REGULATION OF AZOPHENYLARSONATE-SPECIFIC REPERTOIRE EXPRESSION

I. Frequency of Cross-Reactive Idiotype-positive B Cells in A/J and BALB/c Mice*

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Identification of antibody populations that share variable region determinants (idiotypes) among all individuals of an inbred strain has greatly facilitated the study of mechanisms that govern specific immune responses. In almost all cases, such idiotypes constitute a major portion of the antibody response, although the degree of clonal dominance may vary with the number of immunizations or during the course of a response. The observations that (*a*) idiotype expression can be genetically mapped to the heavy chain variable region (V_H)[1] (1), (*b*) DNA sequences for these specificities can be isolated from a sperm genomic library (2), and (*c*) restriction enzyme patterns of V_H correlate with idiotype and allotype inheritance (3) suggest that at least one mechanism of clonal dominance is due to the fact that these clonotypes are the direct expression of germ line genetic information. It is likely that additional mechanisms are involved in this process for several reasons. First, many inherited idiotypes have been found to comprise large families of closely related specificities (4–9), and there can be significantly greater degree of heterogeneity in idiotype-bearing hybridoma proteins characterized than is indicated by analysis of serum idiotype (4, 5). Second, the expression of an idiotype that recurs among all individuals of an inbred strain (cross-reactive idiotype; CRI) may represent the dominant specificity in one strain but constitute a minor clonotype in another (10, 11). Third, genetic information other than that coding for immunoglobulin structural genes can influence the expressed heterogeneity of some antibody responses (11, 12) and can affect the ontogeny of clonotype development (13).

Such observations suggest that there might be a discrepancy between the clonotype diversity present at the precursor cell level (potential repertoire) and what is eventually expressed at the level of the serum antibody response (expressed repertoire). One of the clearest examples of this phenomenon is in the response to phosphorylcholine (PC). The murine serum response to PC is composed almost exclusively of antibodies

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[1] *Abbreviations used in this paper:* ARS, azophenylarsonate; ARS-TGG, 3-(*p*-arsonophenylazo)-*N*-acetyl-L-tyrosyl-glycylglycine; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CRI_A, predominant cross-reactive idiotype in A/J mice; CRI_C, predominant cross-reactive idiotype in BALB/c mice; Hy, hemocyanin; OA, ovalbumin; PBS, phosphate-buffered saline, pH 7.2; PC, phosphorylcholine; V_H, heavy chain variable region.

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corresponding to the myeloma proteins M511, M603, and T15 (14). However, there is considerably more heterogeneity in the IgM precursor cell repertoire analyzed at limiting dilution in the splenic focus system (15) or with monoclonal anti-idiotypic reagents (16, 17). In addition, whereas C57BL/6 mice express only one of two allelic forms of T15 in their serum anti-PC antibody (corresponding to the myeloma protein CBPC3), the other allelic form can be found at the precursor cell level in these mice (18). Indeed, certain BALB/c × C57BL/6 recombinant-inbred strains may have as high a representation of the nonexpressed allele as strains that express it, such as BALB/c.

We have chosen to examine the B cell repertoire specific for azophenylarsonate (ARS) as a model in which to study the regulation of repertoire expression. A large proportion (20–70%) of ARS-specific antibodies from A/J mice share a cross-reactive idiotype (CRI_A) that comprises a family of closely related but nonidentical clonotypes, as shown by amino acid sequence analysis of CRI_A-positive hybridoma proteins (4–6). Anti-idiotypic sera raised against an ARS-specific hybridoma protein unrelated to the major CRI_A can identify a "minor" idiotype population, constituting 5–10% of all anti-ARS antibody in individual A/J mice (10, 19, 20). In all BALB/c mice, this same minor idiotype represents the predominant clonotype family (CRI_C) expressed in the anti-ARS response (10). Individual mice can also produce anti-ARS antibodies with idiotypes that are unique to the individual, and these idiotypes may be in low to undetectable concentrations in other immunized mice of the same strain (6, 21). Therefore, the serum anti-ARS response appears to be extremely heterogeneous, but certain strains express characteristic levels of major and minor idiotype families, making this an excellent choice for analysis of repertoire expression.

In this report, we examine the expression of the predominant CRI_A in the ARS-specific B cell repertoire of A/J and BALB/c mice. Surprisingly, only 2.6% (7 out of 267) of primary A/J ARS-specific monoclonal antibodies generated in the limiting dilution splenic focus system possess the predominant CRI_A, and 2 of 10 BALB/c mice also had CRI_A-positive precursor cells in their nonimmune repertoire. When A/J mice are immunized with ARS-protein conjugates, the serum antibody response and precursor cell population are dominated by CRI_A. The frequency of CRI_A-positive B cells increases over 100-fold after immunization, whereas CRI_A-negative precursor cells may initially decrease, followed by a later rise in frequency. The observation that the CRI_A family can be identified in both BALB/c and A/J mice, along with the striking discrepancy in the expression of this idiotype family at the serum and precursor cell levels, implies the existence of complex regulatory networks that both augment the expression of certain idiotypes and suppress the appearance of others.

Materials and Methods

Antigens. Limulus polyphemus hemocyanin (Hy), ovalbumin (OA), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO. ARS_{20}-OA, ARS_{15}-BSA, and 3-(*p*-arsonophenylazo)-*N*-acetyl-L-tyrosyl-glcylglycine Boc hydrazide-Hy (ARS-TGG-Hy, 10 mol ARS-TGG per 100,000 mol wt Hy) were prepared as described elsewhere (22, 23), and the coupling ratios were determined by methods described for other protein antigens (23).

Animals and Immunizations. 6-wk-old male BALB/c ByJ and A/J mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Neonatal mice were bred in our mouse colony from mice obtained from The Jackson Laboratory. Mice to be used as Hy-primed recipients received

0.1 mg Hy intraperitoneally in complete Freund's adjuvant (CFA) 8–12 wk before use. A/J mice were immunized with 0.2 mg ARS-OA in CFA followed 4 wk later by 0.2 mg ARS-OA in incomplete Freund's adjuvant.

Cell Transfers and Fragment Cultures. Monoclonal antibody responses were obtained in vitro using the splenic fragment culture assay system, as described by Klinman (24). In brief, donor cells were transferred intravenously into syngeneic Hy-primed recipients that had been irradiated with 1,300 rad 4 h previously. Fragment cultures were prepared 16 h after cell transfer and stimulated with ARS-TGG-Hy (1×10^{-6} M hapten) in Dulbecco's modified Eagles medium (Ontario Cancer Institute, Toronto, Ontario), 10% fetal calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), 2 mM glutamine, and 50 μg/ml gentamicin (Schering Corp., Kenilworth, NJ). Culture fluids were changed every 3 d, and fluids collected 9–13 d after stimulation were assayed for anti-hapten antibodies.

Radioimmunoassay. Detection of antibody in culture supernatants was accomplished using a solid-phase radioimmunoassay, as described previously (25). Rabbit anti-mouse $F(ab')_2$ antibody was purchased from N. L. Cappel Laboratories, West Chester, PA., purified by absorption and elution from a mouse IgG + IgM-Sepharose 4B column, and labeled with ^{125}I. Idiotype assays were performed in a competitive radioimmunoassay, as previously described (26). In brief, 0.1 ml of the appropriate dilution of rabbit anti-CRI, kindly provided by Dr. A. Nisonoff, Brandeis University, Waltham, MA, in 0.05 M Tris, pH 9.0, was adsorbed to wells of polyvinyl plates (Dynatech Laboratories, Alexandria, VA). This reagent has been well characterized in Dr. Nisonoff's laboratory (6). After phosphate-buffered saline, pH 7.2 (PBS), containing 1% BSA was added for 1 h and removed, 20 μl of culture supernatant or a serum dilution was added, followed by 2 ng of ^{125}I-labeled hybridoma protein R16.7 or purified A/J anti-ARS antibody, provided by Dr. Nisonoff. After incubation at 37°C for 18 h, the wells were washed with PBS and counted in a gamma counter. Anti-hapten and idiotype radioimmunoassays were run in parallel on culture supernatants and serum samples, using R16.7 as the standard in both assays. Monoclonal antibodies reacting on a 1:1 weight basis in the anti-ARS and idiotype assays were designated CRI_A positive, partial reactivity was defined as anti-ARS/idiotype ratio from 2:1 to 10:1, and CRI_A-negative clones had a ratio greater than 10:1.

Results

Frequency of ARS-specific Precursor Cells in A/J Adult Mice. The data in Table I lists the frequency of ARS-specific precursor cells in 13 nonimmune A/J mice. The average frequency for the group was 1/68,000 splenic B cells, with all donors falling in a relatively narrow range of 6.3 to 30 per 10^6 B cells. Supernatants from fragment cultures containing anti-ARS antibodies were subsequently assayed for CRI_A in a competitive radioimmunoassay. This assay used a rabbit anti-CRI raised against purified A/J anti-ARS antibody and a CRI_A-positive hybridoma R16.7. The results in Table I show that only 7 of the 267 ARS-specific monoclonal antibodies analyzed were reactive on a 1:1 weight basis in this idiotype assay (CRI_A+); another 15 foci could be designated as CRI_A-cross-reactive ($CRI_A\pm$). Thus, the average frequency of CRI_A-positive B cells in this group of nonimmune A/J mice is 1 in 2.8×10^6 splenic B cells. Each donor had, at most, one focus positive for CRI_A, whereas there were no CRI_A+ clones in approximately one-half the individuals. This distribution is consistent with the random distribution of a rarely occurring clonotype.

The surprisingly low frequency of CRI_A+ precursor cells is in marked contrast to its dominant expression in the serum antibody (6) and primary plaque-forming cell responses (27). We questioned whether this finding accurately reflects the representation of this idiotype family in the nonimmune B cell pool. First, it is possible that the competitive radioimmunoassay used may not identify some molecules traditionally considered to be CRI_A+ in other idiotype assays. To test this, we compared the ability of a group of ARS-specific monoclonal antibodies to inhibit the interaction between

TABLE I

Frequency of ARS-specific Precursor Cells in A/J Adult Mice

Donor*	Total number cells transferred ($\times 10^6$)	Positive foci	Precursor frequency (per 10^6 B cells)‡	Percent of clones with CRI_A reactivity§		
				+	±	−
1	74	10	8.8	10	10	80
2	87	11	8.1	0	9	91
3	144	20	8.8	5	5	90
4	93	37	24.4	3	21	75
5	126	26	12.5	0	0	100
6	135	32	14.3	3	3	94
7	100	10	6.3	0	0	100
8	142	27	11.9	4	0	96
9	42	19	28.1	0	0	100
10	60	29	30.0	7	0	93
11	80	27	21.3	0	7	93
12	64	8	8.1	12	0	88
13	79	11	8.8	0	9	91
Totals/average	1,226	267	14.7 ± 8.1	3	6	91

* Spleen cells (15–20 × 10^6) from each donor were transferred into 4–8 Hy-primed recipients.

‡ Calculation based on a cloning efficiency of 4% for the splenic focus assay and the percentage of B cells in the spleen (25).

§ Monoclonal antibodies reacting on a 1:1 weight basis in the anti-ARS and idiotype assays were designated CRI_A positive(+); partial reactivity was defined as an anti-ARS/idiotype ratio of from 2:1 to 10:1; CRI_A-negative clones (CRI_A−) had ratios greater than 10:1.

TABLE II

Correlation between CRI_A Assays Using Hybridoma Protein R16.7 or Purified Anti-ARS Antibodies as Labeled Ligand

Number of clones with CRI_A reactivity using anti-ARS	Number of clones with CRI_A reactivity using R16.7		
	+	±	−
+	2	2	0
±	1	10	6
−	0	0	52

rabbit anti-CRI and labeled serum anti-ARS antibody. This assay may detect broader idiotypic determinants than identified by the use of R16.7 as the labeled ligand. 73 monoclonal antibodies derived from A/J nonimmune spleen fragment cultures were assayed in parallel for both idiotypic specificities. Table II lists the number of clones falling into each reactivity pattern and reveals generally good correlation between the two assays. Approximately 10% (6 out of 58) of the antibodies designated as CRI_A− were found to be cross-reactive in the assay using serum anti-ARS as ligand. All of these antibodies had anti-ARS/idiotype ratios close to 10:1 and never gave >20% inhibition in the radioimmunoassay. Therefore, both assays appear to identify the major CRI_A public specificity and yield equivalent results in the analysis of these ARS-specific monoclonal antibodies.

Effect of Immunization on Precursor Frequency and Representation of CRI_A in A/J Mice. Another possible explanation for the low representation of the CRI_A family in the nonimmune B cell pool is that the spleen fragment culture conditions may not efficiently stimulate the population containing CRI_A+ precursor cells, perhaps because of the form of antigen used or because of the absence of an idiotype-specific helper T cell (28). These questions can be addressed by examining the ARS-specific B cell repertoire of A/J mice immunized with an ARS-protein conjugate. It can be predicted that the dominant expression of CRI_A in the anti-ARS serum response after immunization should be paralleled by a rise in the frequency of CRI_A+ B cells in splenic fragment cultures. Thus, only the source of donor cells would change from the study of the nonimmune repertoire. In addition, these studies can determine the effect of immunization on both the CRI_A+ and CRI_A− precursor cell populations.

10 A/J mice were immunized with 0.2 mg ARS-OA in CFA, and 6 of these mice were boosted 1 mo later with the same antigen in incomplete Freund's adjuvant. At various times after primary or secondary immunization, the mice were bled, their spleen cells transferred into Hy-primed recipients, and fragment cultures prepared and stimulated with ARS-TGG-Hy. As shown in Table III, A/J mice produced low levels of anti-ARS on primary immunization, which increased considerably on reimmunization. The proportion of CRI_A contained in the immune sera ranged from 30–100%; detectable levels of CRI_A were never demonstrated in nonimmune A/J serum. The effect of immunization on precursor frequency and representation of CRI_A is listed in Table III and depicted graphically in Fig. 1. The data demonstrate that, early after immunization, there is a decrease in splenic precursor frequency to undetectable levels (donors 1 and 2) followed by a rise in the frequency of ARS-specific precursor cells. The majority of these B cells produce antibody that is

TABLE III

Effect of Immunization on Precursor Frequency and Representation of CRI_A in A/J Mice

Donor	Number of immunizations*	Time after immunization	Serum		Total number cells transferred ($\times 10^6$)	Positive foci	Percent of clones with CRI_A reactivity‡		
			Anti-ARS	Percent CRI_A			+	±	−
			μg/ml						
1	1	10	21	40	34	0			
2	1	10	6	17	36	0			
3	1	24	22	47	36	3	33	33	33
4	1	24	18	102	39	15	73	0	27
5	2	20	135	114	46	47	68	17	15
6	2	20	150	43	38	57	79	10	11
7	2	34	2,400	30	27	20	70	15	15
8	2	34	1,160	43	27	18	94	0	6
9/10	2	60	930	31	84	72	22	22	55

* 10 A/J adult mice were immunized with 0.2 mg ARS-OA in complete Freund's adjuvant, followed, in some cases, with another immunization 30 d later in incomplete Freund's adjuvant. At the indicated times after primary or secondary immunization, mice were bled, their spleens removed, and 2–8 $\times 10^6$ cells transferred into synergic recipients for assay in the splenic focus system.

‡ Positive foci were assayed for the presence of CRI_A, as described in Table I.

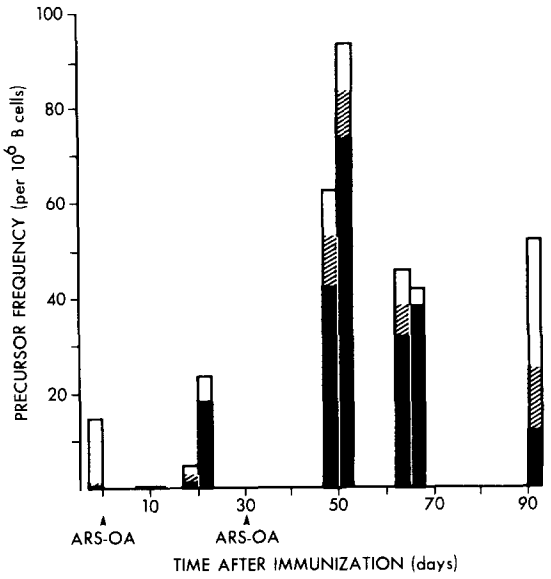

FIG. 1. Effect of immunization on ARS-specific precursor frequency and representation of CRI_A in A/J mice at various times after immunization. 10 A/J mice were immunized with 0.2 mg ARS-OA in CFA, followed by another immunization 30 d later in incomplete Freund's adjuvant. At the indicated times after immunization, spleen cells from the mice were transferred into syngeneic recipients for analysis in the splenic focus assay. Positive foci were assayed for the presence of CRI_A. The figure plots this data as precursor frequency (per 10^6 cells) vs. time after immunization. CRI_A+ clones (■), $CRI_A\pm$ clones (▨), and CRI_A- clones (□).

idiotypically identical to or cross-reactive with the CRI_A. During the peak expansion, the CRI_A+ precursor pool increases, on the average, over 100-fold, from $1/2.8 \times 10^6$ B cells in nonimmune mice to 1/17,500 B cells in donors 5 and 6. The $CRI_A\pm$ population undergoes a less dramatic increase in frequency, and the representation of this group is somewhat more variable from individual to individual.

The frequency of precursor B cells that produe CRI_A- antibodies initially decreases after immunization with ARS-OA and remains lower than preimmune levels for most of the time-course examined. Only at very late times after immunization does the CRI_A- population increase significantly (donors 9/10). Thus, the three idiotype families that can be defined at this level demonstrate independent kinetics after in vivo immunization. In addition, this experiment confirms the prediction that immunization leads to an almost exclusive increase in the CRI_A+ precursor cell population.

Ontogeny of ARS-specific Precursor Cells and CRI_A in A/J Mice. We next wanted to follow the acquisition of ARS-specific precursor cells during ontogeny and, in particular, determine when during development the CRI_A+ precursor cells arise. This experiment addresses whether the predominance of CRI_A in the adult A/J anti-ARS response is accounted for by its early appearance during the development of the B cell repertoire. Because the germ line V_H genes that code for CRI_A have been cloned and sequenced (29), the ontogeny of these specificities may also provide information on the early events that shape antibody diversity. The results shown in Table IV demonstrate that ARS-specific B cells are present in relatively high frequency at birth and in the early neonatal period, but there are no CRI_A+ clones in this population.

TABLE IV

Ontogeny of ARS-specific Precursors and CRI_A in A/J Mice

Age	Number of neonates*	Total number of cells transferred ($\times 10^6$)	Positive foci	Precursor frequency (per 10^6 injected cells)‡	Percent of clones with CRI_A reactivity§		
					+	±	−
days							
0	4	13.6	31	57.5	0	0	100
0	7	18.2	11	15.	0	0	100
1	6	15.4	8	12.8	0	0	100
3	8	30.4	47	37.5	0	0	100
3	4	15.2	18	30.	0	0	100
3	5	18.0	20	27.5	0	0	100
6	12	154	27	4.5	0	11	89
6	6	108	53	12.3	0	4	96

* Spleen cells from individual neonates were transferred into 1–4 syngeneic recipients and assayed for the presence of ARS-specific precursor cells in the splenic focus system.

‡ Calculation based on a cloning efficiency of 4% (25).

§ Positive foci were assayed for the presence of CRI_A, as described in Table I.

TABLE V

Frequency of ARS-specific Precursor Cells in BALB/c Mice

Donor*	Total number cells transferred ($\times 10^6$)	Positive foci	Precursor frequency (per 10^6 B cells)‡	Percent of clones with CRI_A reactivity§		
				+	±	−
1	120	18	9.4	28	6	66
2	50	8	10.0	0	13	87
3	48	3	3.9	0	0	100
4	144	9	3.9	0	22	78
5	100	11	6.9	0	0	100
6	90	5	3.5	0	0	100
7	64	9	8.8	0	0	100
8	72	9	7.8	0	11	88
9	92	6	4.1	17	0	83
10	115	12	6.5	0	0	100
Totals/averages	895	90	6.5 ± 2.5	7	6	87

* Spleen cells (15–20 $\times 10^6$) from each donor were transferred into 4–8 Hy-primed recipients.

‡ Calculation based on a cloning efficiency of 4% for the splenic focus assay and the percentage of B cells in the spleen (25).

§ Positive foci were assayed for the presence of CRI_A, as described in Table I.

CRI_A± clones begin to arise at ~1 wk of age. The ontogeny of ARS-specific precursor cells follows a similar pattern in BALB/c neonates (23), and there is complete absence of CRI_A+ and CRI_A± clonotypes in the early neonatal period (data not shown).

It is interesting to note that the frequency of ARS-specific precursor cells in the

spleen falls during the 1st wk of life. This same phenomenon was observed previously in the development of the BALB/c ARS-specific repertoire, where there is a 20-fold decrease in relative frequency during the 1st wk of life (23). In the previous study, it was shown that the apparent decrease in frequency is due to the fact that the total number of ARS-specific B cells per spleen remains constant during the newborn period, at the same time that the number of B cells responsive to other antigens, and splenic B cells in general, is increasing logarithmically.

Frequency of ARS-specific Precursor Cells in BALB/c Mice. BALB/c mice do not express CRI_A in their anti-ARS immune response. However, as shown in the PC-specific repertoires of some inbred strains (15–18), there can be a dramatic discrepancy between what is present at the clonal precursor cell level and what is expressed in the serum antibody response. Therefore, we wanted to determine whether BALB/c mice have the potential to express CRI_A in their nonimmune repertoire, even though they never use this specificity in the production of anti-ARS antibody. Table V lists the frequency of ARS-specific precursor cells in BALB/c mice and the representation of CRI_A in 90 foci that were analyzed. 2 of 10 BALB/c mice assayed had CRI_A+ clones in their nonimmune precursor cell pools, and several other individuals possessed $CRI_A\pm$ clonotypes among the monoclonal anti-ARS antibodies evaluated. Overall, the representation of these specificities was no different than that observed in nonimmune A/J adult mice, although the distribution of CRI_A+ B cells in BALB/c mice did not fit the random distribution seen in A/J donors. The ARS-specific precursor frequency is similar to that in A/J adult mice (1 per 150,000 B cells) and agrees with previously published data (23).

Discussion

This report examines the frequency and clonal composition of the nonimmune and immune ARS-specific precursor cell pools in A/J and BALB/c mice. In the nonimmune A/J mice assayed in the splenic focus system, only 2.6% (7 out of 267) anti-ARS monoclonal antibodies possess the predominant CRI_A. Because ARS-specific cells are present at a frequency of 1/68,000 B cells in the A/J mouse, the frequency of this entire idiotype family is $1/2.8 \times 10^6$ splenic B cells. Upon immunization with ARS-protein conjugates, the representation of the idiotype increases to 20–80% of the ARS-specific repertoire, and the secondary precursor cell pool now accurately reflects the idiotype composition of the serum antibody response. Thus, there is a striking distinction between the representation of this clonotype family at the precursor cell level in the primary and secondary repertoires. In addition, BALB/c mice have the potential to generate CRI_A-positive precursor cells within their nonimmune repertoire because two of the BALB/c mice analyzed produced CRI_A+ monoclonal antibodies in splenic fragment cultures. The data provide evidence to suggest that a complex set of regulatory phenomena influence precursor cell and serum antibody expression.

Both the low frequency of CRI_A+ precursor cells in nonimmune A/J mice and the identification of CRI_A+ B cells in BALB/c mice were unexpected observations that rely on the sensitivity and specificity of the idiotype assay used. The rabbit anti-CRI used in this study was generously provided by Dr. A. Nisonoff and has been well characterized in his laboratory (6). The inhibition radioimmunoassay requires that anti-ARS antibody displace the strongly CRI_A+ hybridoma protein R16.7 from binding to the anti-CRI. Although it is conceivable that this assay may not identify

some molecules traditionally considered to be members of the CRI_A family in other assays, those culture supernatants observed to inhibit this interaction as well as R16.7, which include the BALB/c clonotypes, must be considered CRI_A+. Table II also shows that there is good correlation in the identification of CRI_A antibodies when either R16.7 or purified A/J anti-ARS antibody is used as the labeled ligand in the idiotype assay. Furthermore, the identification of up to 94% CRI_A+ precursors cells from immunized A/J mice and detection of the expected proportion of CRI_A in the serum of these mice suggest that we are not missing a significant proportion of CRI_A+ monoclonal antibodies in the analysis of the primary repertoire.

The notion that the primary anti-ARS response is heterogeneous has been implied in a number of previous studies and is supported by the data presented in this report. Amino acid sequence analysis of CRI_A+ and CRI_A− hybridoma proteins have shown that variations occur both in framework and hypervariable regions (4–6). Although there appears to be 90% homology between the heavy or light chains of any pair of CRI_A+ hybridoma proteins, identical sequences are rare, and the entire family has been estimated to consist of at least 100 different sequences (21). At the serological level, heterogeneity can be demonstrated by the variation in inhibitory capacity of hybridoma proteins in CRI assays and by the presence of private idiotypic determinants on individual hybridoma proteins (6, 21). Suppression of neonatal A/J mice with anti-idiotypic sera followed by immunization with an ARS-protein conjugate causes complete suppression of that idiotype, with little effect on the total anti-ARS response, and these hyperimmune suppressed mice produce anti-ARS antibodies that also possess private idiotypic determinants (30, 31). In light of the diversity revealed in the expressed ARS-specific repertoire, it is perhaps not surprising to observe the extensive heterogeneity at the precursor cell level in limiting B cell cultures of nonimmune mice.

Although the expressed repertoire may be very heterogeneous, it would appear that individual A/J mice use only a fraction of their available ARS-specific precursor cells. Conger et al. (27) recently examined the kinetics of the primary plaque-forming cell response in A/J mice after in vivo immunization with 500 μg ARS-Hy. They found that ~80% of the IgM plaque-forming cells and close to 100% of the IgG cells were CRI_A+ within the first 1–2 wk after immunization. The kinetic analysis strongly indicated that CRI_A+ IgM plaque-forming cells were preferentially switched to IgG in the primary response, and the authors postulate that CRI_A+ B cells receive more T cell help than CRI_A− ones. Because CRI_A+ precursor cells represent, on the average, only 2.6% of ARS-specific B cells, or ~100 CRI_A+ B cells per mouse, the findings suggest an almost exclusive expansion of this clonotype family on contact with antigen. Using the data of Conger et al. (27) and assuming 100% recruitment of available precursor cells into plaque-forming cells, CRI_A+ B cells must expand with a doubling time of 20 h; the later appearance of CRI_A− IgM plaque-forming cells might represent a slower expansion of the CRI_A− precursor cell pool or the rapid emergence of variants from the CRI_A+ population. Indeed, the CRI_A− clonotypes that are eventually expressed in the primary and secondary responses might represent the expansion of only a small portion of the nonimmune repertoire, perhaps as small as the CRI_A+ family.

At early times after immunization, when the plaque-forming cell response is at its peak, cells that are detectable in the splenic focus assay have been virtually eliminated

from the spleen (Table III, donors 1 and 2). Both CRI_A+ and CRI_A− precursor cells have decreased in frequency at this time point, and when B cells begin to reappear at 24 d after immunization, the CRI_A+ B cells now dominate. The depletion of precursor cells in the spleen is probably due to their differentiation into plaque-forming cells, which would not be "assayable" in the splenic focus system. It is important to note that contact with antigen affects both CRI_A+ and CRI_A− precursor cells in a similar manner. This observation implies that both groups undergo differentiation into plaque-forming cells but that only CRI_A+ B cells preferentially expand and produce the majority of long-lived memory B cells. Although CRI_A− B cells are affected by contact with antigen in vivo, they do not appreciably expand before their differentiation into plaque-forming cells, and they either generate few memory cells or the process proceeds at a much slower rate for this population.

The question of why CRI_A+ B cells preferentially expand to dominate the anti-ARS serum response is not specifically addressed by this study. It is clear that clonal dominance is not due to a high frequency of B cells committed to the production of CRI_A, as observed previously in an analysis T15-positive B cells within the anti-PC repertoire (32). Neither is idiotypic dominance reflected in an early appearance of these clonotypes during B cell development. Although it is possible that CRI_A+ precursors may have a higher affinity for ARS than the CRI_A− B cells in the nonimmune repertoire, immunochemical studies on CRI_A+ and CRI_A− serum antibody (33) and plaque-forming cells (27) would suggest that affinity is not a major determining factor. However, those experiments analyzed the expressed repertoire, not the potential repertoire, so that definitive conclusions cannot be reached about the affinity of CRI_A− monoclonal generated in splenic fragment cultures. The data presented in this report as well as experiments by other investigators (28, 34) are most consistent with the notion that T cells are involved in a regulatory network to control the dominant expression of major idiotype populations. In particular, it would appear that an idiotype-specific T helper cell preferentially expands the small CRI_A+ B cell population in nonimmune A/J mice, resulting in secretion of antibody into the serum, differentiation into plaque-forming cells, and generation of memory cells. Because the representation of CRI_A+ B cells is no different than that of the nondominant clonotypes in the ARS-specific repertoire, it is presumably the presence of these T helper cells that is responsible for the maintenance of CRI_A dominance in A/J mice.

If T cells do play a role in maintenance of clonal dominance, the data also suggest that such a mechanism may influence only rare specificities, whereas most clonotypes are not regulated in this fashion, i.e., the CRI_A− precursor cells. Perhaps idiotype-specific T helper cells exist only with reference to the 200–300 germ line V_H specificities, as found in antibody responses containing predominant idiotypes, such as the anti-ARS, PC, nitrophenyl, and dextran responses (1). The vast majority of clonotypes in the nonimmune repertoire, many of which may have lost their cross-reactive idiotypes due to somatic variation, would remain outside of such network interactions. According to this model, although BALB/c mice can generate the CRI_A specificity at the precursor cell level, perhaps as a somatic variant (see below), they do not normally express this clonotype in their serum anti-ARS response because they lack the appropriate T helper cell. This mouse strain does predominantly express the CRI_C family because it possesses the corresponding helper cell, and it would follow that CRI_C is encoded by a germ line V_H gene in BALB/c mice. Thus, the genetic

linkage of idiotype expression to V_H might not be due to the presence or absence of an immunoglobulin structural gene per se (because all strains might have the ability to carry the clonotype in their nonimmune B cell repertoire), nor would it be due to the ability to maintain a high frequency of the predominant idiotype in the precursor cell pool. Instead, predominant idiotype expression may require the presence of T cells that use or recognize the products of germ line V_H structural genes.

Support for the hypothesis discussed in the preceding paragraphs comes, in part, from the finding of CRI_A+ precursor cells in the BALB/c nonimmune repertoire. This observation mirrors previous experiments in the splenic fragment system, where it was found that C57BL/6 mice can have a significant representation of the T15 "framework" idiotype at the precursor cell level but do not express this idiotype in their serum anti-PC response (18). Lucas and Henry (35) also recently identified CRI_A+ plaque-forming cells in BALB/c mice after in vivo immunization with the T-independent antigen ARS-Brucella abortus. It is highly unlikely that monoclonal antibodies identified as CRI_A+ in BALB/c splenic fragment culture supernatants actually belong to the CRI_C idiotype family. The anti-CRI_A serum used in this study is only weakly cross-reactive with CRI_C, although some of the antibodies identified as $CRI_A\pm$ may be members of the CRI_C family.

Although CRI_A+ precursor cells were found in approximately one-half the individual A/J mice analyzed and were distributed in a random fashion, the BALB/c CRI_A+ clonotypes were clustered in two individuals. Because a V_H gene coding for CRI_A molecules has not been found in the germ line DNA of BALB/c mice (M. Gefter, personal communication), this specificity must have arisen via somatic diversification in those individuals. Given the probability of the somatic events that might be required for the generation of such CRI_A+ B cells, this clonotype might recur relatively rarely in the BALB/c strain as a whole. Indeed, Lucas and Henry (35) identified CRI_A+ plaque-forming cells in 3 of 17 BALB/c mice, which is similar to our detection of CRI_A+ precursor cells in 2 of 10 individuals. Thus, although not all BALB/c mice may possess this idiotype family, because it must arise as a somatic variant in this strain, the critical point remains that these individuals who have generated CRI_A+ B cells do not express this specificity in their serum anti-ARS response when challenged with a T-dependent antigen (6, 10 and N. Sigal, unpublished results). The observation that the CRI_A specificity can be found in the nonimmune BALB/c repertoire is similar to the identification of an M167-like clonotype, also a known somatic variant, among PC-specific IgM-producing precursor B cells (15). Thus, these data support the contention that antibody diversification occurs early in B cell differentiation.

The ontogeny of ARS-specific B cells in A/J and BALB/c mice follow similar kinetics, with relatively high precursor frequencies in the newborn period, followed by a decline in the overall frequency of ARS-specific cells during the first week of life (23). This decline in frequency has been shown to reflect the fact that the total number of ARS-specific B cells per spleen remains constant during this period, at the same time that the number of splenic B cells is increasing. The late appearance of CRI_A+ B cells in the A/J spleen parallels the ontogeny of the T15 clonotype in the BALB/c neonate (26). Whereas the earliest arising PC-specific clonotypes are T15 positive, a large number of ARS-specific precursor cells develop before the appearance of CRI_A+ B cells in the A/J mouse. We have not ruled out the possibility that

CRI_A+ B cells may first develop in the fetal or neonatal liver and never reach the spleen in appreciable numbers. Nevertheless, the findings reported here support previous conclusions that the ontogenetic development of predominant or "germ line" antibody specificities might be no different than the vast majority of nondominant clonotypes (26). Finally, it is interesting to note that monoclonal antibodies designated as $CRI_A\pm$ were identified in 6-d-old A/J spleens. Some of these idiotypic specificities may be generated as somatic variants from the germ line V_H gene pool responsible for the CRI_A+ idiotype family. If this is the case, the results would also suggest that antibody diversification occurs at the stem cell stage and in the absence of antigenic contact. Because the V_H genes coding the CRI_A have been cloned and sequenced (29), mapping the ontogeny of certain private idiotypes known to be present only on variants of the germ line genetic information might provide a more precise understanding of the mechanisms of clonotype diversification.

Summary

A large proportion of *p*-azophenylarsonate (ARS)-specific antibodies from A/J mice share a cross-reactive idiotype (CRI_A) that comprises a family of closely related but nonidentical clonotypes. I determined that only 2.6% (7 out of 267) A/J ARS-specific monoclonal antibodies generated in the splenic focus system possess the predominant CRI_A. Because ARS-specific B cells are present at a frequency of 1/68,000 B cells, the frequency of the entire idiotype family is 1 per 2.8×10^6 splenic B cells. Thus, there is a striking discrepancy between the representation of this idiotype at the clonal precursor cell level and the serum antibody response. In addition, BALB/c mice have the potential to generate CRI_A-positive precursor cells within their nonimmune repertoire. When A/J mice are immunized with ARS-protein conjugates, the serum antibody response and precursor cell population are both dominated by CRI_A. The frequency of CRI_A-positive B cells increases over 100-fold after immunization, whereas CRI_A-negative precursor cells may initially decrease, followed by a later rise in frequency. Finally, although ARS-specific precursor cells are present in high frequency at birth, CRI_A-positive monoclonal anti-ARS antibodies are not observed during the early neonatal period. These data provide evidence to suggest that complex regulatory networks influence precursor cell and serum antibody expression.

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