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Repetitive usage of immunoglobulin V_H and D gene segments in $CD5^+$ Ly-1 B clones of (NZB × NZW)F_1 mice

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The usually small Ly-1 B cell population is markedly increased in older mice by expansion of certain clones. This results in a cellular picture very similar to human B chronic lymphocytic leukemia. Here we report a molecular analysis of the immunoglobulin gene rearrangements of the Ly-1 B cell populations in (NZB × NZW)F_1 females. We find that (i) the number of clones found in the peritoneum (a major tissue source of Ly-1 B cells) decreases with age till mono- or biclonality is common by ~6 months, (ii) many clones from different mice show the same size rearrangements at both the Ig heavy and light chain loci and (iii) the IgH rearrangements found in a clone isolated from the spleen of one mouse are a subset of those found in the peritoneum of the same mouse, implying migration occurs from the peritoneum to the spleen. Molecular cloning and sequencing of the IgH rearrangements from the peritoneal clones of one B/W mouse revealed that all productive rearrangements used the identical unmutated V_H and D elements joined to different J_Hs. Indeed, two VDJ_H4 rearrangements were recovered which were identical but for six junctional (N region) nucleotides. The conservation of V_H and D segment usage in the rearrangements of these Ly-1 B cell clones could indicate some strong selective pressure for clonal expansion (for example antigen selection) operates via the immunoglobulin molecules of these cells. Southern analyses of other (NZB × NZW)F_1 mice with this cloned V_H and the usage of the same or similar V_H genes among a number of Ly-1 B origin tumors in other mouse strains indicate the generality of this repetitive V_H gene usage in individual mice.

Key words: Ly-1 B cells/V_H usage/clonal populations/FACS

Introduction

The molecular basis for the generation of diversity among immunoglobulin genes is now well understood (reviewed in Tonegawa, 1983; Yancopolous and Alt, 1986). Diversity at the heavy chain locus is due in part to combinations of V, D and J_H segments being used, with the extent of this contribution being determined by the large number of each of these segments in the germline. Using even conservative estimates of the number of germline V_H segments (Brodeur *et al.*, 1984), the probability that two IgH chains will use the same V_H and D elements is exceedingly low (1:1000–1:5000). This assumes a stochastic process of rearrangement, which may not be true at all stages of development (Yancopolous *et al.*, 1984; Perlmutter *et al.*, 1985; Schroeder *et al.*, 1987) but does appear to be the case in the adult (Dildrop *et al.*, 1985; Manser *et al.*, 1984). To date measurement of the V_H gene segment repertoire has used retroviral transformed lines (Yancopolous *et al.*, 1984), B cell hybridomas (Perlmutter *et al.*, 1985) and bulk splenocytes (Schroeder *et al.*, 1986). The recent description of the Ly-1 B cell lineage with its unique functional characteristics (reviewed in Herzenberg *et al.*, 1986) raises the possibility that V_H gene segment usage will be different in this population relative to the conventional B cells.

As a first step in analysis of the V_H repertoire of cells of the Ly-1 B lineage we set about characterizing the IgH rearrangements of the $CD5^+$ oligoclonal B cells found in (NZB × NZW)F_1 (B/W) females (Wofsy and Chang, 1987; Stall *et al.*, 1988). While these hyperplastic Ly-1 B cells appear in older mice of all strains so far examined, their development is accelerated in the B/W females such that they are readily detected by Southern and FACS analysis in peritoneal cells by 3 months of age (Stall *et al.*, 1988). The clones continue to proliferate until, later in the life of the mouse, they are the predominant cell type in the peritoneum, and appear in the spleen, lymph nodes and peripheral blood. Preliminary Southern analysis of the Ig loci of clonal populations isolated from the peritonea of different B/W female mice revealed a striking restriction in the sizes of J_H and J_κ hybridizing rearrangements (Stall *et al.*, 1988). Although identity cannot be concluded from comigration on a Southern, this result made it unlikely that the IgH rearrangements of these Ly-1 B cells represent a random sampling of all possible IgH rearrangements. The question then becomes what is the nature of these immunoglobulin rearrangements such that a restricted size distribution is observed and does this have any bearing on the ontogeny of these Ly-1 B cell clones.

In this report we describe the molecular cloning of all of the detectable IgH rearrangements in the Ly-1 B clonal populations of a B/W female mouse. While this revealed that only normal Ig gene segments were involved, sequence analysis of the IgH rearrangements gave the surprising result of a complete bias in V_H and D usage: only a single V_H and a single D element was used in all three of the productive rearrangements. Three of the four J_H elements were used, and the N region also differed among rearrangements. Further only a single light chain rearrangement is detected in these cells. Because of this very restricted H and L chain segment usage a considerable degree of homogeneity in structure is expected for the Ig molecules on the surface of the clonally expanded $CD5^+$ B cells in this mouse. Southern analysis of other B/W mice using this cloned V_H as a probe showed repetitive usage of this or a highly related V_H in the Ly-1 B clones of a second mouse. The relation-

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ship between repetitive usage and the expansion of these clones is discussed.

Results

Southern analysis reveals a restriction in rearrangements at both the IgH and IgL loci

We used Southern analysis of DNA derived from total peritoneal cells of mice from three age groups to determine the relationship between the extent of clonality in B/W females and age. DNA from mice 2 months of age or less had only the germline band visible when probed with either heavy chain or light chain J region probes (Stall *et al.*, 1988; and data not shown), indicating that the B cells in the peritonea of these mice have rearranged randomly, at least at the level of detection by Southern. When we examined mice of 3 months of age or greater, however, it was apparent that several clones were being expanded in each mouse. Furthermore, as shown in Figure 1, in the older mice the number of Ig rearrangements decreased; that is, the mice appear to become more clonally restricted as they age. This clonal dominance could arguably be due either to an intrinsic growth rate advantage of certain clones within each mouse, or to some selective process acting from outside the clones to either enhance or reduce the growth rate of each clone.

Examining the rearrangements at both the heavy and light chain loci in the greater than 6-month-old mice shows that several of the bands are the same in different mice. This comigration of fragments does not prove segment identity but it is unlikely that we would observe this level of similarity if the clones detected on these Southern blots were using V_H and V_L gene segments randomly selected from all those available. One explanation for this repetition is that the IgH bands in general, and the shared bands in particular, represent common DJ_H joins. Since several of the D elements are in roughly the same position on the same size *Eco*RI restriction fragments (Kurasawa and Tonegawa, 1982), any of them joining to the same J_H will produce a fragment of about the same size. While such an argument cannot be used at the κ chain locus, it does raise the possibility that the J_H bands we see are all incomplete or non-productive rearrangements and that the expressed alleles are randomly distributed throughout the V segment repertoire. The arguments against this interpretation are that firstly some of the bands we have cloned and sequenced are complete VDJ_H joins (see below) and, secondly, both the heavy chain and the κ chain rearrangements are clonal implying that expansion occurred after the completion of both heavy and light chain gene segment rearrangement.

Ly-1 B clones found in the spleen are a subset of those in the peritoneum

In previous studies we have documented the tissue distribution of these clonal populations by FACS analysis. They are first detected in the peritoneum and only later in the spleen, lymph nodes, bone marrow and peripheral blood (Wofsy and Chang, 1987; Stall *et al.*, 1988). On the basis of these results we proposed that the clonal populations were initially expanded in the peritoneum and then migrated to the other organs, rather than the various organs were seeded independently and developed separate clones. As a means of testing this proposal of peritoneal derivation, we have analysed molecularly the Ly-1 B cell clonal population in the spleen of one B/W female. In this particular animal, the

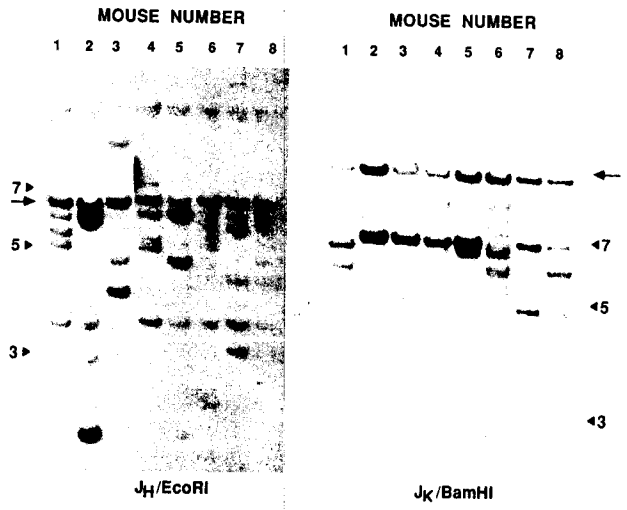

Fig. 1. Southern blot analysis of the immunoglobulin loci of peritoneal cells from B/W females. DNA extracted from the peritoneal cells of greater than 6-month-old B/W females was digested with either *Eco*RI or *Bam*HI and, after electrophoresis and transfer, probed with the J_H region and the J_κ probe respectively (see Materials and methods for a description of the probes used). Samples from the same mouse are indicated by the number above the lane. The positions of germline fragments were obtained from B/W liver DNA samples and are indicated by arrows. Mol. wt markers (in kb) are indicated by the numbers alongside the autoradiographs.

Ly-1 B clone represented ~10% of all B cells in the spleen (Figure 2). The splenic Ly-1 B cell population was isolated by FACS sorting using the gates indicated in the figure. A control population of conventional B cells was also sorted from the same spleen cell suspension, with the sort parameters again indicated by a box in Figure 2. DNA was extracted from these sorted cells, from unseparated spleen and from peritoneal cells from the same animal, digested with *Eco*RI and analysed by Southern blot for IgH rearrangements. As one can clearly see from the autoradiograph in Figure 2, the rearrangements detected in the sorted splenic clone are a subset of those in the peritoneum. Of the three non-germline J_H hybridizing fragments visible in the total peritoneal sample, only the doublet at 4.4 kb is seen in the clonal population sorted from the spleen. In the total spleen sample we can detect a faint 4.4 kb doublet and a much more intense germline band, reflecting the low ratio of the clonal population to the non-B cell splenocytes. In both the total spleen cell lane and the adjacent sorted conventional B cell lane faint, slightly dispersed bands are visible. These bands are most probably due to the contribution of common DJ_H joins from many different cells, as described above. The faint germline band visible in the sorted B cell lane is presumably derived from the IgM negative cells included in the left hand edge of the sort window.

These results support our hypothesis that, at least in this one mouse, the clonal populations develop primarily in the peritoneum and then migrate to the other lymphoid organs. Interestingly enough, only one pair of bands visible in the peritoneal sample is found in the splenic clone. This segregation of two bands in what is apparently one phenotypic clone might indicate that these bands represent the two IgH alleles of one clone of the oligoclonal population. The finding that the two bands of this segregating 4.4 kb doublet are a potentially functional VDJ_H and an incomplete

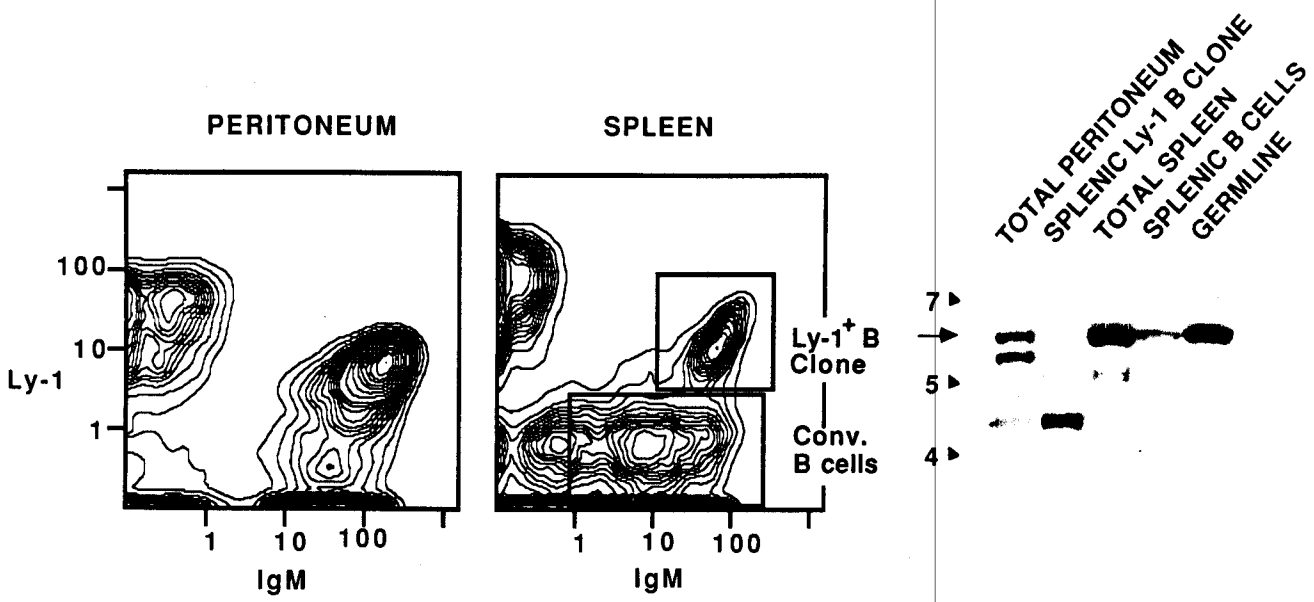

Fig. 2. Two color FACS analysis, sorting and Southern analysis of the Ly-1 B cell clone from the spleen and peritoneum of a single B/W female. Cells were prepared, stained and analysed by standard procedures. FACS results are presented as 5% probability plots. Gates used for sorting spleen cells are indicated by boxes. High mol. wt DNA, extracted from each of the cell fractions, was digested with *Eco*RI and hybridized with the J_H probe after transfer. Position of the germline J_H containing fragment is indicated by the arrow and shown in the lane marked L. Mol. wt markers are in kb.

DJ_H rearrangement (Figure 3) is consistent with this proposal.

Sequencing shows repetitive use of the same V_H and D segments in different clones

In order to determine the exact nature of the IgH rearrangements detected in the peritoneal clonal populations, we cloned the predominant J_H hybridizing fragments from one B/W mouse. The mouse used to provide cells for this study is the one whose spleen was analysed in Figure 2. That is, the IgH rearrangements which we have cloned are shown in the Southern blot of that figure. J_H hybridizing clones were isolated from size enriched λgt10 libraries as described in Materials and methods. That the cloned fragments were representative of the bands seen by Southern was determined by obtaining several clones with identical restriction maps from one of the size enriched libraries and/or by using the 5′ segment of a molecular clone to detect the clonal IgH band on a Southern. From the 4.4 kb doublet we recovered two distinct types of clones identified by restriction mapping, hereafter referred to as 4.4.1 and 4.4.2. Each corresponds to one band of the doublet and both involve J_H4 joinings. For the 5.4 kb band, however, we recovered two clones, either of which could have given rise to the clonal band because restriction mapping showed that the same element had been rearranged to J_H1 in one clone and to J_H2 in the other. These clones will be referred to as 5.4.1 for the J_H1 and 5.4.2 for the J_H2. Since these two types of clone were recovered in equal numbers from the library and since a probe derived from the 5′ end of both hybridizes to the clonal band, we cannot say which of these two actually gives rise to the original 5.4 kb band. Surprisingly, we also observed that the 5′ probe from the 5.4.1 and 5.4.2 clones detected the lower rearrangement of the 4.4 kb doublet and that the restriction map of the rearranged element of these clones

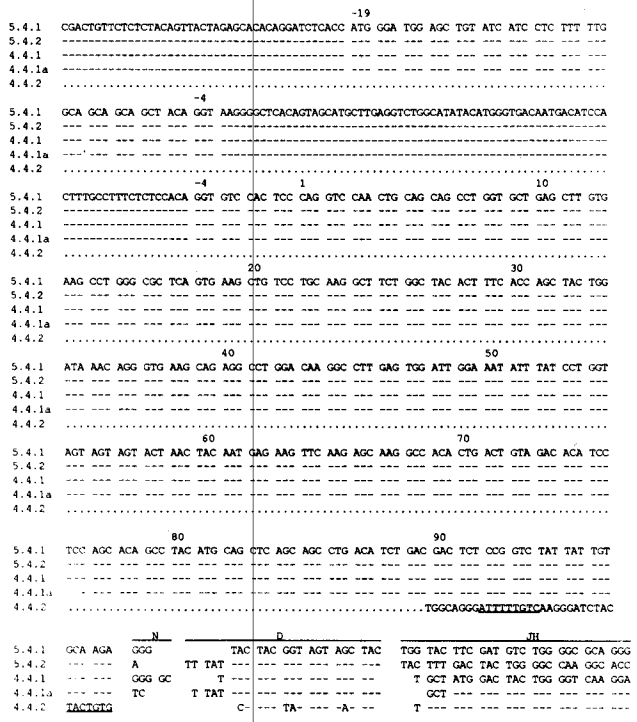

Fig. 3. Nucleotide sequence of the IgH rearrangements cloned from the Ly-1 B cell clones of one mouse. A dash indicates identity and a dot is undetermined. Numbering is by the codon. The N and D regions are as indicated and the consensus heptamer/nonamer of the DJ_H4 rearrangement is underlined.

was identical to that of the 4.4.1 rearrangement, except that the 4.4 kb band was due to a J_H4 joining.

Sequencing of these cloned rearrangements revealed several remarkable things. First, clones 5.4.1, 5.4.2 and

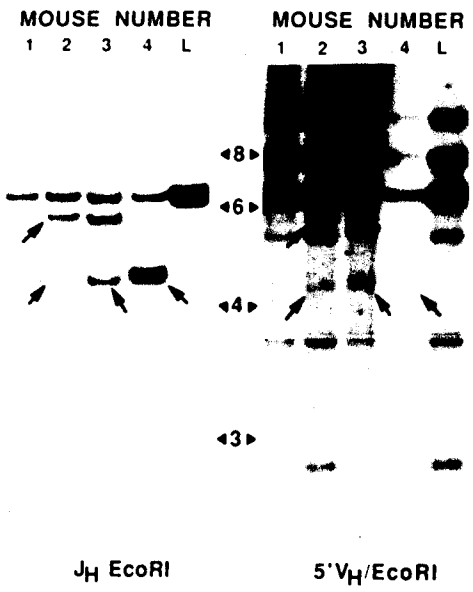

Fig. 4. Comparative Southern analysis of Ly-1 B cell peritoneal clones with J_H and 5′ V_H probes. *Eco*RI digested DNA from the peritonea of the indicated mice were probed with either the J_H probe or a fragment located 5′ of the cloned V_H coding sequence (see Materials and methods). Non-germline 5′ V_H hybridizing bands and their corresponding J_H hybridizing bands are indicated by arrows. The same lane number in the two panels indicates digested peritoneal DNA from the same animal except in lane 4, which is DNA from spleen cells from a mouse reconstituted with the Ly-1 B clone from which all the rearrangements were cloned. The lower intensity of the V_H hybridizing bands in lane 4 relative to its J_H bands is due to a difference in the amount of DNA loaded.

4.4.1 did in fact involve the identical V_H element (Figure 3). This V_H has no exact homologue published as yet, but it is 95% similar to V_H 186.2 (Bothwell *et al.*, 1981), a Group I member (Dildrop, 1984). Second, these three rearrangements all use the identical D element, DFl16.1 (Kurasawa and Tonegawa, 1982), each however with a slightly different junction point (Figure 3). Third, all of these VDJ_H rearrangements are potentially translatable and have the correct reading frame for splicing to the μ constant region. Fourth, sequencing of four independent clones from the VDJ_H4 band (i.e. the 4.4.1 type) showed no nucleotide differences throughout the VDJ_H segment, implying that there was no somatic diversification during the expansion of this clone. Comparison of the VDJ_H junctions of these four clones to that of a fifth independent clone of the 4.4.1 type, however, did reveal differences in its N region sequence between V and D, and different recombination sites at both the V to D and D to J_H4 junctions. This clone is labeled 4.4.1a in Figure 3. This meant that what appeared by Southern as an homogeneous J_H hybridizing band contributed by a single clone was, in fact, produced by the unequal contribution of at least two clones, differing from each other by only six nucleotides which results in two amino acid differences between the two clone types. The majority 4.4.1 type is Gly Ala at positions 99 and 100, while the minor 4.4.1a type is Ser Tyr at these positions. The single base difference at the DJ_H junctions of these two rearrangements does not result in an amino acid difference in the deduced protein sequence. The 4.4.2 clone corresponding to the upper band in the 4.4 kb doublet was shown by sequence to be a DJ_H4 join involving a D element which is probably DSp2.7 (Kurasawa and Tonegawa, 1982). As noted above, only the 4.4 kb doublet is found in the spleen of this mouse (Figure 2), but we are currently unable to state whether this includes both the 4.4.1 and 4.4.1a rearrangements.

The clonal rearrangements of a second mouse show repetitive use of the cloned V_H or a highly related V_H

In order to get an indication of how often the V_H gene segment which we have cloned is used in these Ly-1 B cell clones and how often clones within the one mouse repeatedly use this V_H in combination with different J_Hs, we analysed several mice by Southern using a fragment 5′ of the cloned V_H as a probe. DNA extracted from the peritoneal cells of seven 6-month-old B/Ws was probed with the 5′ flanking probe and, in parallel, with the J_H intron probe. Data are shown for three of these mice, two in which we detected usage of the cloned V_H and a representative negative mouse. The 5′ V_H probe detects three predominant germline bands of 9.5 kb, 7.5 kb and 6.3 kb and three faint cross-hybridizing bands (Figure 4, lane L, 5′ V_H panel). These germline bands are visible in all of the peritoneal samples (lanes 1–3, 5′ V_H panel), reflecting the contribution of peritoneal T cells, macrophages, etc. The positive control, shown in lane 4, is DNA derived from the spleen cells of a B/W mouse reconstituted with the clone found in the spleen of the mouse in Figure 2. Consequently this clone should contain the sequenced VDJ_H4 and DJ_H4 rearrangements. J_H intron hybridization shows the germline 6.2 kb band and the expected 4.4 kb doublet (Figure 4, lane 4, J_H panel). A parallel sample probed with the 5′ V_H fragment shows the three germline V_H bands and a single rearranged band at 4.4 kb (Figure 4, lane 4, 5′ V_H panel), corresponding to the VDJ_H4 rearrangement. Of the test mice shown, numbers 2 and 3 clearly show rearranged 5′ V_H hybridizing bands that also hybridize with the J_H intron probe (indicated by the arrows in Figure 4). Indeed in mouse number 2 there are two 5′ V_H hybridizing bands that have corresponding J_H hybridizing bands, one at 4.4 kb and the second at 5.4 kb corresponding to J_H4 and J_H1 joins respectively, indicating that the same V_H or one of three highly related J558 V_H segments is rearranged in two of the Ly-1 B cell clones of this one mouse. Thus even though only a small proportion of the IgH rearrangements of the Ly-1 B clones utilize the cloned or a highly related V_H, we are still able to detect repetitive use of this V_H in an individual mouse. It would therefore appear that the repetitive usage of the same V_H in the different clones of the one mouse is not uncommon.

Discussion

The development of a leukemia usually results from expansion of a single malignant cell. This view is strengthened by the almost routine finding of only monoclonal lymphomas. In some instances the original clone has undergone diversification by somatic mutation of its Ig genes during expansion (Cleary *et al.*, 1988), but we are unaware of confirmed examples of oligo- or biclonal B cell tumors. In this report we have described how in a Ly-1 B cell leukemia clonality is reached by the ability of one or several clones to outgrow the other cells in an originally multiclonal population. This process of clonal reduction in the peritoneal

Ly-1 B cells was shown by Southern analysis of the Ig heavy and light chain loci. As such, we have seen that young B/W mice are polyclonal since we see no individual rearranged bands by Southern analysis, while in older mice the number of clones per mouse has decreased to one or two (for example, see mice numbers 2 and 3 in Figure 1). While the developmental data we have presented are based on observations made in unrelated mice at these different ages, we are confident that repetitive sampling from a single mouse over the same time span would produce identical conclusions about the process of clonal development.

This clonal reduction can be explained as a consequence of the characteristics of the population from which these cells are drawn, namely the Ly-1 B cells. Hayakawa *et al.* (1985, 1986) have previously noted that the Ly-1 B cells are replenished from Ly-1 B precursors in the adult mouse, not from bone marrow B cell precursors, since transfer of sorted peritoneal (IgM positive) Ly-1 B cells along with IgH allotype congenic adult bone marrow into an irradiated recipient results in the lifelong reconstitution of Ly-1 B cells of only the peritoneal Ly-1 B allotype. The relevant implication from these experiments is that the total Ig repertoire of the Ly-1 B cells is established by the time the mouse reaches adulthood, although the relative frequency of clones may change. The only specificities and rearrangements found in the population are those present at the time when the IgM negative precursors disappear, with the caveat that the V_H gene segment usage could change if V_H gene segment replacement (Reth *et al.*, 1986; Kleinfield *et al.*, 1986) occurs in Ly-1 B cells *in vivo*. This has not been demonstrated. Consequently the population consists of a finite number of clones, and not a random representation of all possible rearrangements, as occurs with bone marrow derived conventional B cells since they are continuously replenished from IgM negative precursors (reviewed in Osmond, 1986).

Since in any population of cells there will be a distribution of growth rates, certain clones will predominate after a large number of cell divisions provided that growth rate is a clonal property. As the clone expands, the possibility of it undergoing a neoplastic or hyperplastic transformation presumably increases. In this view increased clonal restriction with age would be a consequence of the longevity and self-replenishment rate of clones in the Ly-1 B population. One might therefore expect to find restricted clonality in the Ly-1 B cells of all mice, their development and dominance being simply a function of age. In fact, we have seen exactly such a phenomenon in the peritonea of old mice from 'normal' inbred strains (Stall *et al.*, 1988). Clonality is observed in B/W females by around 3 months of age while in a BALB/c congenic strain it is not seen until around 12 months of age.

The observation of repetitive usage of the same V_H and D elements in the Ly-1 B clones of one mouse is not consistent with these clones being chosen at random from the population but rather with their being selected on the basis of their immunoglobulin. One hypothesis to explain the development of these clones is that a selective force such as antigenic stimulation acts on these cells. Clones could be preferentially expanded by repeated or continual exposure to antigen and subsequently transformed to an antigen independent state. The antigen could be from either a common environmental source, such as an infectious agent, or an endogenous antigen and given the apparent prediliction of Ly-1 B cells for autoantibody production (Hayakawa *et al.*, 1984; Herenberg *et al.*, 1986), this may be a more likely source in the B/Ws. Furthermore, the difference between strains in the age at which clones become detectable may be related to the permissiveness of autoantibody production the NZB related strains. Many Ly-1 B cells actively produce autoantibodies (Hayakawa *et al.*, 1984; Herzenberg *et al.*, 1986), a process that is presumably highly regulated in non-autoimmune mice. In B/W mice, however, the same constraints on autoantibody production do not seem to exist, a situation that results in the B/W mice dying early of the complications arising from autoimmune disease (Theofilopoulos and Dixon, 1985). The greater responsiveness of B/W mice to self antigens may explain the more rapid development and selection of the CLL like Ly-1 B clones.

Our data also show that repetitive use of the same V_H with different J_Hs in the Ly-1 B cell clonal populations may happen quite often. These results show that the V_H described in this report (or a closely related V_H) is able to detect three of the 28 J_H hybridizing bands in the Ly-1 clones of seven B/W mice. Two of these bands are in the same mouse, separated by a distance equal to that between J_H1 and J_H4 (see Figure 4). Thus while this V_H is unable to detect all of the rearrangements in the Ly-1 B clones it does detect a surprisingly high proportion. This result implies that only a small number of V_H segments are used in these clones. Pennell *et al.* have recently reported the V_H gene sequences for several of the Ly-1 B CH lymphomas (Pennell *et al.*, 1988)—a group of spontaneously arising tumors which are idiotypically cross-reactive and have a restricted set of specificities (reviewed in Haughton *et al.*, 1986). The authors report finding a significant bias in V_H usage with only five segments represented in the 10 sequences. While the predominant type is an anti-bromelain treated MRBC associated V_H (Reininger *et al.*, 1987), the CH12 nucleotide sequence is 95% similar to ours (Pennell *et al.*, 1988). This small set of V_H segments to a large extent may define the V_H usage in the Ly-1 B cell clones.

Similarly, Rajewsky *et al.* have reported the partial V_H sequences of several Ly-1 B cell hybridomas which were derived from the transfer of adult peritoneal cells into neonates (Rajewsky *et al.*, 1987). These authors made the remarkable observation that in three instances the same V_H was used in combination with different D and J_H segments in peritoneal and splenic derived hybridomas. The repetitive V_H usage in these hybridomas and the homogeneous distribution of IgM on the surface of the transferred cells (Forster and Rajewsky, 1987) suggests that these results are derived from Ly-1 B cell clonal populations. This would suggest that even at a relatively early age in 'normal' mice, strong selective pressure can be exerted on the Ly-1 B cell population such that the V_H gene repertoire is greatly influenced. This implication is strongly and independently supported by the data presented in this report.

Materials and methods

DNA extraction and Southern blotting

DNA was extracted from peritoneal cells by standard methods (Maniatis *et al.*, 1982), digested with the appropriate restriction endonucleases according to the conditions recommended by the manufacturer (Biolabs), fractionated by electrophoresis through 0.7% agarose gels and immobilized by transfer to nitrocellulose membranes (Southern, 1975). Hybridizations

were carried out as described (Stall *et al.*, 1988) with the J segment probes described below. Probes were labelled by random hexamer priming (Feinberg and Vogelstein, 1983) to a specific activity of $\sim 10^8$ c.p.m./μg.

DNA probes

The J_H region probe, pJ11, consists of a 2 kb *Bam*HI/*Eco*RI fragment containing the J_H3 and J_H4 gene segments subcloned into pBR322. The J_κ region probe is a 1 kb *Xba*I/*Hin*dIII fragment derived from the $J_\kappa - C_\kappa$ intron. The 5′ V_H probe is a 1.5 kb *Eco*RI/*Sac*I fragment located ~ 1 kb 5′ of the V_H coding sequence.

FACS analysis and sorting

Preparation of cells, antibodies, staining, FACS analysis and sorting were as previously described (Parks *et al.*, 1984; Stall *et al.*, 1988).

Animals

B/W mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained at Stanford.

Cloning and sequencing of Ig V_H segments

Eighty micrograms of genomic DNA from the peritoneal cells of the relevant mouse were digested to completion with *Eco*RI and size fractionated by agarose gel electrophoresis. Those fragments between the sizes of 5.2 and 5.6 kb and 4.2 and 4.6 kb were electroeluted and ligated to λgt10 arms which had been previously digested with *Eco*RI and dephosphorylated (Stratagene). The resultant ligation mixture was then packaged *in vitro* to produce recombinant phage. Approximately 100 000 and 300 000 primary recombinant plaques were screened from the 5.4 kb and 4.4 kb libraries respectively by probing with the J_H probe. The inserts from J_H positive phage were subcloned into the SP65 plasmid vector (Promega) to facilitate exhaustive restriction mapping. Those V, D and J_H containing restriction fragments were further subcloned from each of the SP65 subclones into the pTZ sequencing vectors (Pharmacia) and subjected to nucleotide sequencing using the dideoxy chain termination method (Sanger *et al.*, 1980). All fragments were sequenced in both directions to obtain unambiguous results, and the rearrangements from all positive plaques were sequenced.

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