responses of spleen, bone marrow, and sIg^- bone marrow populations derived from (CBA/N × BALB/c)F_1 male and female mice injected into carrier-primed BALB/c recipients. It can be seen in Table II that the three cell populations derived from (CBA/N × BALB/c)F_1 female mice mounted vigorous responses to PC. The frequency of responding cells of all three subpopulations was somewhat lower than that observed for homozygous BALB/c mice and a somewhat lower proportion of monoclonal antibodies bore the T15 idiotype as assessed by the AB1-2 antibody. The response of cell populations obtained from (CBA/N × BALB/c)F_1 male mice was markedly different. Consistent with the findings of several investigators (17–20), spleen cells derived

TABLE II

Frequency and Idiotype of PC-responsive Cells in Various Lymphoid Cell Populations of (CBA/N × BALB/c)F_1 Female and Male Mice

Source	Total No. of cells injected* (×10⁶)	Clones per 10⁶ injected cells[‡]	T15[+§]	T15⁻
			%	
(CBA/N × BALB/c)F_1 Female				
Spleen	290	0.46	46	54
Bone marrow	410	0.16	42	58
sIg^- bone marrow	720	0.04	40	60
(CBA/N × BALB/c)F_1 Male				
Spleen	710	0.01	0	100
Bone marrow	630	0.04	30	70
sIg^- bone marrow	1140	0.04	38	62

* As in footnote * to Table I.
‡ As in footnote ‡ to Table I.
§ As in footnote § to Table I. At least 20 monoclonal antibodies from each set, chosen at random, were analyzed except for antibodies derived from spleen and bone marrow cells of F_1 male mice, of which 7 and 10 monoclonal antibodies were analyzed, respectively.

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from these mice displayed a very low frequency of precursor cells responsive to PPC-TGG-Hy. Only seven monoclonal antibodies were obtained and none bore the T15 marker nor was their binding to PC-BSA inhibited by 8×10^{-4} M PC. Since the vast majority of monoclonal anti-PC antibodies are readily inhibited by this concentration of PC (10), it is likely that these antibodies were not directed at PC per se.

Importantly, responses of sIg^- B cells derived from (CBA/N × BALB/c)F$_1$ male mice were similar in frequency and idiotypic composition to those obtained from sIg^- populations of (CBA/N × BALB/c)F$_1$ female mice. Indeed, an analysis of the intact bone marrow indicates that probably all responsive cells in the bone marrow of (CBA/N × BALB/c)F$_1$ male mice could be accounted for by the sIg^- cells within this population. Thus, it appears that mice bearing the CBA/N immunologic deficit have a normal capacity to generate cells potentially responsive to PC and that the environment present in splenic fragment cultures derived from carrier-primed recipient mice permits the maturation and stimulation of these cells. It is apparent that, in situ, such cells would have been eliminated late in their maturation, presumably upon acquisition of their immunoglobulin receptors.

Responses to PC of Cell Populations Derived from Individual Bone Marrows. The apparent absence of PC-specific cells from sIg^+ B cell populations of (CBA/N × BALB/c)F$_1$ male mice provides an unique opportunity to carry out a relatively unambiguous analysis of generative cell pools. For this purpose, 20 2–3-mo-old (CBA/N × BALB/c)F$_1$ male mice were sacrificed and cells prepared individually from each femur and from a pooled population of their two tibias. Each of these cell populations contained $\sim 10^7$ cells. Since no sIg^+ PC-responsive cells were likely to be present in any of these preparations, each individual preparation could be examined without prior treatment to remove sIg^+ cells. The results of this analysis are presented in Table III. In this table, each bone marrow preparation is listed individually both for the frequency of PC-responsive cells observed and the idiotype of those cells, where sufficient antibody was produced to enable a quantitative idiotypic analysis. Of the total of 60 individual preparations analyzed, only 15 yielded clones producing anti-PC antibody. Of the total of 28 clones observed, 8 could be demonstrated to be T15 positive while 14 could be demonstrated to be T15 negative. Both the overall frequency and idiotype distribution obtained from this set of experiments were similar to those obtained using large pools of sIg^- and total bone marrow cells derived from (CBA/N × BALB/c)F$_1$ male mice (presented in Table II). Assuming that these responses were derived from sIg^- precursors within these bone marrow populations, several conclusions can be made. First, it can be seen that both $T15^+$ and $T15^-$ PC-specific precursor cells were generated in each of the different bone marrow sources. Thus, the generation of T15 B cells is not solely accomplished in one generative cell pool, such as the right femur. Second, the occurrence of $T15^+$ or $T15^-$ precursor cells within individual bone marrow populations was proportionate to the expression of $T15^+$ or $T15^-$ B cells in mature (female F$_1$ controls) and immature B cell populations. Since $T15^-$ populations include >100 clonotypes (10), the relative high frequency of $T15^+$ precursor cells would seem to be the result of an unusually frequent recurrence of $T15^+$ B cell clones within the

TABLE III

PC-specific Responses from Individual Bone Marrow Isolates of (CBA/N × BALB/c)F₁ Male Mice

Mouse No.	Source*	Total responses‡	T15+‖	T15⁻	Mouse No.	Source	Total responses	T15+	T15⁻
1	RF	0	—	—	11	RF	0	—	—
	LF	0	—	—		LF	0	—	—
	T	0	—	—		T	0	—	—
2	RF	0	—	—	12	RF	1	—	—
	LF	0	—	—		LF	3	—	1
	T	1	—	1		T	0	—	—
3	RF	0	—	—	13	RF	0	—	—
	LF	1	1	—		LF	0	—	—
	T	0	—	—		T	0	—	—
4	RF	2	2	—	14	RF	0	—	—
	LF	0	—	—		LF	0	—	—
	T	3	—	3		T	0	—	—
5	RF	0	—	—	15	RF	0	—	—
	LF	2	—	1		LF	0	—	—
	T	0	—	—		T	0	—	—
6	RF	0	—	—	16	RF	0	—	—
	LF	0	—	—		LF	0	—	—
	T	3	3	—		T	0	—	—
7	RF	0	—	—	17	RF	1	1	—
	LF	0	—	—		LF	0	—	—
	T	0	—	—		T	2	—	2
8	RF	0	—	—	18	RF	0	—	—
	LF	0	—	—		LF	0	—	—
	T	0	—	—		T	0	—	—
9	RF	2	—	2	19	RF	1	1	—
	LF	0	—	—		LF	0	—	—
	T	2	—	1		T	2	—	2
10	RF	0	—	—	20	RF	0	—	—
	LF	2	—	1		LF	0	—	—
	T	0	—	—		T	0	—	—

* 20 individual 2–3-mo-old (CBA/N × BALB/c)F₁ male mice were sacrificed and cells were prepared individually from their femurs (F) and tibias (T). Cells from each femur (R, right; L, left) were injected into separate recipients and cells from both tibias were pooled and injected into a third recipient. Each of these three sources provided ~10⁷ cells.

‡ Responses are reported as the total number of fragment cultures derived from each recipient spleen containing a clone producing >1 ng of anti-PC antibody per day over two collection periods.

‖ As in footnote ‖ to Table I. In general, monoclonal antibodies that could not be designated as T15+ or T15⁻ were available in insufficient amounts for accurate quantitation of the T15 idiotype.

generative cell population. A third important finding was the appearance of both T15+ and T15⁻ cells as isolated events in any given bone marrow and the frequent expression of multiple copies of cells of a given specificity within that individual bone marrow. This finding is indicative of the fact that precursor cells of a given specificity already exist, at the prereceptor B cell level, as expanded clones, and that the cells within such clones are already committed to their ultimately expressed specificity before receptor acquisition or stimulation in fragment cultures. While the idiotypic analyses carried out in this study do not permit any conclusions as to the identity of the clonotypes of precursor cells derived from a single bone marrow, particularly with respect to those which are T15⁻, the independent segregation of T15+ and T15⁻ clonotypes is consistent

with the interpretation that sIg⁻ precursor cell clones arise as independent events in individual bone marrow populations.

Discussion

Among the most striking aspects of B cell expression is the reproducible appearance of B cells representing certain clonotypes in high frequency in all individuals of an inbred murine strain. Since such clonal predominance is superimposed on an extraordinarily diverse B cell repertoire, it presents the paradox of disproportionate representation of clonotypes within the repertoire. To address this issue, we have analyzed the response to PC of bone marrow cells in the B cell lineage before their expression of sIg. It is assumed that at the time of isolation these cells would not yet have interfaced with the environment and, thus, should be representative of the B cell repertoire as it emerges from bone marrow stem cells; it should thereby be reflective of the readout of expressed immunoglobulin variable region genes. Additionally, it is assumed that, within the context of the fragment culture system, such cells can acquire their surface receptors but do so in the presence of antigen and excess carrier-specific T cells that provide a stimulatory milieu and avoid tolerance induction of cells that would otherwise be obligately tolerance susceptible (13, 26).

Several findings attest to the validity of the assumption that the responsive cells in bone marrow populations depleted of sIg⁺ cells are truly sIg⁻ precursors to B cells: (*a*) FACS analysis and fluorescence microscopy show background levels of sIg⁺ cells in these preparations; (*b*) the responsive cells yield very few IgG-producing progeny, which is characteristic of immature cells (14, 23); (*c*) in other antigen systems, this cell population has been shown to be comprised of the vast majority of bone marrow cells that are tolerance susceptible, which is also characteristic of immature cells (12, 13); (*d*) in responses to several antigens, this population contains a disproportionately high frequency of specificities that are rare in mature B cell populations either in the bone marrow or spleen (16, 29, 30); (*e*) in previous studies, it was demonstrated that this cell population remained intact in mice that were profoundly tolerant to 2,4-dinitrophenol (13); (*f*) in the present report this cell population has been shown to remain uniquely unaffected after neonatal antiidiotypic suppression; (*g*) this population is uniquely impervious to the mechanism responsible for elimination of PC-responsive cells in mice bearing the CBA/N immunologic deficit; and (*h*) the independent segregation of T15⁺ and T15⁻ precursor cell clones in individual bone marrow preparations would likely be a characteristic unique to in situ generated cell clones.

The response to PC in mature BALB/c mice is generally characterized by the dominant expression of antibodies of the T15 clonotype both at the serum and plaque-forming cell level (5–10). In this case, clonal dominance is reflective of the fact that a majority of PC-responsive B cells express the T15 idiotype, whereas the minority of the response, which is T15⁻, represents the expression of B cells of >100 distinct clonotypes (10). The results presented in this report demonstrate that the extraordinarily high frequency of mature B cells of the T15 clonotype can be accounted for by an equally high frequency of B cells of this clonotype that emerge from the generative cell pool in the bone marrow. Thus, while these findings do not rule out idiotype-specific or antigen-specific

selection as contributors to the high frequency of T15 B cells (31, 32), they obviate the necessity of such explanations to account for the high frequency of this clonotype. It should be noted that recent investigations using polyclonal stimulation of splenic and immature bone marrow cell populations have led to similar conclusions concerning cells expressing the NP^b idiotype in C57BL/6 mice (33) and the 460 idiotype in BALB/c mice (34). It is of additional interest that PC-specific B cells of clonotypes other than T15 are also expressed at the generative cell level approximately in proportion to their representation in the mature B cell repertoire. This finding is consistent with studies of responses of sIg$^-$ bone marrow cells to the influenza hemagglutinin wherein repertoire diversity of the pre-B cell population appears equivalent to that of mature splenic primary B cell populations (15).

Since it is possible, by this experimental approach, to evaluate repertoire expression at the generative cell level, we have also been able to address the basis of the finding that mice bearing the CBA/N X-linked immunologic deficit do not express B cells in their mature primary B cell pool responsive to PC (17–20). In particular, B cells of (CBA/N × BALB/c)F$_1$ male defective mice neither respond to PC nor express the T15 idiotype in their primary B cell repertoire, whereas these specificities are abundant in mature B cells of their female littermates. The studies presented above demonstrate that the absence of PC-specific mature primary B cells in male defective mice must result from elimination of these cells after sIg expression and cannot be attributed to defects associated with generation of the specificity repertoire.

Several years ago, it was noted that one of the immunologic abnormalities associated with the CBA/N defect is an unusual degree of tolerance susceptibility of B cells obtained from defective mice (26, 27). Thus, whereas in normal and conventional mice, <10% of splenic B cells are tolerance susceptible in vitro, >50% of splenic B cells in F$_1$ male defective mice remain tolerance susceptible (26). Since PC is a ubiquitous environmental antigen, it is an interesting possibility that the absence of mature PC-specific B cells in (CBA/N × BALB/c)F$_1$ male defective mice is the result of tolerance induction by a normal environmental antigen. This would be consistent with the finding that such mice are similarly defective in their responsiveness to other known environmental antigens (reviewed in 28). This may represent a novel demonstration that tolerance induction to a normally abundant environmental antigen can be an important contributor to the shaping of the expressed B cell repertoire. Additionally, since the present experiments indicate that carrier-primed T cells can "rescue" developing PC-specific B cells and permit their stimulation, it is not unexpected that the multiple immunizations of (CBA/N × BALB/c)F$_1$ male mice with T cell-dependent forms of PC would permit the generation of secondary PC-specific B cells in these mice, as has been previously demonstrated (19).

The absence of sIg$^+$ cells responsive to PC in the bone marrow of (CBA/N × BALB/c)F$_1$ male mice has provided a unique opportunity for further evaluation of several important aspects of repertoire generation. The higher frequency of cells of the T15 clonotype, even in the generative cell pool described above, could have been the result of either (a) the expression of the T15 variable region as a unusually frequent event during immunoglobulin gene rearrangements, or

(b) the generation of the T15 variable region at a frequency that is not unusually high for clonotypes in general, but the expression of T15 as unusually large clones within the prereceptor cell pool. By analyzing independently the population of bone marrow cells obtained from each femur and pooled cells from the tibias of individual mice, we have been able to demonstrate that the former explanation is largely responsible for the high frequency of expression of T15 B cells. That is, when T15 is expressed, it is not represented as inordinately large cell clones, but rather the expression of B cells bearing the T15 idiotype is an extraordinarily frequent event during repertoire generation. Thus, the dominance of T15 antibodies in the serum of PC-immunized BALB/c mice is apparently the result of a high frequency of T15-expressing B cells within the mature B cell pool, and this high frequency of B cells, in turn, is the result of a high frequency of expression of the T15 variable region during repertoire generation.

The analysis of precursor cells obtained from individual bone marrows reveals another highly interesting aspect of B cell repertoire generation. It can be seen from Table III that, although only 1 in 4 bone marrow populations yield PC-responsive cells, the bone marrow populations that contain a PC-specific B cell often contain multiple copies of PC-specific B cells. The fact that these multiple B cells from within a given marrow are generally either all $T15^+$ or all $T15^-$ implies that prereceptor B cells exist in situ as expanded clones. Given the efficiency of cells in homing to the spleen and in stimulation in fragment cultures, it must be assumed that if the 2–3 precursor cells identified are progeny of the same clone, then such clones are the product of 5–7 cell divisions. Thus, it would appear that a considerable amount of clonal expansion may precede sIg expression. This finding is particularly interesting in light of observations of others (35, 36) that indicate that B cells do not ordinarily divide after sIg expression. Indeed, some investigations have implied that extensive division does not occur after heavy chain gene rearrangement and cytoplasmic μ expression (36). Thus, if B cells are expressed as expanded clones, it is not surprising that most clonal division antedates sIg expression. However, the expression of multiple identical sister cells of a given clone at the prereceptor B cell level would likely necessitate the identical rearrangement of light chain genes and the expression of a common light chain among each of the members of that clone. If so, then it is unlikely that light chain gene rearrangement is a totally random process during clonal expression. Alternatively, it is possible that clones are massively expanded at a time when only heavy chain genes have rearranged. In this case, any of several potential light chain gene rearrangements would yield expanded functional clones. Nonetheless, the finding of clonal expansion and commitment before sIg expression introduces the possibility that variable region specificity determination antecedes variable region gene rearrangements and that such rearrangements may be accomplished in a nonrandom, nonstocastic manner.

The findings of this report, in concert with other recent studies of pre-B cells, enable several tentative conclusions concerning the basis of repertoire expression. While it is likely that antigenic stimulation and subsequent environmental selection may alter or increase repertoire diversity and permit the expression and selection of mutations within variable region gene segments (37, 38), the primary B cell repertoire itself emerges from the generative cell pool within the bone

marrow of adult individuals as an already highly diverse population of clonotypes
(15). Additionally, clonotype distributions (such as the expression of high fre-
quency predominant specificities) that characterize the mature primary B cell
repertoire also characterize the prereceptor B cell repertoire. Thus, neither
antigenic stimulation nor idiotypic selection are required for repertoire diversi-
fication (15) or predominant clonotype expression (16, 33, 34). It also appears
that clonal expansion precedes sIg expression, so that expressed specificities are
represented by clones of multiple pre-B cells and B cells. This would imply that
environmental influences, such as tolerance and idiotypic selection, operate on
clones of B cells rather than individual B cells. Thus, the relative representation
of any given clonotype in the mature primary B cell repertoire would be a
function of both environmental selective processes operating at the clonal level
and the frequency with which a clonotype's variable regions recur within the
generative cell pool. Finally, it has been estimated that 5×10^7 B cells emerge
from the bone marrow of adult mice per day (35). If these emerging cells are
comprised of clones already expanded to $\sim 10^2$ cells, then only 5×10^5 clonotypes
may emerge per day. Since a fully mature repertoire emerges within 2 mo after
birth, this may set a limit of 10^7–10^8 clonotypes for potential primary B cell
repertoire diversity in mature individuals of a given murine strain.

Summary

To evaluate the role of environmental selective processes, as opposed to
variable region gene expression, in the determination of B cell repertoire
expression, we have assessed the phosphorylcholine (PC)-specific repertoire of
precursor cells that remain in bone marrow cell populations after the removal of
surface immunoglobulin (sIg)-bearing cells. Such cells are assumed to represent
a stage in B cell maturation before the expression of sIg, and thus at a time when
they have not as yet interfaced with environmental influences that operate
through sIg receptors such as antigenic stimulation, tolerance, or antiidiotypic
regulation. The repertoire as expressed in these cells, therefore, should reflect
the readout of immunoglobulin variable region genes as they are expressed in
progenitors to B cells. The results of these studies indicate that, as in mature
primary B cell pools of BALB/c mice, the majority of PC-responsive sIg$^-$ bone
marrow cells are of the T15 clonotype. Thus, environmental selective mecha-
nisms would not appear to be required for the high frequency of B cells of the
T15 idiotype in the primary B cell repertoire of BALB/c mice. Analysis of the
sIg$^-$ bone marrow cells in (CBA/N $\times$ BALB/c)F$_1$ male mice demonstrated that
the deficit of PC-responsive mature B cells, which is a characteristic of this
murine strain, must occur after receptor expression, since a normal frequency
of PC-responsive and T15-expressing cells is present in their sIg$^-$ bone marrow
population. Finally, these same mice were used to obtain bone marrow cell
preparations from individual leg bones, so as to permit an analysis of the
occurrence of T15$^+$ and T15$^-$ clonotypes within individual bone marrow popu-
lations. The findings from these studies indicate that T15$^+$ B cells occur as a
high frequency event within bone marrow generative cell pools. Furthermore,
bone marrow populations that are positive for PC-responsive precursor cells
often display multiple copies of such precursor cells that are exclusively either

T15$^+$ or T15$^-$. This finding indicates that clonal expansion of cells within the B cell lineage apparently occurs before immunoglobulin receptor acquisition.

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