GENOTYPIC ANALYSIS OF B CELL COLONIES BY IN SITU HYBRIDIZATION

Stoichiometric Expression of Three V_H Families in Adult C57BL/6 and BALB/c Mice

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Diversity in the antibody repertoire is generated by the fusion of five genetic elements: V_H, D, J_H, and V_L and J_L to form the variable regions of the active immunoglobulin (Ig) heavy- and light-chain genes, respectively (1, 2). The combinatorial possibilities for the known V-region gene segments could generate a repertoire of at least 10^7 specificities.

Evidence from studies of hybridomas and virally transformed cell lines suggests that the expression of V-region gene segments may be stochastic in the adult mouse (>2 wk) (3, 4), but biased in the fetus and neonate (5, 6). The implied developmental program may be significant in determining the quality of the mature immune system (7).

Here we report that the method of B cell cloning on filter paper discs (8) can be used to census populations of normal B lymphocytes for V_H gene family expression. By this method, splenocytes plated onto filter paper discs along with thymocyte feeder cells are activated by lipopolysaccharide (LPS) to proliferate and differentiate, forming pure colonies of antibody-secreting daughter cells immobilized within the paper (8–10). Each colony then, represents a single founder B cell and contains sufficient Ig-specific message for detection by in situ Northern hybridizations. Our results demonstrate that, for three representative V_H families, V_H X-24, -Q52, and -J558, expression within populations of splenic lymphocytes from C57BL/6 and BALB/c mice is similar; the expression of each family among LPS-inducible B cells is proportional to each family's size, as estimated by Southern analysis (11). These data, beyond creating a detailed description of V_H expression in adult mice, provide direct evidence that the basis of specific humoral immunity is indeed a process of random genetic combinatorics.

Materials and Methods

Lymphocyte Cloning on Filter Paper Discs. The technique of cloning mitogen- or antigen-reactive B lymphocytes on filter paper discs and methods for the phenotypic and genotypic

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analysis of colonies have been described in detail (8, 9). Briefly, colony-forming precursor cells are plated at low densities ($\leq 10^6$ splenocytes/disc) and cultured for 5–6 d with Ig^- thymic feeder cells and appropriate concentrations of mitogen or antigen. Inducible precursor cells are stimulated to proliferate and differentiate into pure colonies (clones) of antibody-secreting daughter cells immobilized within the disc's matrix of cellulose fibers. Colonies are firmly adherent and may be visualized directly by microscopy or indirectly by immunoblotting, in situ labeling and in situ Northern hybridization (8, 9). Replica immunoblots and hybridizations are efficient, permitting multiple phenotypic (10) and genotypic (8) analyses of individual colonies.

Mice. C57BL/6J, BALB/cJ, and BALB/cByJ (used only for analyses of V_H X-24 and -Q52) mice were purchased (The Jackson Laboratories, Bar Harbor, ME) and maintained at the University of Texas Medical Branch Animal Care Center through four to six generations of randomized mating. Donors of splenic lymphocytes were age- (within 2 wk) and sex-matched adults (14–36 wk). Thymocyte donors were sex-matched young mice (5–8 wk).

Cell Lines. Five cell lines were used to demonstrate V_H specificity. The fusion-cell line P3-X63-Ag8.653 (P3) has lost the ability to express endogenous immunoglobulin heavy and light chain genes (12). The Ig-producing lines B1-8 (μ; λ_1) and TEPC-15 (α; κ) secrete antibodies that use the J558 and S107 V_H families, respectively (13, 14). A line of TEPC-15 adapted to propagation in vitro was generously provided by Dr. J. Cerny. The 28.23 and 28.120 hybridoma lines secrete IgM using V_H Q52 (28.23) or V_H X-24 (28.120) (D. H. Schulze, manuscript submitted for publication).

Media. Cells were cultured in RPMI-1640 medium (K.C. Biologicals, Lenexa, KS) supplemented with 10% (vol/vol) FCS (Hyclone, Logan, UT), 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO), 2 g/liter Na_2HCO_3 (Sigma Chemical Co.), and penicillin and streptomycin (50 U/ml and 50 μg/ml, respectively) (Flow Laboratories, McLean, VA). A washing medium (WM)[1] was prepared by diluting culture medium 1:10 with RPMI-1640 medium.

Mitogen. LPS (*E. coli* 0127:B8; Difco Laboratories, Detroit, MI) was prepared by dialysis against distilled water, lyophilization, and reconstitution to ~10 mg/ml in HBSS. This stock LPS solution was filter sterilized and used at final concentrations of 10–16 μg/ml.

Filter Paper Discs. Whatman 54 filter paper discs (8.26-cm diam; Whatman Paper Division, Clifton, NJ) were used. Each disc was keyed in pencil and notched, ensuring correct orientation of sequential hybridizations. Discs were sterilized by autoclaving. Before culture, sterile filter paper discs were placed numbered-side up into petri dishes (100 × 200–mm; Falcon Labware, Oxnard, CA) and washed in 5–10 ml of WM. After 20–30 min, the WM was removed by suction.

Preparation of Splenocytes and Thymocytes. Spleens and thymuses were taken aseptically from mice killed by cervical dislocation. Single-cell suspensions were prepared by standard methods, and the cells were washed twice in cold WM. Thymocytes were then resuspended to 5×10^7 cells/ml in an ice-cold rinse medium (RM) of equal parts HBSS and RPMI-1640, while splenocytes were resuspended in cold culture medium to 2×10^4 cells/ml.

Thymocytes were depleted of contaminating B cells by treatment with sterile rabbit anti–mouse Ig serum (Cedarlane Laboratories, Hornby, Ontario) and complement (C) (Low-Tox-M rabbit C; Cedarlane Laboratories). Routinely, washed thymocytes were cooled on ice for 15 min and cold anti-Ig serum was added to a final dilution of 1:50. The suspension was mixed and held on ice for 1 h. The cells were then washed twice in cold RM and resuspended to the same volume in RM containing fresh adsorbed C (final dilution, 1:15). The suspension was mixed and incubated at 37°C for 1–1.5 h. Residual Ig^- thymocytes were recovered after two washes in WM and were resuspended to 6×10^6 cells/ml in culture medium. Generally, 25–35% of the starting thymocytes are killed by this treatment.

Culture Method. 5 ml of culture medium containing splenocytes (10^5 cells) were pipetted evenly over each washed filter paper disc, and the cells were allowed to settle for

[1] *Abbreviations used in this paper:* RM, rinse medium; WM, wash medium.

20–30 min. Next, 5 ml of culture medium containing B cell–depleted thymocytes (3×10^7 cells) and LPS (20–32 μg/ml) were gently added. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 5–6 d.

V_H Family- and C_μ-specific DNA Probes. Hybridization probes were the gifts of Drs. F. Alt (Columbia University, College of Physicians and Surgeons, New York), (pNP.B4, specific for V_H J558 [15], and pμ8, specific for the μ constant region [13]), S. Riley (Scripps Clinic and Research Foundation, La Jolla, CA) (pV_H X-24, specific for V_H X-24 [16]) and R. Riblet (Medical Biology Institute, La Jolla, CA) (pV_H Q52N, specific for V_H Q52 [11]). Plasmids were digested with restriction endonucleases as described (above), and the appropriate fragments were electroeluted from agarose gels. Eluted fragments used in hybridizations were nick-translated to specific activities of $\sim 8 \times 10^7$ cpm/μg DNA. The specificity of each probe was verified in hybridizations to genomic Southern blots (11).

In Situ Hybridization to Fixed B Cell Colonies. On day 5 or 6 of culture, medium was removed from each petri dish by gentle suction and 5 ml of 10% neutral-buffered formalin (37% formaldehyde solution [Sigma Chemical Co.] diluted 1:10 in 0.29 M $NaH_2PO_4 \cdot H_2O$ and 0.46 M Na_2HPO_4] were gently added. Discs were fixed for 5 min at room temperature and then washed twice with PBS diluted 1:10 with distilled water (0.1 × PBS). Washed discs were air-dried and immediately prehybridized for ≥3 h at 43°C. Hybridization followed in solutions containing $\sim 2 \times 10^5$ cpm/ml of denatured, nick-translated V_H- or C_μ-specific probes. Prehybridization and hybridization followed the methods of Wahl et al. (17). Discs were hybridized for 2 d at 43°C and subsequently washed three times (5 min) at room temperature in 2 × SSC (0.3 M NaCl and 0.03 M sodium citrate) containing 0.1% SDS, and once (15 min) at 43°C in 0.1 × SSC/0.1% SDS. After washing, discs were air-dried and autoradiographed at −70°C with intensifying screens. Colonies of ≥100 cells produce a good signal after 3–6 d of exposure (8).

Discs were rehybridized to a second DNA probe by the same procedure after washing for 1.5 h at 70°C in 0.1 × SSC/0.1% SDS to reduce the original signal.

Calculation of Frequencies of V_H Family Expression. The frequencies of B cell colonies expressing particular V_H families were determined by sequential hybridizations (V_H followed by C_μ). In all cases, V_H^+ colonies formed a congruent subset of C_μ^+ colonies. The frequency of colonies expressing a V_H family (as percentage of total C_μ^+ colonies) was calculated as 100 × (number of V_H^+ colonies)/(number of C_μ^+ colonies).

Results

Specificity of IgV and C_μ Probes. To ensure the specificity of the molecular probes, each nick-translated fragment was hybridized to an Eco RI digest of BALB/c genomic DNA that had been transferred to nitrocellulose paper. The pattern of hybridization and numbers of hybridizing bands agreed with the results of Brodeur and Riblet (11) (data not shown). Specificity was confirmed in hybridization analyses of characterized cell lines. Fig. 1 demonstrates hybridization with V_H and C_μ probes to colonies of two Ig-producing lines, B1-8 and TEPC-15, and the nonproducer fusion-cell line, P3. V_H probes recognize only the appropriate hybridoma lines. The C_μ probe hybridizes to the IgM producer, B1-8, but not to TEPC-15 (IgA) or P3 (Fig. 1).

Reduction of Ig^+ Thymocytes. Although the frequency of Ig^+ thymocytes is low, $1–5 \times 10^{-5}$ (18), as many as 1.5×10^3 contaminating B cells would be expected among the plated thymocyte feeder cells. These contaminating cells may be reduced ≥100-fold by treatment with anti-Ig and C (data not shown). Virtually all colonies arise from splenic B lymphocytes.

Efficiency of Sequential Hybridizations. Discs hybridized multiply with the C_μ probe indicate that second hybridizations detect 90–95% of all colonies positive initially (data not shown). Thus, no correction factor for reduced hybridization

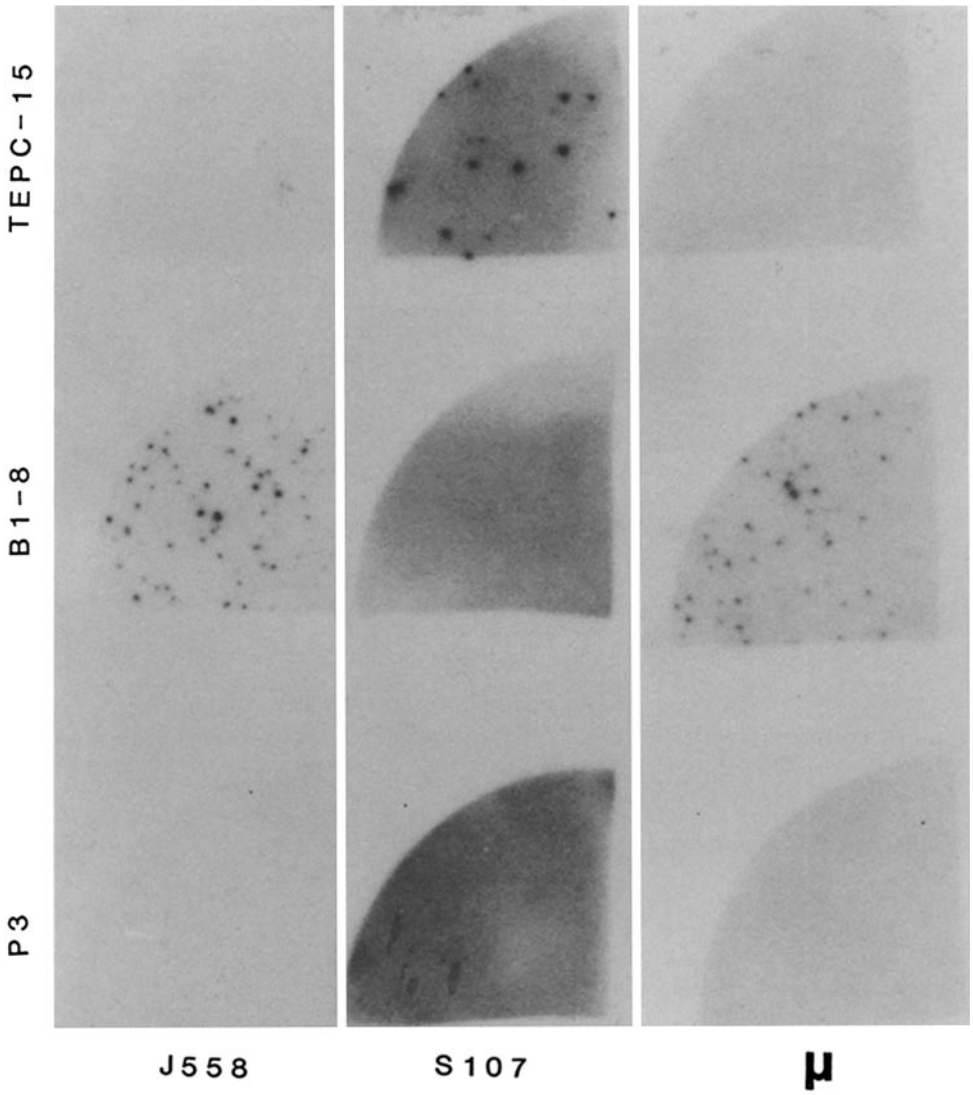

FIGURE 1. V_H and C_μ probes hybridize specifically to characterized hybridoma and myeloma colonies. Colonies (80–140 cells) of TEPC-15, B1-8, and P3 (*top* to *bottom*) cells were fixed and hybridized with ^{32}P-labeled fragments of the pNP.B4, pS107V1 (the gift of Dr. R. Perlmutter), and pμ8 (*left* to *right*) plasmids (see Materials and Methods). V_H-specific probes hybridize and label only the appropriate colonies (pNP.B4/B1-8; pS107V1/TEPC-15). The C_μ probe identifies the IgM-producing B1-8 colonies but does not label the IgA-secretor, TEPC-15, or the nonproducer, P3. Specificity of pV_H X-24 and pV_H Q52N-derived probes was confirmed by dot-blot hybridization with hybridoma lines 28.23 and 28.120, respectively (data not shown).

efficiency was used. Pretreatment of filter paper discs with 0.1 M NaOH for 20 min prevented any subsequent hybridization, whereas DNase treatment for 30 min at 37°C did not alter hybridization results.

Kinetics and Efficiency of LPS-induced Colony Growth. Formation of antibody-secreting cell colonies depends absolutely upon the presence of mitogen (8–10).

The relatively low concentrations of LPS (10–16 μg/ml) used in these experiments favor cellular proliferation over rapid differentiation to produce colonies sufficiently large (>100 cells) to detect by in situ hybridization (8). Typically, microscopic examination reveals that by day 5 of culture, 40–60% of colonies contain ≥100 cells, consistent with an average division time of 12–14 h (8).

At low plating densities (≤10^4 splenocytes/disc), the frequency of LPS-inducible, colony-forming precursor cells is about one-half that reported for LPS-reactive splenocytes (8, 19). Colony formation is less efficient at higher plating densities; at 10^5 splenocytes/disc, the frequency of colony-forming precursors among C57BL/6 splenic B-cells is 1 in 23, representing an efficiency of 13–14% (8).

Efficiency of Colony Detection by In Situ Hybridization. The frequencies of LPS-reactive C57BL/6 and BALB/c splenocytes in limiting dilution culture are reported as 1 in 6 and 1 in 20, respectively (19). Thus, among the 10^5 splenocytes plated onto each filter paper disc, a maximum of 1.67×10^4 C57BL/6 and 5×10^3 BALB/c cells should be sensitive to LPS stimulation. The numbers of hybridizable colonies, however, are much lower. In a representative experiment, 10^5 C57BL/6 or BALB/c splenocytes were plated in tandem using identical reagents and cultured for 6 d. C57BL/6 splenocytes generated 1,103 ± 123 C_μ^+ colonies/disc, while BALB/c splenocytes produced only 388 ± 60 C_μ^+ colonies/disc. Nonetheless, because of the lower frequency of LPS-reactive BALB/c splenocytes, the response of both strains represents an efficiency of 7–8%. This result is consistent with the requirement of ≥100 cells/colony to generate a hybridization signal and the distribution of colony sizes at day 5–6 of culture (see above).

Frequency of B Cell Colonies Expressing the V_H X-24, -Q52, or -J558 Families. Fig. 2 illustrates typical hybridizations of filter paper discs with the V_H J558 or -Q52 probes followed by subsequent hybridization with the C_μ probe. The results of 10 independent determinations, in which almost 6.7×10^4 C_μ^+ colonies were screened, are summarized in Table I. Among some 3.6×10^4 C_μ^+ colonies arising from C57BL/6 splenocytes, 3.4% were V_H X-24$^+$, 17.3% were -Q52$^+$, and 55.5% expressed V_H J558 (Table I). Each V_H family was comparably expressed among BALB/c-derived colonies as well; V_H X-24$^+$, -Q52$^+$, and -J558$^+$ colonies constituted 2.5, 21.7, and 48.3% of the C_μ^+ clones screened (Table I). Consistently, BALB/c-derived splenocytes generated slightly higher numbers of V_H Q52$^+$ colonies and lower numbers of V_H J558$^+$ colonies than did equivalent numbers of C57BL/6 cells (Table I). While this may represent real differences between the Igh^a and Igh^b loci, this strain-associated variability is not statistically significant.

V_H expression among lymphocyte colonies varied between discs even though colonies arose from pooled splenocyte populations. For example, when V_H frequencies are determined for individual discs, rather than summed as in Table I, the mean (± SD) frequencies of V_H X-24$^+$, -Q52$^+$, and -J558$^+$ C57BL/6-derived colonies are: 3.3 ± 2.6, 16.9 ± 4.5, and 49.5 ± 16.7%, respectively. These harmonic means differ from the arithmetic means calculated in Table I, as expected. Although the number of C_μ^+ colonies per disc varies (678 ± 281, n = 56), all V_H frequencies are normalized with respect to C_μ^+ hybridization.

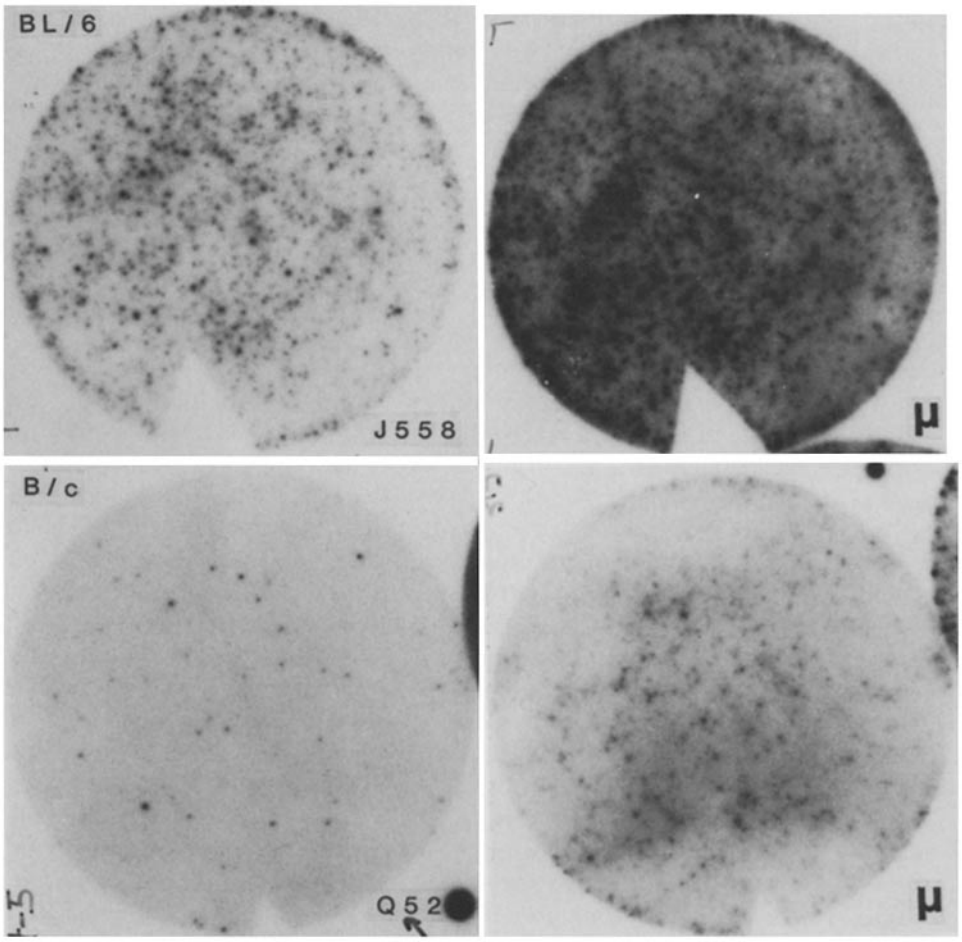

FIGURE 2. Typical sequential hybridizations of single discs with V_H and C_μ probes. Equal numbers (10^5) of C57BL/6 (*top*) and BALB/c splenocytes (*bottom*) were cultured, fixed, and hybridized with V_H- (*left*) and subsequently C_μ-specific (*right*) cDNA probes as described (Materials and Methods). Every V_H^+ colony may be mapped to a congruent C_μ^+ colony. The frequency of LPS-inducible, colony-forming precursor cells among BALB/c splenocytes is approximately one-third that of C57BL/6.

Because the pμ8 probe appears to hybridize with an efficiency comparable to that of the V_H probes (Fig. 1), the variability we observe is probably due to sampling errors (population fluctuations on single discs) compounded by the sequence diversity within V_H families, especially J558 (11).

Discussion

Generally, the frequencies at which V_H segments are expressed in hybridomas derived from mitogen-stimulated splenocytes of adult mice have been proportional to estimates of V_H number or V_H complexity (3, 4). These findings, confirmed by our analysis of C57BL/6 and BALB/c mice, are consistent with models of the stochastic assembly of Ig V regions. Among the some 6.7×10^4

TABLE I

Frequencies of B Cell Colonies Expressing V_H X-24, -Q52, or -J558

Responder strain	V_H^+ colonies per C_μ^+ colonies*			C_μ^+ colonies screened
	X-24	Q52	J558	
C57BL/6	412/12,012 (3.4)	2,163/12,479 (17.3)	6,512/11,723 (55.5)	36,214‡
BALB/c	432/17,408 (2.5)	2,327/10,739 (21.7)	1,152/2,385 (48.3)	30,532§
Combined	844/29,420 (2.9)	4,490/23,218 (19.3)	7,664/14,108 (54.3)	66,746

* Numbers in parentheses are percentages.
‡ Data from six independent determinations, *n* (discs) = 56.
§ Data from four independent determinations, *n* (discs) = 60.

TABLE II

Stoichiometric Expression of V_H Families among LPS-inducible Splenocytes

V_H family	Complexity*	Expression ratios			
		Expected	C57BL/6	BALB/c	Combined
X-24	2	1 (2/2)	1‡	1‡	1‡
Q52	15	7.5 (15/2)	5.1	8.7	6.7
J558	36	18 (36/2)	16.3	19.3	18.7

* Defined as the number of genomic DNA fragments formed by Eco RI digestion that hybridize to single V_H-specific probes (11).
‡ Values from Table I.

C_μ^+ C57BL/6- and BALB/c-derived colonies screened, ~3% expressed the V_H X-24 family, 19% expressed V_H Q52, and some 54% transcribed J558 V_H segments (Table I). Although fine differences in expression may exist between the two murine strains (Q52, BALB/c ≥ C57BL/6; J558, C57BL/6 ≥ BALB/c), within both the Igh-V^b and -V^a loci, V_H expression correlates well with V_H complexity.

Complexity, the number of genomic DNA fragments formed by Eco RI digestion that hybridize to specific V_H probes (11), is a relative measure of the numbers of V_H gene segments, not an enumeration. Therefore, the complexities of the V_H families X-24 (= 2), Q52 (= 15), and J558 (= 35–40, single probe; ~60, multiple probes) only estimate the number of homologues. Despite this, unless these estimates are strongly biased (e.g., the density of V_H gene segments in family A ≫ family B), comparison of ratios of V_H complexity should be a robust measure of comparative size.

The approximate ratio of complexities for the X-24, Q52, and J558 V_H families determined by single V_H-specific probes is 1:7.5:18 (11). If the expression of V_H gene segments were indeed stochastic, the same ratio should describe the ratio of frequencies of B cell colonies expressing the X-24, Q52, and J558 families. Among some 6.7×10^4 LPS-induced, C_μ^+ colonies derived from C57BL/6 or BALB/c splenocytes, ~3% expressed V_H X-24 segments, ~19% were $Q52^+$, and ~54% were $J558^+$, a ratio of 1:6.7:18.7 (Table II). Thus, the expression of the V_H X-24, -Q52, and -J558 families among splenic B lymphocytes is consistent

with a process of equiprobable expression of individual V-region genetic elements. Although this survey was not exhaustive, the X-24, Q52, and J558 families include the full range of structural variability among the known V_H groups: X-24 and J558 are the smallest and largest of the V_H families (11); Q52 is proximal to the J_H locus, while J558 and X-24 are increasingly distal (20). There seems no good reason, a priori, to expect bias in the expression of those V_H families not tested.

Manser et al. (3) screened almost 9×10^3 unspecifically induced, A/J-derived hybridoma cultures for the expression of a single V_H gene segment, obtaining an average frequency of 1 in 328; a result in good agreement with the expected total number of V_H gene segments. Dildrop and her colleagues (4) determined V_H expression in 51 LPS-induced, C57BL/6-derived hybridomas for all nine V_H families and found a reasonable correlation between complexity and the frequency at which each family was expressed. Our experiments with normal splenic B lymphocytes confirm these earlier reports; the antibody repertoire in these mice appears to be generated by equiprobable expression of V-region gene segments.

Recently, Livant et al. (21) have presented hybridization data suggesting that the J558 V_H family may contain as many as 10^3 members. These authors hypothesize that each of the 60 or so bands that define J558 complexity must represent 10–15 individual V_H segments. However, the simplest interpretation of these findings, that the number of functional J558 segments is much greater than all other V_H segments, is inconsistent with our results and those of Manser et al. and of Dildrop et al. (3, 4). In terms of V_H use, complexity appears to be the best estimator of the frequencies at which V_H families (including J558) are expressed. This discrepancy implies either that few of the 500–1,000 J558 segments detected by Livant et al. (21) are capable of productive rearrangement, or that all V_H families contain ($10 \times V_H$ complexity) members.

Somewhat paradoxically, specific humoral immunity appears to be founded upon random processes: genetic combinatorics, junctional artefacts, and somatic hypermutation. What then is the role of germline V_H diversity in the antibody repertoire? Do V_H families represent phenotypically neutral libraries of gene segments, or have they been selected for the utility of a specific product? Experiments to map V_H families to antigen-specific paratopes are in progress.

Summary

The filter paper disc method for cloning inducible lymphocytes was used to census the splenic B cell population of C57BL/6 and BALB/c mice for the expression of three V_H gene-families, V_H X-24, -Q52, and -J558. B cell colonies, arising from single founder lymphocytes, were identified by in situ hybridization with V_H family- and C_μ-specific cDNA probes. Some 6.7×10^4 C_μ^+ colonies were screened. Among C57BL/6- or BALB/c-derived colonies, ~3% were V_H X-24$^+$, ~19% were V_H Q52$^+$, and ~54% were V_H J558$^+$. These frequencies are consistent with a process of equiprobable expression for individual V_H segments, and provide direct evidence that normal splenic B lymphocytes use a process of random genetic combinatorics to generate the antibody repertoire.

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