THE DIVERSITY OF THE INFLUENZA-SPECIFIC PRIMARY B-CELL REPERTOIRE IN BALB/c MICE*

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Interest in the basis and extent of Ig variable region diversity has resulted in the development of experimental systems with which the component parts of heterogeneous antibody populations may be examined. Considerable progress has been made in the establishment of markers for Ig variable regions using isoelectric focusing, anti-idiotypic sera, and fine specificity measurements. Such markers have now been described for components of several murine immune responses, including those to phosphorylcholine (1–5), dextran (6, 7), *p*-azophenyl-arsonate (8, 9), streptococcal group A carbohydrate (10, 11), and (4-hydroxy-3-nitrophenyl)acetyl (NP)[1] and its derivatives (12, 13). Although these systems have provided valuable information with respect to the invariant expression of certain specificities within a murine strain, their ability to approach more subtle questions regarding the total antibody repertoire is severely limited. For example, many of these systems have examined serum antibody, which is heterogeneous in composition and is therefore subject to interpretive ambiguities regardless of the methods employed to define a given subpopulation of antibody molecules. Although these ambiguities may be overcome through the study of homogeneous antibodies (5, 14, 15), most systems employed to date have been restricted to a relatively narrow range of definable clonotypes. Finally, few of these systems have examined complex protein antigens (16), which presumably comprise a substantial portion of the normal antigenic exposure of an individual.

Recent studies (17–19) have shown that secondary monoclonal anti-influenza hemagglutinin (HA) antibodies may be characterized by their reactivities toward heterologous HAs. Because a large number of antigenically related influenza strains exist, this system provides an opportunity to extensively dissect the response to a complex protein antigen. However, previous investigations have examined the secondary response, and therefore cannot be used to assess the size or diversity of the primary repertoire. In the present study, the primary anti-HA response in BALB/c mice has therefore been examined. The results indicate that: (*a*) the frequency of HA-specific precursors is surprisingly

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[1] *Abbreviations used in this paper:* CHC, chicken host component; DNP, 2,4-dinitrophenyl; HA, hemagglutinin; HAU, hemagglutinating unit; NA, neuraminidase; NP, 4-hydroxy-3-nitrophenyl acetyl; NIP, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl; PBS, phosphate-buffered saline; RIA, radioimmunoassay; RP, reactivity pattern.

low when compared to those obtained with many small haptenic determinants; (*b*) the primary response to HA is at least as diverse as the secondary response when analyzed by reactivity with heterologous viruses; and (*c*) based upon an estimate of the minimum number of anti-HA clonotypes, the average representation of a given clonotype in the primary BALB/c repertoire is <1 in 10^7 splenic B cells. This suggests that the representation of a given specificity in the primary repertoire may be quite limited. Furthermore, in conjunction with previously published data (19), the results indicate that those B-cell populations which express these sparsely represented clonotypes expand substantially upon primary antigenic exposure.

Materials and Methods

Mice. BALB/c mice were obtained from the Institute for Cancer Research, Philadelphia, Pa. B10.D2 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Germ-free BALB/c mice were obtained from the National Institutes of Health, Bethesda, Md., and they were used immediately after removal from shipping tubes.

Antigens and Immunizations. The influenza virus strains PR8 [A/PR/8/34 (H0N1)], WSE [A/WSE/33 (H0N1)], MEL [A/Mel./35 (H0N1)], BEL [A/Bel./42 (H0N1)], CAM [A/Cam./46 (H1N1)], and the influenza B virus, LEE, were originally obtained in the form of high infectivity stocks from Dr. S. Fazekas de St. Groth, Commonwealth Scientific and Industrial Research Organization, Molecular Genetics, Sydney, Australia. The hybrid virus Eq-PR8 [A/equine/Miami/1/63 (Heq 2)-A/PR/8/34 (N1)] was kindly provided by Dr. R. Webster, St. Jude Children's Research Hospital, Memphis, Tenn. The virus strains BH [A/BH/35 (H0N1)], WEISS [A/Weiss/43 (H0N1)], HICK [A/Hickcox/40 (H0N1)], and FM1 [A/FM/1/47 (H1N1)], were obtained through the generosity of Dr. A. P. Kendal from the Department of Health, Education, and Welfare, Atlanta, Ga. All viruses were grown in the allantoic cavity of 11-day-old chicken eggs and purified by adsorption to and elution from human erythrocytes followed by velocity sedimentation in a linear sucrose gradient (20). The HA activity of the virus preparations was determined as described by Fazekas de St. Groth and Webster (21).

B10.D2 mice were immunized intraperitoneally with $\cong$1,250 hemagglutinating units (HAU) of purified PR8 virus in phosphate-buffered saline (PBS) 6–8 wk before use as recipients in adoptive transfer experiments.

Splenic Focus Technique. Monoclonal antiviral antibodies were produced in the splenic fragment culture system (22, 23) as previously described (17, 18). Briefly, donor spleen cell preparations were transferred intravenously to PR8-primed B10.D2 recipient mice which had received 1,300 rad of whole-body irradiation from a cesium source 4 h earlier. Fragments were prepared from recipient spleens 14–18 h later, and stimulated individually in vitro with 40 HAU of PR8 in 0.2 ml of Dulbecco's modified Eagle's medium. Fragment cultures were maintained at 37°C in a humidified atmosphere of 10% CO_2 in oxygen. Medium was changed at 3-day intervals, and culture fluids were collected individually beginning on day 9.

Radioiodination of Antisera. Rabbit anti-mouse Fab and goat anti-μ, -γ_1, -γ_2, or -α antibodies were purified and then radioiodinated according to the chloramine-T procedure as previously described (22–24).

General Protocol for Radioimmunoassay for Anti-Influenza Antibody. Culture fluids were assayed for the presence of antiviral antibodies by solid-phase radioimmunoassay (RIA) as previously described (18). Briefly, 20 μl of culture fluid were added to wells of polyvinyl microtiter "V" plates (Cooke Laboratory Products, Div. Dynatech Laboratories Inc., Alexandria, Va.) to which 20 HAU of purified virus had been previously adsorbed. After 4 h, the culture fluids were removed, the plates washed twice with PBS made 1% with horse serum, and 0.1 ml of radioiodinated rabbit anti-mouse Fab added. For isotype analyses, purified, radioiodinated goat-anti-mouse-μ, -γ_1, -γ_2, or -α were used. After 4 h, the label was withdrawn and the plates washed 10 times with water. The plates were dried at room temperature, and individual assay wells were subsequently removed and the bound radioactivity counted in a gamma counter.

For reactivity pattern (RP) analyses, radioiodinated, isotype-specific antisera were employed. Monoclonal anti-PR8 antibodies were tested in the RIA against at least six heterologous influenza A viruses. All tests were done in duplicate. Although the vast majority of reactions of

monoclonal antibodies with a heterologous virus was either close to 100% or nil, the lower limit for a positive reaction was set at 10% of the binding observed with the homologous (PR8) virus. This figure was chosen on the basis of previous work from this laboratory which has shown that if the reactivity of a monoclonal antibody exceeds 10% with a heterologous virus, that virus is generally capable of adsorbing all anti-HA activity present (17, 18).

Results

Frequency of Primary HA-Specific B Cells. Previous adoptive transfer experiments have shown that carrier-specific enhancement is maximized for B cells from nonimmune donors when they are transferred to immunized recipients which are syngeneic at the major histocompatibility complex (*H-2*), but allogeneic for Ig allotype markers (25). This combination apparently circumvents an antibody-specific immunoregulatory mechanism present in immunized recipients which suppresses the stimulation of syngeneic primary B cells (25). Preliminary analysis of the primary anti-PR8 response confirmed this finding by demonstrating a severalfold increase in the observed primary influenza-specific B-cell frequency when BALB/c B cells were transferred to PR8-immunized B10.D2, as opposed to BALB/c recipients. Therefore, the frequency of HA-specific B cells in the spleens of both conventionally reared and germ-free adult BALB/c mice was assessed in the splenic focus assay by using irradiated B10.D2 recipients, which had been immunized intraperitoneally with PR8 6 wk previously.

Fragment cultures derived from these recipients were always stimulated with PR8, and the collected culture fluids were initially analyzed in the RIA against PR8 with labeled rabbit anti-mouse Fab antibody. Culture fluids from fragments which produced anti-PR8 antibodies were subsequently analyzed for anti-HA activity because two additional viral surface components could also elicit an immune response, the neuraminidase (NA) and chicken host component (CHC), a carbohydrate moiety found on viruses grown in chicken eggs. Anti-HA specificity of a particular monoclonal antibody could be delineated by analysis against a panel of three viruses: PR8; LEE, an influenza B virus which shares only CHC with PR8; and equine PR8, which shares CHC and all viral proteins except HA with PR8. In such an analysis, only those antibodies found to react with PR8 alone were considered HA specific. In general, ≅30% of monoclonal antibodies obtained were HA specific. Of the remaining antibodies, ≅40% were directed against NA or an internal viral component, and 30% were directed against CHC. These proportions are in contrast to observations in the secondary response, where >80% of antiviral antibodies are HA specific.

The results presented in Table I indicated that the frequency of primary HA-specific precursors in conventionally reared BALB/c mice varies from 8.7 to 14.9 per 10^6 splenic B cells. In two experiments which employed germ-free donors, the frequencies obtained were not appreciably different. The mean frequency obtained for all experiments was 13.0 ± 2.3 HA-specific precursors per 10^6 B cells. This frequency is 10- to 20-fold lower than that reported for secondary HA-specific precursors (17, 18), and it suggests that a substantial increase in the precursor cell pool occurs upon primary antigenic exposure.

Isotype Distribution of Monoclonal Anti-HA-Specific Antibodies. Monoclonal HA-specific antibodies obtained in the above experiments were analyzed

TABLE I
Frequency of Viral HA-Specific B Cells in Adult BALB/c Mice

Experiment*	Donor mice‡	Total cells injected $\times 10^{-7}$	HA-specific foci per 10^6 cells injected	HA-specific cells per 10^6 splenic B cells§
1	C	24	0.14	8.7
2	C	24	0.19	11.8
3	C	24	0.23	14.3
4	C	20	0.24	14.9
5	C	20	0.20	12.4
6	GF	20	0.25	15.5
7	GF	20	0.22	13.7

Mean HA-specific precursors per 10^6 splenic B cells = 13.04 ± 2.3. Total number of cells analyzed = 152×10^7.

* In each experiment, 2×10^7 donor cells were administered per recipient.

‡ C, conventionally reared; GF, germ free.

§ Values given are after correction for homing efficiency and cloning efficiency (14).

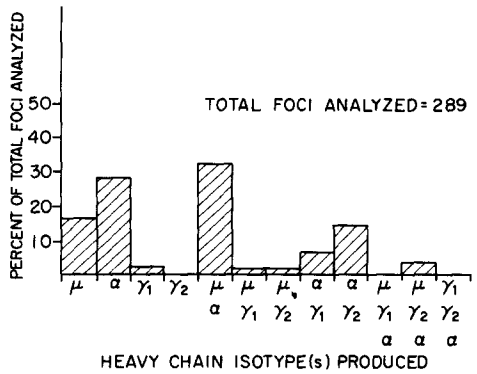

FIG. 1. The relative frequencies of heavy chain isotypes produced by foci in the anti-HA response in vitro. Anti-HA antibodies were analyzed with radiolabeled isotype-specific goat anti-mouse antibodies in the solid-phase RIA against PR8. The relative frequency of each isotype or combination of isotypes is expressed as the percent of total foci analyzed.

for Ig isotype with class-specific goat anti-mouse Ig sera. The results of this analysis are shown in Fig. 1. Of 289 clones analyzed, 81 produced IgA alone and 98 produced both IgM and IgA. These two groups comprised the majority (60%) of anti-HA clones, whereas the IgG_1 and IgG_2 classes were less frequently observed. It should be noted that ≅15% of the clones produced IgM only, which is similar to findings in other primary responses in the splenic focus system (24).

Specificity Analysis of Monoclonal Anti-HA Antibodies. The monoclonal HA-specific antibodies obtained in the above experiments were tested in the RIA for reactivity against at least six heterologous viruses. Purified, isotype-specific antisera were used in this analysis. In cases where more than one isotype had been produced, antisera to the predominant isotype was used. Only monoclonal HA-specific antibodies from foci which had produced a total of at least 80 ng of antibody were utilized, since lesser amounts precluded testing

TABLE II

*Relative Frequencies of Reactivity Patterns in Primary and Secondary Anti-PR8 HA Responses**

Heterologous virus											
WEISS	CAM	BEL	BH	+	+	+	−	+	−	−	−
			WSE	+	+	−	+	−	+	−	−
			MEL	+	−	+	+	−	−	+	−
+	+	+		7.0‡ / 4.5	1.6 / 5.3	*§ / *	1.6 / 1.5	* / *	* / *	* / *	* / *
−	+	+		3.1 / *	* / 3.0	* / *	0.8 / *	* / *	0.8 / *	* / *	2.3 / 0.8
+	−	+		1.6 / 3.8	* / 0.8	0.8 / 0.8	* / 0.8	0.8 / *	* / 6.0	* / *	1.6 / 0.8
+	+	−		2.3 / 12.0	* / *	2.3 / 5.3	* / 3.0	* / 0.8	* / 3.0	* / 0.8	* / *
−	−	+		* / *	* / 0.8	0.8 / 0.8	* / *	* / *	2.3 / *	0.8 / 0.8	2.3 / 1.5
−	+	−		0.8 / 0.8	0.8 / 0.8	0.8 / 0.8	* / *	* / *	2.3 / 0.8	4.7 / 0.8	1.6 / 1.5
+	−	−		1.6 / *	* / 0.8	* / *	* / 0.8	0.8 / 0.8	5.5 / 0.8	1.6 / 2.3	1.6 / 1.5
−	−	−		5.5 / 1.5	2.3 / *	1.6 / *	7.8 / *	3.1 / *	6.2 / *	5.5 / 12.8	14.0 / 23.3

* Relative frequencies are given as percent of total response and are based upon 129 primary and 134 secondary (19) monoclonal anti-HA antibodies.

‡ Upper number refers to primary response; lower number refers to secondary response.

§ *, RP not observed.

against all heterologous viruses. In a given experiment, these represented approximately half of the anti-HA antibody-forming clones obtained. The relative frequencies of each RP in the primary anti-HA response are shown in Table II. The relative frequencies of RPs observed in a previous analysis of secondary anti-HA antibodies are also shown for purposes of comparison. 36 of the 64 possible RPs were realized in an analysis of 112 primary monoclonal anti-HA antibodies from conventionally reared mice. No additional RPs were observed when 17 monoclonal antibodies from germ-free mice were included in the analysis. Anti-PR8 antibodies reacted more frequently with MEL, WSE, and BH than with BEL, WEISS, or CAM. Interestingly, this pattern closely parallels the isolation dates of these viruses and is in agreement with previous findings obtained with secondary monoclonal antibodies (19).

Estimation of the Minimum Number of BALB/c HA-Specific Clonotypes. Inasmuch as the donor cells of individual mice were treated separately, an estimate of the total number of anti-HA clonotypes in the BALB/c population could be made. This type of estimate employed the method described by Wybrow and Berryman (26), which has been used by Kreth and

TABLE III
*RP Analysis in Individuals**

	RP							Absolute RP frequencies in individuals											
								Conventionally reared mice									Germ-free mice		RP totals
	BH	WSE	MEL	WEISS	CAM	BEL	PR8	A	B	C	D	E	F	G	H	I	A	B	
1	+	+	+	+	+	+	+	‡	1	2	1	·	2	1	1	·	1	·	9
2	−	+	+	+	+	+	+	·	·	1	·	·	·	·	·	1	·	·	2
3	+	+	−	+	+	+	+	·	·	·	·	1	·	·	·	·	·	1	2
4	+	+	+	−	+	+	+	·	1	1	1	·	·	·	1	·	·	·	4
5	+	+	+	+	−	+	+	·	·	1	·	·	·	·	·	1	·	·	2
6	+	+	+	+	+	−	+	·	·	·	1	·	·	·	·	·	2	·	3
7	−	+	+	−	+	+	+	·	·	·	·	·	·	1	·	·	·	·	1
8	+	−	+	+	−	+	+	·	·	·	·	·	·	·	1	·	·	·	1
9	+	−	+	+	+	−	+	·	1	1	·	1	·	·	·	·	·	·	3
10	+	+	+	−	+	−	+	·	·	·	·	·	1	·	·	·	·	·	1
11	+	+	+	+	−	−	+	·	·	·	·	·	·	·	·	1	·	1	2
12	+	+	−	−	+	−	+	·	1	·	·	·	·	·	·	·	·	·	1
13	+	−	+	−	−	+	+	·	·	·	·	·	1	·	·	·	·	·	1
14	+	−	+	−	+	−	+	·	·	·	·	·	·	1	·	·	·	·	1
15	−	+	−	−	+	+	+	·	1	·	·	·	·	·	·	·	·	·	1
16	+	−	−	+	−	+	+	·	1	·	·	·	·	·	·	·	·	·	1
17	+	+	+	−	−	−	+	1	·	·	2	·	1	·	·	·	·	3	7
18	−	−	+	−	−	+	+	·	·	·	·	·	·	·	·	1	·	·	1
19	−	−	+	−	+	−	+	·	2	·	·	·	1	·	1	1	1	·	6
20	−	−	+	+	−	−	+	·	·	·	·	·	1	·	·	·	·	1	2
21	−	+	−	−	+	−	+	1	·	·	·	1	·	1	·	·	·	·	3
22	−	+	−	+	−	−	+	2	1	1	·	·	1	·	·	2	·	·	7
23	−	+	−	−	−	+	+	·	·	·	·	·	·	·	1	1	·	1	3
24	+	+	−	−	−	−	+	·	·	·	1	·	·	·	·	·	2	·	3
25	+	−	−	+	−	−	+	·	·	·	·	·	1	·	·	·	·	·	1
26	+	−	+	−	−	−	+	·	·	·	·	1	·	·	1	·	·	·	2
27	−	−	−	−	+	+	+	·	·	·	1	·	·	·	1	·	1	·	3
28	−	+	+	−	−	−	+	·	2	2	1	·	2	1	1	1	·	·	10
29	−	−	−	+	−	+	+	1	·	·	·	·	·	1	·	·	·	·	2
30	+	−	−	−	−	−	+	·	·	1	·	·	·	1	·	1	1	·	4
31	−	+	−	−	−	−	+	·	1	2	1	1	·	2	1	·	·	·	8
32	−	−	+	−	−	−	+	2	1	2	·	1	·	1	·	·	·	·	7
33	−	−	−	+	−	−	+	1	·	·	1	·	·	·	·	·	·	·	2
34	−	−	−	−	+	−	+	·	1	·	·	1	·	·	·	·	·	·	2
35	−	−	−	−	−	+	+	1	·	1	·	·	1	·	·	·	·	·	3
36	−	−	−	−	−	−	+	3	1	3	1	2	2	1	3	·	1	1	18

* RP was determined in the RIA (as described in Materials and Methods) for monoclonal anti-HA antibodies from individual BALB/c mice.
‡ RP not observed.

Williamson (27) in a similar analysis of anti(4-hydroxy-5-iodo-3-nitrophenyl)-acetyl (NIP) antibodies. The data used for this analysis are shown in Table III, where the absolute frequency of each observed RP is presented on an individual basis. In this estimate, each RP has been considered a single clonotype. Because it is possible that several clonotypes might exhibit the same RP, this analysis provides a minimum estimate of clonotypes in the BALB/c repertoire. This evaluation assumes that: (*a*) all clonotypes are equally probable; (*b*) all mice are identical in their clonotype composition; and (*c*) the number of clonotypes that may be discriminated by the method employed is substantially larger than the actual number of total clonotypes. In the present study, the third assumption was not met until at least six heterologous viruses were employed in the analysis, and would probably not be entirely fulfilled until greater than nine heterologous viruses are used. This is evident from Fig. 2, where the predicted

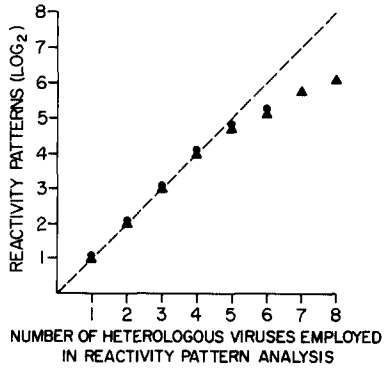

FIG. 2. The number of HA-reactive clonotypes predicted for the BALB/c mouse population. The number of clonotypes (RPs) predicted for the BALB/c population by the method of Wybrow and Berryman (26, 27) is plotted against the number of heterologous viruses used in the reactivity pattern analysis. The dashed line indicates the maximum number of reactivity patterns definable with a given number of heterologous viruses. Closed circles are the calculated values for conventionally reared mice; triangles are values for germ-free mice. The order in which the various heterologous viruses were arranged had little effect upon this type of calculation. In addition to the viral strains shown in Table III, monoclonal antibodies from germ-free mice were also tested against HICK and FMI (data not shown).

population clonotype repertoire is plotted against the number of heterologous viruses used in the RP analysis. With the present data, only antibodies from the germ-free group were analyzed against eight heterologous viruses. The extrapolated results of this analysis indicate that the predicted total number of clonotypes will probably become constant at a value slightly >100. Although the antibodies from conventionally reared mice were analyzed against only six heterologous viruses, the total predicted clonotypes for this group appears to follow a pattern similar to that of the germ-free animals (Fig. 2).

Table III also allows a comparison of clonotype (RP) frequencies and diversity among individuals. Although the number of RPs obtained from each donor was relatively constant (ranging from 6 to 13), the frequency of a particular RP varied considerably among individuals. Further, in several individuals, some RPs appeared more frequently than might have been expected based upon the frequency of that RP in the entire sample population. It is thus possible that only a portion of the HA-specific clonotype repertoire present in the BALB/c population is expressed in an individual at a particular time.

Discussion

The present study allows an assessment of: (*a*) the overall frequency of BALB/c B cells that participate in the primary response to a biologically relevant protein antigen and the comparison of this frequency to those obtained with small haptenic determinants in previous studies; (*b*) the increase in precursor frequency that occurs after immunization; (*c*) the relationship of clonotypes present in the primary B-cell repertoire to those observed in secondary responses to the same antigen; and (*d*) the average frequency of a clonotype (or set of clonotypes) that comprises a single RP within this response.

The frequency of B cells responsive to influenza HA in BALB/c is 1 per 1.0–

1.5×10^5 splenic B cells. This value is substantially lower than that observed with certain simple haptenic determinants, such as dinitrophenyl (DNP), trinitrophenyl (both 20 per 10^5) (14, 28, 29), fluorescein (14 per 10^5) (14, 28), and dimethyl amino napthalene-sulfonyl (9.4 per 10^5) (30). Thus, the representation of HA-specific clonotypes appears comparatively small, especially when the large number of available determinants on such a molecule is considered.

It is possible that the methods used herein to determine the precursor frequency of B cells responsive to PR8 failed to produce optimal stimulatory conditions and thus provided an artificially low frequency estimate. This could result from the suppression of BALB/c clonotypes by immunoregulatory mechanisms directed against primary B10.D2 antibodies and generated during recipient priming. Such suppression would presumably require significant repertoire similarities between the BALB/c and B10.D2 strains, and it apparently does not pertain to the present studies because (*a*) Pierce and Klinman (25) have shown that the frequency of primary BALB/c DNP-specific B cells observed with DNP-hemocyanin-primed B10.D2 recipients is identical to that observed with BALB/c or B10.D2 recipients primed with hemocyanin (carrier) alone; and (*b*) the array of reactivity patterns obtained with monoclonal antibodies generated by primary BALB/c HA-specific B cells in virus-immunized B10.D2 recipients is comparable to that observed in the BALB/c secondary anti-HA repertoire. Thus, there is no evidence that any clonotypes are suppressed in this recipient environment.

A second, and perhaps more probable reason for the comparatively low frequency of precursors specific for PR8 HA is that foreign protein antigens present surface structures that differ only minimally from the vast array of "self" determinants encountered by an individual's immune system, and thus only a limited subset of clonotypes may recognize and be stimulated by those spatial configurations which contain structural nuances distinguishable as "non-self." Small haptenic determinants, on the other hand, may be less rigorously selective in their stimulation since they present clearly novel and readily accessible structures which enable interactions of sufficient energy to allow the stimulation of multiple and diverse clonotypes.

A comparison of the primary and secondary HA-specific precursor frequencies indicates that the precursor population expands substantially after antigen exposure, increasing from 1 in 10^5 to 1 in 5×10^3 splenic B cells or more. These newly generated PR8-specific precursors may be shown to be secondary cells by the following criteria. First, a portion of this population will respond in both unprimed and nu/nu syngeneic recipients.[2] Second, they also respond in syngeneic, PR8-immunized recipients, an environment suppressive to primary B cells. This 10- to 50-fold increase in HA-specific B cells after immunization contrasts markedly with the 2- to 4-fold increase in DNP-specific cells after immunization (23).

A major advantage provided by reactivity-pattern analysis of the anti-HA repertoire is that it allows both the comparison of RPs present in the primary repertoire to those observed in the secondary response, and an estimate of the frequency of primary B cells available for a response against a single determi-

[2] M. P. Cancro, and W. Gerhard. Manuscript in preparation.

nant. The RPs observed among primary BALB/c B cells are quite similar if not equivalent to those present in the secondary repertoire (see Table II). It should be noted that although a few RPs have been observed only in the secondary response, the majority of these are quite infrequent in the secondary response and therefore may only be observed after a more extensive analysis of the primary repertoire. These observations suggest that it is unlikely that immunization leads to the recognition of novel determinants on the immunizing antigen, as has been suggested by several investigators (31, 32). Instead, the secondary repertoire appears to reflect primarily an expansion of the clonotypes present in the primary repertoire. In conjunction with the observation that the primary B-cell frequency is similar in conventionally reared and germ-free mice, this finding argues strongly against the role of antigen in the generation of novel specificities. Immunization may, however, lead to differential expansion of clonotypes within the primary repertoire. The data presented here suggest that this may indeed be the case since the apparent expansion of a given RP varies from <1 to >30-fold.

In Results, we have presented an extrapolated estimate of over 100 RPs in the primary BALB/c clonotype repertoire specific for PR8 HA. This estimate was obtained by a statistical procedure based upon the enumeration of repeats in RPs observed as increasing numbers of primary monoclonal antibodies were analyzed. This is consistent with a recent estimate of 64–128 RPs present in the secondary BALB/c HA-specific repertoire, which was based upon the number of novel RPs revealed as the number of heterologous viruses included in the assay was increased. The close agreement of these two estimates may imply that the upper limit of RPs is actually dictated by the number of distinct antigenic determinants on PR8 HA, and that several clonotypes may exhibit the same RP. If there are, in fact, 100 or more distinct anti-HA clonotypes, the frequency of a given clonotype in the BALB/c primary anti-HA pool would average fewer than 1 in 10^7 splenic B cells. This frequency would predict that each clonotype is represented by 20 or fewer B cells in the primary population of a single mouse. This estimate is based upon the entire BALB/c population, however, and the analysis of individual donors (Table III) suggests that it is possible that only a portion of the total BALB/c anti-HA repertoire is expressed within the primary precursor pool of an individual at a given time. If this is the case, then single clonotypes may be disproportionately represented in the B-cell population of an individual at any one time, and all clonotypes may not be simultaneously available at the individual level.

These studies indicate that the B-cell repertoire, even in individual mice, is probably too diverse to be accounted for by any current estimate of the number of V_H region genes inherited in the germ line (33). The present analysis of the primary anti-HA B-cell repertoire is thus consistent with a somatic diversification process independent of extrinsic antigenic influences (34).

Summary

The primary immune response of BALB/c mice to influenza (PR8) hemagglutinin (HA), a complex protein antigen, has been examined by the splenic focus assay, and the resulting monoclonal anti-HA antibodies have been character-

ized by their reactivity with heterologous viruses. The analysis of the primary B-cell response to HA revealed marked differences from responses previously defined for haptenic determinants. There were the following differences: (*a*) the frequency of HA-specific B cells in both conventional and germ-free BALB/c mice was 1 in 1.0–1.5 $\times$ 10^5 splenic B cells, which is substantially lower than the frequency of B cells responsive to various simple haptenic determinants; (*b*) monoclonal anti-HA antibodies were predominantly of the IgA or IgM isotypes instead of IgG, which dominates antihapten responses; and (*c*) after immunization, the frequency of anti-HA-specific B cells increases by 10- to 50-fold, which is a much greater increase than that observed after immunization with haptenic determinants. Fine specificity analysis of primary monoclonal HA-specific antibodies revealed extensive diversity and a considerable overlap with the specificities obtained from immune mice. Given the low overall frequency of HA-specific B cells, it could be calculated that the representation of most HA-specific clonotypes within the B-cell repertoire could not exceed 1 in 10^7 B cells. These findings indicate that the primary B-cell clonotype repertoire is extremely diverse and largely antigen independent in its generation.

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