B-1 cell origins and V_H repertoire determination

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We show here that antigen selectively stimulates the progressive increase of B cells expressing a particular V_H gene in the B-1 repertoire. However, the frequencies of cells expressing a series of other V_H genes in antibodies with the same antigen specificity remain constant in the same animals. To establish context for these findings, we first review several key studies that bear on the origins of B-1 cells and the mechanisms that shape the B-1 repertoire.

Distinct Origins of B-1 and B-2 cells

Much has been said and written about the origins of B-1 cells and whether they constitute a distinct lineage. We originally proposed that progenitors for B-1 and B-2 are distinct, and hence that these B cells belong to two distinct lineages[1-6]. Wortis, Haughton, and colleagues[7, 8] later argued that the specificity of the receptor (Ig) expressed by a given B cell determined whether it would differentiate into a B-1 or B-2 cell. Variants of this argument persist today despite consistent evidence, gathered by our laboratory and others, that directly demonstrates that the progenitors for B-1 and B-2 cells are distinct[5, 6, 9-16].

There is little question that under some circumstances, cells whose phenotype classifies them as bone marrow derived follicular B cells can be stimulated to assume phenotype(s) that would classify them as B-1 cells. However, these findings do little more than testify to the plasticity of B cell phenotypes and perhaps the mechanisms that define these phenotypes. Basically, the issue is not whether a B-2 cell can be stimulated to adopt a B-1 phenotype but whether such phenotype shifts occur normally and reflect the origins of substantial numbers of B-1 cells. The collective data from several studies, including our own, argue strongly against this latter hypothesis. These studies, outlined below, show that B-1 and B-2 cells differentiate from distinct progenitors that arise at different points during the ontogeny of the immune system.

Independence of B-1 and B-2 progenitors. Two seminal studies, by John Kearney[12], Miguel Marcos[17] and their colleagues, have shown that progenitors for B-1 cells exist at early fetal sites that do not contain progenitors for B-2 cells. Although these studies did not isolate or phenotypically characterize the B-1 progenitors at these sites, the functional existence of these progenitors independent of progenitors for B-2 cells argues strongly for the existence of separate B cell lineages.

Both the Kearney and the Marcos studies are based on transfer of undisrupted embryonic tissue (fetal omentum in the Kearney paper; splanchnopleura in the Marcos

paper) to a protected site under the kidney capsule in SCID mice. Arguments could be made, therefore, that only the B-1 progenitors can function under these conditions. However, a note added in proof to the Kearney paper[12] removes this objection by stating that the same result (B-1 but not B-2 reconstitution) was obtained by transferring a single-cell suspension of cells harvested from omental tissue. Since single-cell suspensions from fetal liver readily reconstitute B-2 cells in similar (SCID) recipients, the demonstration that omental cells transferred in the normal manner reconstitute B-1 but not B-2 cells provides clear evidence that progenitors for B-2 cells are not present in the fetal omentum.

Our studies, which complement these findings (or *vice versa*), show that although functional progenitors for B-2 cells are abundant in adult bone marrow, functional progenitors for B-1 cells are rare at this site[5, 6, 18]. In essence, transfers of adult bone marrow to lethally-irradiated recipients readily reconstitute B-2 cells but reconstitute only a small population of B-1 cells, largely composed of B-1b (CD5⁻) cells. In contrast, transfers of fetal liver (which usually includes a portion of the fetal omentum) to irradiated or SCID recipients fully reconstitutes both B-1 and B-2 cells. Thus, since functional progenitors of B-1 cells can fully reconstitute the B-1 population in transfer recipients, the minimal reconstitution obtained in typical recipients indicates a paucity of B-1 progenitors in adult bone marrow.

This evidence falls short of conclusively demonstrating that functional progenitors for B-1 cells are rare in adult bone marrow because the transferred bone marrow could either contain an inhibitor of B-1 progenitor differentiation and/or could lack cells necessary to support this differentiation. To test these possibilities, we prepared and mixed single-cell suspensions from bone marrow and allotype-
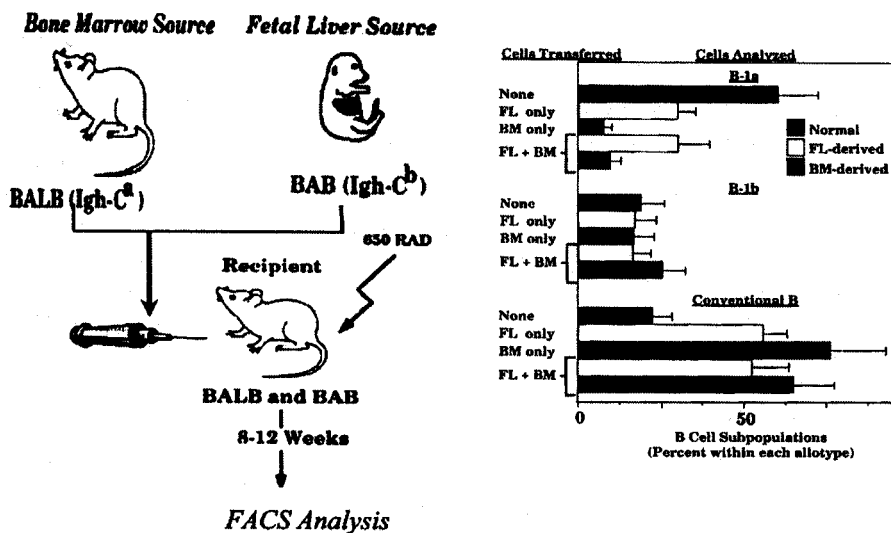

Fig 1. Developmental potential for B cell progenitors for from fetal liver and bone marrow is the same whether transferred alone or together into irradiated recipients[5]. B-cell reconstitution in cotransfer recipients is evaluated by calculating the IgH^a allotype (BM-derived) B-la, B-lb, and B-2 (conventional B) cell populations as percent of total IgH^a B cells and by calculating IgH^b allotype (FL-derived) B-la, B-lb, and B-2 cell populations as percent of total IgH^b allotype B cells. Analyses were done 8-11 weeks after transfer.

congenic fetal liver and transferred the mixture to lethally irradiated recipients (Fig. 1)[5]. As controls, we transferred each cell suspension alone to similar recipients.

Comparison of the B cells that developed in recipients 8 weeks (or more) after transfer demonstrates that B-1 cells derived from fetal liver develop equivalently in the mixture recipients and the recipients of fetal liver alone, thus ruling out the presence of an inhibitor of B-1 development in bone marrow (Fig. 1). Furthermore, the minimal B-1 population derived from bone marrow is equivalent in the mixture recipients and recipients of bone marrow alone, demonstrating that the failure to reconstitute B-1 cells from bone marrow is not due to lack of support for B-1 progenitors. Thus, with the caveat that these mixture experiments were done with fetal liver and adult bone marrow that express different allotypes, we conclude that progenitor activity for B-1 cells is selectively lacking (although not entirely absent) in adult bone marrow.

Importantly, our studies demonstrated progenitors for both B-1 and B-2 cells in fetal liver from embryos as early as day 12. In contrast, Kearney *et al*, recovered progenitors for B-1 but not B-2 cells from day 13 fetal omentum[12]. Therefore, bridging the results from the two studies, progenitors for B-2 cells are already present in fetal liver at a time when they are not detectable in fetal omentum.

In fact, the B-2 progenitors detected in murine fetal liver may be anatomically separated from the B-1 progenitors. Solvason, Kearney and co-workers have pointed out that suspensions of fetal liver cells always contain the endoderm-derived cells that form the bulk of the liver and the mesoderm-derived liver capsule that is contiguous with the omentum. Thus, B-2 progenitors may reside within the liver while B-1 progenitors reside in the liver capsule and related mesoderm tissues.

Together, these studies demonstrate that progenitors for B-1 and B-2 cells are distinct and hence that B-1 and B-2 cells as belong to separate developmental lineages. This conclusion can still accommodate the idea that B-2 progenitors or their progeny contribute to the B-1 compartment during adulthood. Cells that express certain Ig receptors, for example, could differentiate from B-2 progenitors but be triggered to express (or mimic) the B-1 phenotype. This pathway can never be ruled out entirely. However, data from two types of studies put a very close limit on the extent to which it is used:

1) Multiple studies in which adult bone marrow and mature B-1 cells were co-transferred to irradiated mice collectively show that the transferred B-1 cells reconstitute a normal-sized B-1 population that persists indefinitely by self-replenishment. Although a small bone marrow derived B-1 population (mainly B-1b) often develops shortly after transfer, its size, like the size of the majority B-1 population derived from the transferred B-1 cells, remains constant thereafter. Thus, once stabilized, the B-1 population in irradiated recipients is neither replaced nor progressively increased by bone marrow derived cells.

2) Feedback regulation studies show that *de novo* development of B-1 cells is blocked by the presence of mature B-1 cells during the first 3-4 weeks of life[19, 20]. In neonates treated from birth until 4-6 weeks of age with anti-IgM antibodies that remove all endogenous B cells, early introduction of allotype-congenic B-1 cells (that do not react with the treatment antibody) selectively blocks recovery of endogenous B-1 cells when the treatment is terminated. Similarly, in

allotype heterozygotes treated neonatally with anti-allotype antibodies that remove all B cells that express one of the IgM allotypes, recovery of the depleted B-1 population is selectively blocked by the remaining B cells. In both cases, the B-2 population recovers completely and small numbers of B-1 cells (mainly B-1b) may also recover. However, the size of the recovered B-1 population remains constant throughout life, i.e., cells from bone marrow do not add to it.

Collectively, the findings discussed above constitute a solid body of data indicating that B-1 and B-2 cells are derived from different progenitors and have different developmental patterns. Comparative studies of the B cell developmental pathway support this two-lineage model of B cell development. Differential expression of at least two genes distinguish these developmental pathways (PLRLC-myosin-like light chain and MHC I-A [21-23]). Terminal deoxynucleotidyl transferase (TdT) gene expression also distinguishes B cell progenitors in fetal liver and adult bone marrow[24], indicating precommittment to independent development pathways (even if, as has been proposed[7, 8], low TDT predisposes to expression of the B-1 phenotype). Finally, studies by Hardy, Hayakawa and colleagues indicate that B-1 and B-2 progenitors have dramatically different mechanisms for determining which V_H genes ultimately appear in the respective mature B cell repertoires[25].
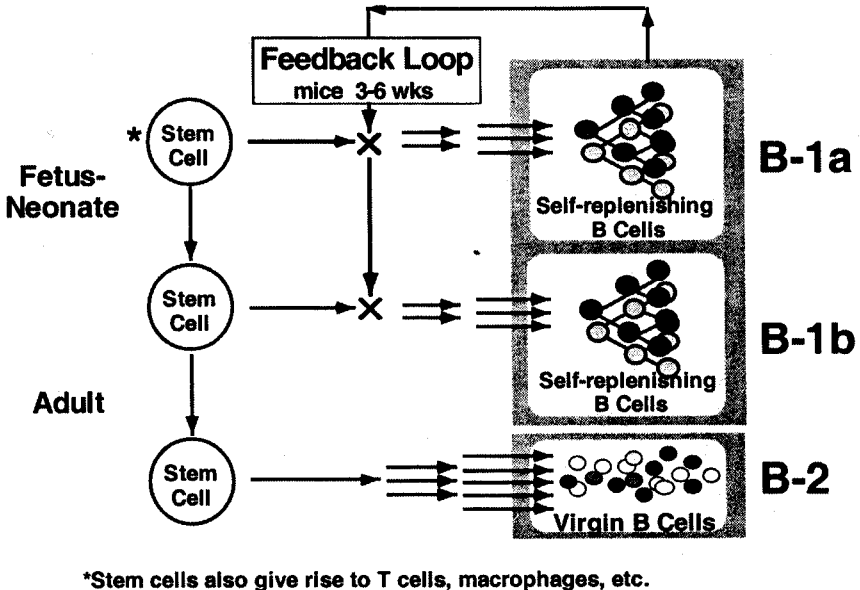

Fig 2. Three B cell lineages: B-1a and B-1b are found in the same locations and are phenotypically similar. However, CD5 is expressed on B-1a but not B-1b. B-2 ("conventional") B cells have a strikingly different phenotype and are distributed differently from B-1 and B-1b.

A third B cell lineage. Current data actually define three murine B cell lineages (Fig. 2), two of which (designated B-1a and B-1b) are similar enough to be treated collectively for most purposes as B-1 cells [26]. B-1a, which normally comprise the majority of B-1 population, express CD5. B-1b do not express detectable surface CD5 but share most of the other properties of B-1a cells, including self replenishment, sensitivity to feedback regulation and localization to the peritoneal and pleural cavities. Nevertheless, studies in which B-1a and B-1b were sorted and transferred to irradiated recipients demonstrate clearly that mature B-1a and B-1b cells are committed to replenish only their respective populations[27].

B-1a also differ from B-1b in that B-1b are somewhat more efficiently reconstituted by bone marrow transfers and tend to recover somewhat better after neonatal depletion in feedback regulation studies[19, 20]. Furthermore, at a functional level, B-1a and B-1b express different antibody repertoires[28], respond to different cytokines and switch to different Ig isotypes[1], i.e., B-1a respond to IL-5[29]and tend to spontaneously produce IgG3, IgG2a and IgG2b[6] while B-1b respond to IL-9 and tend to spontaneously produce IgE and IgG1 [30].

Antigen Selection in the B-1 repertoire

The antibody repertoires produced by B-1 and B-2 cells contain mutually exclusive specificities for antigens[31, 32] and also show differences in antibody variable region (V_H) usage[28]. In perhaps the best studied difference between these repertoires, a significant fraction of B-1 cells (5-15%) produce antibodies that bind phosphatidyl choline (PtC) while B-2 cells that produce antibodies specific for this antigen are not found in normal mice[33].

Antibodies that bind PtC, a ubiquitous membrane phospholipid found in both mammalian and bacterial membranes, are natural antibodies. PtC binding cells, detectable by FACS as cells that bind fluorochrome-labelled liposomes[34], are present at normal numbers in germfree mice[35, 36]. Furthermore, the frequency of PtC-binding cells B cells is not altered by injection of PtC antigen into normal mice[37, 38]. Consistent with this evidence, which suggests that cells producing anti-PtC antibodies play an important role in innate immunity, J. Chen and colleagues[39], using genetically altered mice that cannot secrete IgM antibodies, have shown that injection of anti-PtC antibodies protects against bacterial sepsis induced by cecal ligation and puncture.

The immunoglobulin heavy chain variable (V_H) gene families that encode anti-PtC antibodies are mainly restricted to three V_H gene families ($V_H 11$, $V_H 12$ and $V_H Q52$)[33, 40]. These three genes collectively encode the bulk of the anti-PtC antibodies produced in all mouse strains tested. The BALB/c and C.B-17 strains, however, differ with respect to which V_H gene family dominates the anti-PtC repertoire (Fig. 3). For example, BALB/c anti-PtC antibodies are predominantly encoded by the $V_H Q52$ family, while C.B-17 anti-PtC are predominantly encoded by the $V_H 12$

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[1] Note that although there is a common tendency to think that B-1 cells do not undergo isotype switching, B-1 cells as a whole can switch to produce all of the advanced isotypes.

family. Both strains produce V_HQ52 and V_H12 anti-PtC; it is only the representation of these antibodies that differs[40].

To determine the V_H representation in the anti-PtC repertoires in these strains, we used multiparameter FACS analysis and single-cell RT-PCR and sequencing[40]. We co-stained cells with PtC-liposomes and a combination of monoclonal antibody reagents detecting IgH allotypes, B cell surface markers and antibodies encoded by V_H11 or V_H12[41, 42] (The latter reagents were kindly supplied by Geoffrey Haughton and colleagues). Among cells expressing antibodies encoded neither by V_H11 nor V_H12 (collectively designated as "Other"), single-cell RT-PCR and sequencing shows that the V_HQ52 family predominates. In fact, among the large proportion of cells that express neither V_H11 nor V_H12 in BALB/c mice, roughly 70% express V_HQ52 and many of these express a single member of the V_HQ52 family (MMU53526, V_HOx-1)[40].
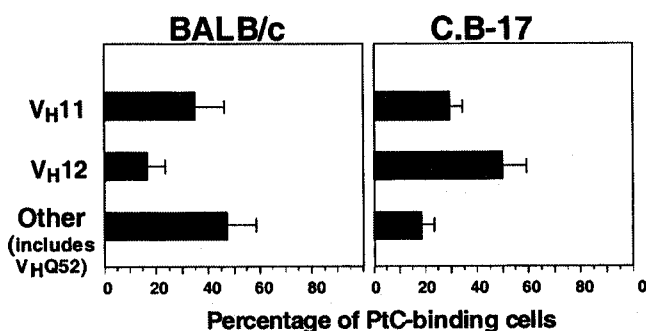

Fig 3. V_H gene family repertoire of PtC-binding B cells. Peritoneal B cells analysed from 5 month old animals. Bars represent the V_H family frequency among PtC-binding cells determined by flow cytometry for individual mice and averaged for 6-12 mice. Standard deviations are shown for each bar. V_H genes other than V_H11 and V_H12, which are individually recognized by monoclonal antibody FACS reagents, are designated "Other".

C.B-17 is a BALB/c IgH allotype congenic strain generated (by Michael Potter) by mating and successively backcrossing a (BALB/c x C57BL/Ka)F_1 hybrid to BALB/c while selecting for maintenance of the C57BL-derived (IgH^b) chromosome region. Thus, this congenic strain carries the IgH^b allotype chromosome region on the BALB/c genetic background. The difference in V_H predominance between the anti-PtC repertoires of BALB/c (IgH^a) and C.B-17 (IgH^b) is linked to the IgH chromosome region (confirmed by backcross analysis, Wilshire et al, in preparation).

The association between the IgH allotype and V_H expression patterns in the BALB/c and C.B-17 anti-PtC repertoires is maintained in the F_1 hybrid between these strains. Staining with monoclonal anti-allotype reagents to distinguish the anti-PtC V_H repertoire encoded by each parental chromosome in the F_1 demonstrates that the IgH^a and IgH^b allotype B cell repertoires in the F_1 are comparable to their corresponding parental repertoires. For example, comparison of the "Other" (mainly V_HQ52) anti-PtC data for 5 month old animals in figures 3 and 4 shows that in the F_1, "Other" predominates among IgH^a PtC-binding cells as it does in the

BALB/c parental animals. Furthermore, IgH^b V$_H$12 anti-PtC increases with age in the F$_1$ as it does in C.B-17 (see below).

Analysis of V$_H$ expression during the course of development of the F$_1$ mice from 3 weeks to 8 months of age demonstrates a striking difference between V$_H$12 anti-PtC encoded by the IgH^b chromosome region (derived from C.B-17) and all of the other predominant V$_H$ genes in the anti-PtC repertoire. With the exception of IgH^b V$_H$12, the anti-PtC V$_H$ genes (including IgH^a V$_H$12) represent a fixed percentage of peritoneal B cells at 3 weeks that remains fixed over time (Fig. 4). Only IgH^b V$_H$12 selectively increases with age.
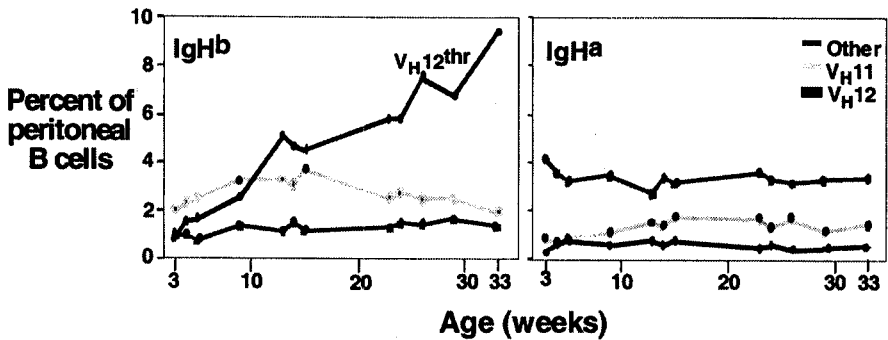

FIG 4. Only cells expressing the V$_H$12 anti-PtC encoded by the Igh^b chromosome (V$_H$12^{thr}) increase with age in (C.B-17 x BALB/c)F$_1$ hybrid mice. **Left panel:** V$_H$ encoded by IgH^b chromosome derived from C.B-17 parent. **Right panel:** V$_H$ encoded by the IgH^a chromosome derived from the BALB/c parent. V$_H$ expression on PtC-binding cells was determined by multiparameter FACS. Each point represents the average of 5-20 mice.

Collectively, studies with F$_1$ mice described above demonstrate that the difference between the parental strain anti PtC repertoires is not due to the presence of different extracellular antigens or cell surface molecules that operate to select the V$_H$ genes. In the F$_1$ mice, selection should operate equally on V$_H$ encoded by both parental chromosomes. However, we find that the V$_H$ expression pattern encoded by each of the chromosomes in the F$_1$ mimics the pattern in the corresponding parental animal. Therefore, selection due to the presence of different antigens cannot account for the difference between the IgH^a and IgH^b anti-PtC repertoires.

In fact, the selective increase in IgH^b V$_H$12 traces to an amino acid difference between IgH^a and IgH^b V$_H$12. Comparison of the V$_H$12 gene sequence from BALB/c and C.B-17 mice demonstrates that there is a single amino acid difference at codon 21 in the framework 1 (FR1) region[40, 43]. There is an alanine residue encoded at this position in BALB/c (V$_H$12^{ala}) and a threonine residue encoded in C.B-17 (V$_H$12^{thr}). Importantly, C57BL/10-related mice, including the "2a4b" mice studied by Haughton and colleagues[44], also carry V$_H$12^{thr}. However, C57BL/6J mice, which have the same IgH^b constant region as C57BL/10 and C.B-17, nonetheless express the V$_H$12^{ala} allele found in BALB/c. Six additional silent nucleotide differences between V$_H$12^{ala} and V$_H$12^{thr} indicate these genes are well separated in evolution.

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In contrast to the age-dependent increase in cells expression V_H12^{thr} anti-PtC antibodies, B cells expressing V_H12^{thr} antibodies that do no bind PtC do not increase in frequency over time (Fig. 5). Thus, the specificity of V_H12^{thr} for PtC (and/or related antigens) is required for the selective increase of cells expressing these antibody molecules. In other words, expansion of the V_H12^{thr} anti-PtC population is depend-
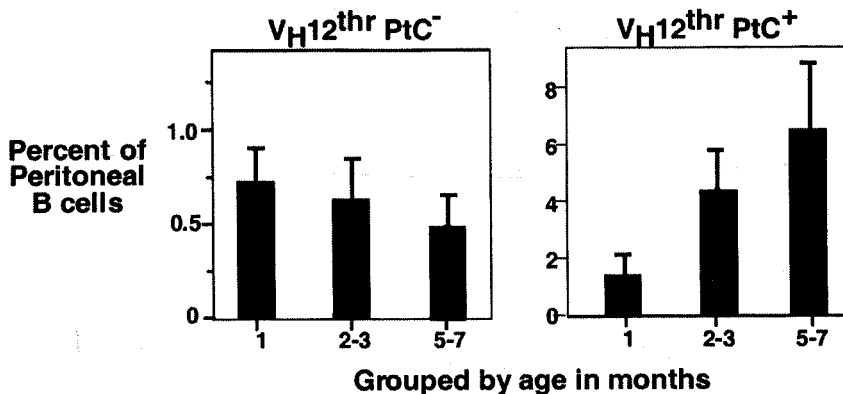

FIG. 5. Cells expressing V_H12^{thr} antibodies that do not bind PtC do not increase in frequency with age. Flow cytometry was used to determine the percent of B cells in C.B-17 mice which express antibodies encoded by V_H12. **Left panel:** Percentage of B cells that *do not* bind PtC. **Right panel:** Percentage of B cells expressing V_H12 encoded antibodies that *do* bind PtC. Points represent the average and standard deviation of the percentage of B cells from 5-20 mice each.

ent on its antigen specificity.

In principle, clonal expansion could explain the V_H12^{thr} anti-PtC findings. However, since 24/25 sequences obtained from V_H12^{thr} PtC-binding cells that were FACS-sorted from 5-month old F_1 mice were unique, and since the frequency of V_H12^{thr} increases with age in all animals tested, these findings rule out both clonal expansion and neoplastic or pre-neoplastic events underlying the increased V_H12^{thr} anti-PtC frequency.

CDR3 sequences associated with V_H12^{thr} anti-PtC could also, in principle, explain the antigen-dependent selective expansion of IgH^b cells expressing this V_H gene. However, sequence data shows that there are no systematic differences between the IgH^a and IgH^b CDR3 sequences, e.g., IgH^a and IgH^b encoded anti-PtC antibodies both have the typical 10/G4 CDR3 region sequences (10 amino acid length with glycine in the fourth position) shown by Mercolino et al[45] to be characteristic of V_H12 anti-PtC antibodies. Thus, although the CDR3 region must contribute to the specificity of these antibodies for PtC, there is no evidence that it is responsible for selective expansion of the IgH^b V_H12. The substitution of threonine for alanine at codon 21 in FR1 therefore emerges as the single defining difference between IgH^a and IgH^b V_H12 anti-PtC.

Since codon 21 occurs in a beta-pleated sheet that is not in proximity to any CDR region (antigen binding site), it seems an unlikely candidate to alter antigen binding avidity. However, several studies indicate that this region may help to increase

binding avidity for unusual antigens[46]. For example, human $V_H 3$ encoded antibodies bind to Staphylococcal Protein A using the FR1 region as well as the CDR2 and FR3 regions (reviewed in [47]). Our studies in which B-1 cells were stained graded amounts of PtC-liposomes are also consistent with the idea that the alanine/threonine difference alter avidity. At the same PtC-liposome concentration, $V_H 12^{thr}$ B cells bind more PtC-liposomes than $V_H 12^{ala}$ cells (Fig. 6). Thus, we propose that the single amino acid difference between the IgH^a and IgH^b allotype encoded $V_H 12$ antibodies alters avidity for PtC and results in the age-dependent increase in $V_H 12^{thr}$.
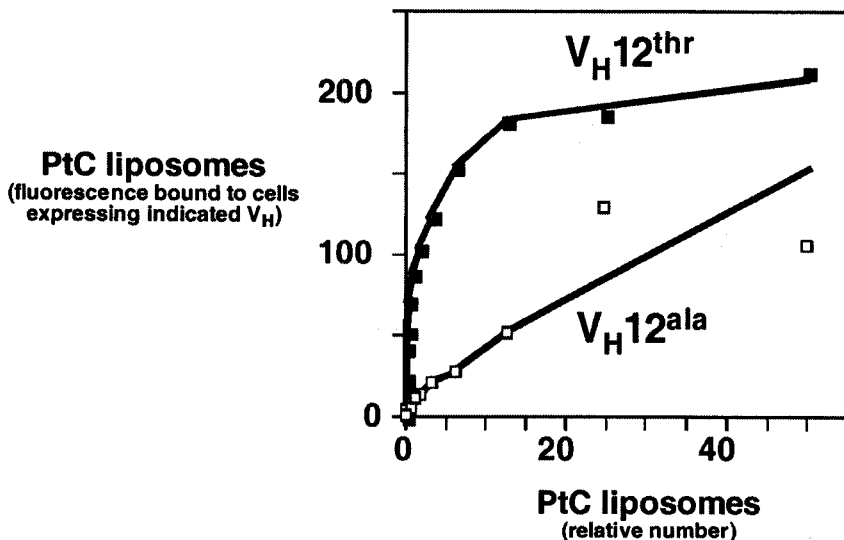

Fig 6. Cells expressing $V_H 12^{thr}$ bind PtC-liposomes more efficiently than cells expressing $V_H 12^{ala}$. Peritoneal B cells from BALB/c ($V_H 12^{ala}$) and C.B-17 ($V_H 12^{thr}$) were co-stained with monoclonal antibodies that recognize $V_H 12$ and graded numbers of fluorescent PtC-liposomes. The figure shows liposomes bound (median liposome-derived fluorescence) at each "concentration" of liposomes for the two cell types.

Our findings confirm the age-dependent increase in $V_H 12^{thr}$ detected by Clarke and colleagues, who showed that the vast majority of the $V_H 12$ rearrangements isolated from cDNA libraries of adult "2a4b" mice (which express $V_H 12^{thr}$) are productive (rather than non-productive) rearrangements[48]. These investigators reasoned that antigen selection accounts for the increased frequency of the productive rearrangements. In our studies, we show that $V_H 12^{thr}$ antibodies that bind PtC increase with age while $V_H 12^{thr}$ antibodies that do not bind this antigen remain constant. Thus, we conclusively demonstrate that the increase in $V_H 12^{thr}$ is antigen-driven.

Finally, our studies show that except for cells expressing $V_H 12^{thr}$, V_H gene frequencies among PtC-binding B cells within the peritoneal B cell population remain constant from 3 weeks until at least 8 months of age. Thus, although antigen sometimes selectively stimulates continued expansion of components of the B-1 repertoire, the repertoire as a whole does not change dramatically with age.

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