Brief Definitive Report

B CELL REPERTOIRE DIVERSIFICATION PRECEDES IMMUNOGLOBULIN RECEPTOR EXPRESSION*

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It is now generally accepted that the expressed adult murine B cell repertoire is extremely diverse and probably consists of $>10^7$ unique antibodies (clonotypes) (1–4). However, it is not yet clear to what extent the expression of B cell repertoire diversity is limited or expanded by the environment within which developing B cells mature. The imposition of tolerance to self-antigenic determinants (5–7) and the effects of idiotypic recognition (8–11) may have significant impact in shaping the B cell repertoire. These processes must exert their effects, both positive and negative, through interaction with the V regions of B cell surface immunoglobulins (sIg). Therefore, we have attempted to evaluate the contribution of such environmental influences upon the establishment of the B cell repertoire by assessing repertoire expression in bone marrow–derived sIg^- B cell precursors. Presumably, the majority of these responsive B cell precursors consist of sIg^- pre–B cells that have not yet been subjected to environmental regulatory processes (12, 13). We have analyzed the monoclonal responses of these B cell precursors to the hemagglutinin (HA) of the PR8 influenza virus, an antigen that has proven uniquely useful in assessing repertoire diversity (3, 4, 14, 15). The results indicate that the HA-specific repertoire of sIg^- B cells is comparable in diversity to the primary anti-HA repertoire of splenic B cells. Thus, extensive diversification and repertoire establishment per se need not depend upon either previous interaction with antigen or regulatory mechanisms operating within the milieu of developing B cells.

Materials and Methods

Mice. 1–4-mo old BALB/c and DBA/2 mice were obtained from the mouse breeding colony at Scripps Clinic and Research Foundation.

Viruses. Preparation and purification of viruses have been previously described in detail (3, 14, 15). Strains employed in the current studies were: PR8(A/PR/8/34(H1N1)); WSE(A/WSE/33(H1N1)); MEL(A/Mel/35(H1N1)); BH(A/BH/35(H1N1)); BEL(A/Bel/42(H1N1)); WEISS(A/Weiss/43/(H1N1)); CAM(A/Cam/46(H1N1); the recombinant virus, EQ-PR8(A/Equine/Miami/1/63(Heq2)-A/PR/8/34(N1); and the B virus B/LEE.

Preparation of Surface Immunoglobulin-Negative Bone Marrow Cells. Surface Ig-positive cells were depleted from bone marrow by rosetting with goat anti-mouse (IgG + IgM) coated sheep erythrocytes (13, 16). In some experiments, sIg^- bone marrow cells were

*Supported by grant CA 25803 and AI 15797 from the National Institutes of Health.

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prepared by panning on anti-murine Ig–coated plates as previously described (17, 18). Using either method, at least 90% of sIg^+ lymphocytes were depleted and the resulting sIg^+ bone marrow cell preparations possessed <2% sIg^+ lymphocytes as assessed by staining cells with fluorescein-labeled rabbit anti-mouse Ig antibody and analysis with a Becton-Dickinson FACS IV (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) (13).

Fragment Cultures. Splenic fragment cultures were performed as described in detail elsewhere (3, 9, 12–15). $\sim 30 \times 10^6$ BALB/c donor sIg^- bone marrow cells were transferred intravenously into PR8-primed DBA/2 recipients that had received 1,300 rads of total body irradiation. DBA/2 recipients were immunized 6–8 wk before use with ~1,280 HAU of purified PR8 in phosphate-buffered saline. In vitro stimulation of splenic fragment cultures was achieved with 200 HAU/ml purified PR8 virus. Culture supernatants collected between days 9 and 21 of culture were assayed for anti-viral antibody by solid phase radioimmunoassay (RIA) (3, 12–15).

Results

Frequency of Anti-PR8 Specific B Cells in sIg^- Bone Marrow. In experiments using a total of 1.69×10^9 sIg^- bone marrow cells, the average frequency of anti-PR8 specific B cell responses was 1.7 ± 0.3 (SEM) per 10^7 sIg^- bone marrow cells injected. Given the previously established frequency of ~1.6 per 10^6 transferred B cells for responses of primary sIg^+ B cell populations (3, 15), a maximum of 20% of the observed anti-PR8 responses could have resulted from the <2% contaminating Ig^+ B cells. Additionally, studies in our laboratory have indicated that >90% of B cell clones derived from sIg^- bone marrow cells specific for 2,4-dinitrophenyl (DNP) produce IgM and/or IgA, and <10% produce IgG (12, 13). Consistent with these results, <10% of anti-PR8 monoclonal antibodies derived from sIg^- bone marrow cells were IgG.

Specificity Analysis of Monoclonal Anti-HA Antibodies. As previously observed with mature splenic B cells (3), ~25% of the monoclonal antibodies derived from sIg^- bone marrow B cell precursors were reactive with PR8 (H1N1), but not EQ-PR8 (Heq2N1), and were thus assumed to be potentially HA specific. These antibodies were tested for their reactivity on a panel of six heterologous H1N1 viruses. Previous experiments have shown that this panel of viruses is sufficient to permit a comprehensive delineation of the anti-HA repertoire (3, 14, 15). 29 reactivity patterns (RP) were detected of 64 possible in analyses of 68 monoclonal antibodies (Table I). Most of these RP had been observed previously in experiments with primary adult splenic B cells (3, 15). Fig. 1 presents a statistical analysis (19, 20) which demonstrates that the set of monoclonal antibodies derived from sIg^- bone marrow B cells display a similar extent of diversity when compared with monoclonal antibodies derived from primary adult splenic B cells. In this analysis, we have excluded clones reactive with all tested H1N1 strains, since they presumably include the recognition of multiple determinants including potential non-HA determinants. Monoclonal antibodies that bound only PR8 were also excluded since this RP has previously been shown to include multiple determinant specificities (14). When less than five heterologous H1N1 viruses were employed in RP analysis, the number of observed RP was essentially equivalent to the total possible number of RP defined by the virus panel. When larger virus panels were utilized, the total number of clonotypes expressed by BALB/c sIg^- bone marrow cells and splenic B cells was estimated by a statistical analysis of single vs. recurrent clonotypes (19, 20). It should be noted that the extrapolated number of clonotypes obtained by such analyses always represent minimum estimates of the anti-HA repertoire.

TABLE I

*Relative Frequencies of PR8-HA Specific RPs in Adult sIg⁻ Bone Marrow Cells and Primary Splenic B Cells**

Heterologous virus										
		BH	+	+	+	−	+	−	−	−
		WSE	+	+	−	+	−	+	−	−
		MEL	+	−	+	+	−	−	+	−
WEISS	CAM	BEL								
+	+	+	10.3 / 7.0	4.4 / 1.6	1.5 / x	x / 1.6	x / x	x / x	1.5 / x	x / x
−	+	+	x / 3.1	4.4 / x	x / x	x / 0.8	x / x	x / 0.8	x / x	x / 2.3
+	−	+	2.9 / 1.6	2.9 / x	1.5 / 0.8	x / x	x / 0.8	x / x	x / x	x / 1.6
+	+	−	1.5 / 2.3	2.9 / x	x / 2.3	1.5 / x	2.9 / x	2.9 / x	x / x	1.5 / x
−	−	+	x / x	2.9 / x	x / 0.8	x / x	4.4 / x	x / 2.3	x / 0.8	1.5 / 2.3
−	+	−	x / 0.8	2.9 / 0.8	1.5 / 0.8	x / x	x / x	x / 2.3	x / 4.7	1.5 / 1.6
+	−	−	1.5 / 1.6	1.5 / x	1.5 / x	x / x	x / 0.8	x / 5.5	1.5 / 1.6	2.9 / 1.6
−	−	−	x / 5.5	5.9 / 2.3	x / 1.6	x / 7.8	x / 3.1	2.9 / 6.2	1.5 / 5.5	23.5 / 14.0

* Relative frequencies are given as the percent of the total anti-HA response and are based on 68 adult sIg⁻ bone marrow and 129 primary splenic anti-HA monoclonal antibodies (3). Numbers above the line refer to sIg⁻ bone marrow responses; numbers below the line refer to primary splenic responses. *x*, RP not observed.

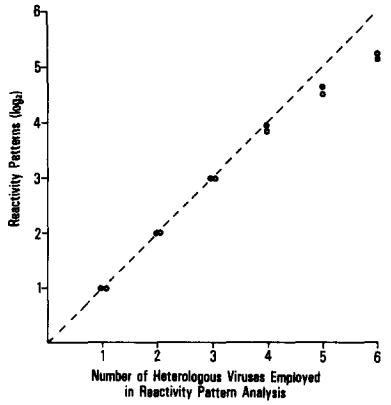

FIGURE 1. Estimation of the number of HA-reactive clonotypes for adult primary spleen and sIg⁻ bone marrow cells. The number of anti-HA clonotypes (RP) expressed by BALB/c B cell populations is plotted against the number of heterologous H1N1 viruses used in the reactivity pattern analysis. When 5 or more viruses were used in the panel, the number of RP was estimated by the method of Briles and Carroll (19). Closed circles represent the number of anti-HA RP for primary adult spleen; open circles are values for sIg⁻ bone marrow cells. The broken line indicates the maximum number of RP definable with a given number of heterologous viruses. The order in which the heterologous viruses were compared had little effect upon the calculated values.

Discussion

The organization of immunoglobulin heavy and light chain variable region genes into multiple DNA segments and recombination among these segments to form functional V genes (21, 22) as well as potential somatic mutations (23) suggests that an enormous B cell repertoire could be obtained in the absence of any interaction of the developing B cells with their environment. However, much evidence suggests that environmental influences are important in shaping the B cell repertoire. It is likely that tolerance to self antigens acts to limit B cell repertoire expression (5–7). In addition, recognition of immunoglobulin idiotype by an individual's immune system may also affect the establishment and subsequent expression of the B cell repertoire (8–11). Thus, determining the contribution of selective processes within the environment to the ultimate expression of the B cell repertoire remains a fundamental unresolved issue.

It has recently been demonstrated that adult murine bone marrow, depleted of sIg^+ lymphocytes, retains a population of sIg^- B cell precursors that are apparently capable of expressing sIg upon adoptive transfer, and responding to challenge with hapten-protein conjugates in the splenic focus assay (12, 13, 24). The responses of these sIg^- B cell precursors are similar to those of very immature fetal and neonatal B cells with respect to both the heavy chain isotypes of antibodies synthesized by responding B cells, as well as their susceptibility to tolerance in vitro (6, 12, 13, 17, 24–26). Additional evidence that this population is distinct from sIg^+ populations comes from the demonstration that sIg^- cells display a disproportionately high representation of certain specificities that are rare in mature B cell populations (17, 24, 27). Thus, the sIg^- bone marrow preparations provide a source of immature B cell precursors that presumably have not been subjected to the range of selective environmental influences.

Previous experiments have shown that the anti-HA B cell repertoire of adult BALB/c mice extrapolates to well over 100 different specificities (3, 4, and Fig. 1). In contrast, 7-d old and 12–14-d old neonates express at least a 10-fold smaller anti-HA repertoire (14, 15). The results presented in this report indicate that the distribution and estimated diversity of anti-HA clonotypes of sIg^- bone marrow B cell precursors is comparable to that of mature primary splenic (sIg^+) B cells and is considerably greater than that of neonatal B cell populations. Since the proportion of B cell precursors responsive to HA in comparison with other tested antigens (e.g. DNP and phosphorylcholine) is similar in sIg^- bone marrow and mature sIg^+ B cell pools (12, 13, 17, 24), it is likely that diversification equivalent to that of mature splenic primary B cells is achieved in bone marrow B cell precursors before sIg acquisition. Since these immature sIg^- B cell precursors have presumably not yet encountered environmental antigens or immunoglobulin-specific regulatory mechanisms, such processes would not seem to be required for the generation of a vast array of antibody specificities.

It remains a possibility that idiotype-specific mechanisms and/or antigen may interact with emerging immature sIg^+ B cells to enhance (10) or diminish (8, 9) the representation of particular clonotypes within the established B cell repertoire. However, it should be noted that this and other laboratories have recently demonstrated that certain clonotypes that are present in high frequency in mature B cell populations are also expressed in relatively high frequency within the sIg^- bone marrow cell population (24, 28, 29). Taken together with the

present report, these findings would indicate that both repertoire diversification and predominant clonotype expression occur in the absence of environmental selective mechanisms. All of these findings, however, address the establishment of the *primary* B cell repertoire and do not address the effects of antigenic stimulation of mature B cells, which appears to disproportionately increase the subsequent representation of certain clonotypes (3) and may permit the expression and selection of totally new clonotypes (4).

Summary

68 monoclonal antibodies specific for the hemagglutinin (HA) of the influenza virus, PR8, were obtained from sIg^- bone marrow B cell precursors stimulated in splenic fragment cultures. Reactivity pattern (RP) analysis demonstrated that these anti-HA antibody responses included at least 29 distinguishable clonotypes. Comparison of the specificities of anti-HA antibodies obtained from sIg^- bone marrow cells with those obtained from adult spleen cells indicates that the anti-HA repertoires of the two populations are comparable in diversity. Since the sIg^- bone marrow B cell precursor pool presumably has not encountered V region–specific regulatory mechanisms in vivo, our data suggest that substantial diversification of the B cell repertoire precedes surface immunoglobulin (sIg) expression and subsequent interaction with environmental regulatory processes.

We thank Mr. David Finney for performing the FACS analyses, Ms. Maya Kluysner and Ms. Pamela Skeen for their technical assistance, and Ms. Carol Wood for preparation of the manuscript.

Received for publication 18 July 1983 and in revised form 16 August 1983.

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