GENETIC AND TEMPORAL CONTROL OF NEONATAL ANTIBODY EXPRESSION*

By KATHLEEN A. DENIS‡ and NORMAN R. KLINMAN

From the University of California at Los Angeles, Los Angeles, California 90024; and the Department of Immunology, Scripps Clinic & Research Foundation, La Jolla, California 92037

Studies of B lymphocytes at the nucleic acid level have confirmed the potential for the expression of an extremely diverse antibody repertoire and have provided insights into the molecular processes involved in this expression (1–7). In addition, studies at the phenotypic level have increased our understanding of the mechanisms that regulate this genetic information and determine the expression of each of the many antibody permutations. These mechanisms include tolerance induction (8–14) and antiidiotypic suppression (15, 16) of developing B cell clones, as well as idiotype-specific mechanisms that may be responsible for clonal expansion (17, 18).

Among the most striking features of the immune mechanism is the extraordinary reproducibility of repertoire expression (19–24). Thus, in spite of the extremely complex mechanisms available for diversification and clonotype selection, repertoire sharing appears to be the rule among genetically identical individuals. This is particularly true in situations where the repertoire is limited such as in genetically identical frogs (19), bursectomized chickens (20), and, most importantly, neonatal mice (21–24). Additionally, where it has been possible to evaluate the expression of clonotypes that occur in high frequency, or even rare clonotypes that are idiotypically identifiable, adult mice of the same strain apparently share repertoire (25–33).

To initiate an evaluation of the mechanisms, both genetic and regulatory, that may be responsible for the reproducible acquisition and expression of the antibody repertoire, we have undertaken to establish highly specific idiotypic assays that can identify early neonatal clonotypes. This has been accomplished by producing hybridomas with antibody-producing cells derived from dinitrophenyl (DNP)[1]-responsive neonatal B cells. The use of these monoclonal antibodies and antiidiotypic reagents has confirmed several previously established basic tenants of neonatal responses (21). In addition, the availability of large quantities of monoclonal neonatal antibodies has enabled a careful characterization of neonatal antibodies in terms of their physicochemical and antigen-binding properties. Most important, the availability of anti-idiotypic assays for neonatal antibodies has enabled a more precise definition of the neonatal DNP specific B cell repertoire of BALB/c mice and an evaluation of the expression and control of these clonotypes, in both developing and mature mice, at

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

‡ Supported by National Institutes of Health training grant CA-09140 from the Dept. of Pathology, University of Pennsylvania.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DNP, dinitrophenyl; Hy, hemocyanin; IEF, isoelectric focusing; PBS, phosphate-buffered saline; PC, phosphorylcholine; pI, isoelectric point; RIA, radioimmunoassay.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/83/04/1170/14 $1.00
Volume 157 April 1983 1170–1183

the level of clonal precursors and serum antibodies. These assays have permitted an evaluation of the expression of these clonotypes during the lifetime of allotype allogeneic murine strains and F_1 hybrids.

Materials and Methods

Animals. Pregnant BALB/c mice were obtained from Ace Animals, Philadelphia, PA. Neonatal and adult BALB/c, B10.D2, (BALB/c × B10.D2)F_1, (BALB/c × A/J)F_1, CB.20, and (BALB/c × CB.20)F_1 mice were obtained from the colony of M. P. Cancro, University of Pennsylvania, or the colony of Scripps Clinic and Research Foundation. New Zealand red rabbits were obtained from Burley Ranch, Bonita, CA.

Antigens. Hemocyanin (Hy) (Worthington Biochemicals, Freehold, NJ) and bovine serum albumin (BSA) (Calbiochem-Behring Corp., San Diego, CA) were coupled to 2,4-DNP sulfonate (Eastman Laboratory and Specialty Chemicals, Rochester, NY) using previously described methods (8, 34). The unbound hapten was removed by column chromatography and the substitution ratio of the coupled protein was determined. All haptenated proteins used in this study had a substitution ratio of ~10 mol of DNP/100,000 g of protein.

Splenic Fragment System. The splenic fragment system has been described in great detail elsewhere (21, 34). Briefly, limiting numbers of spleen cells ($2–4 \times 10^6$) obtained from neonatal or adult donors were injected into lethally irradiated BALB/c adult mice that had been immunized by the intraperitoneal injection of 0.1 mg Hy 2 mo previously. The following day, the recipient spleen was removed asceptically and chopped into 1-mm cubes. These fragments were individually placed in culture and stimulated with DNP-Hy (10^{-6} M for DNP) for 3 d. The culture fluids were then changed and collected every 3 d and were assayed for anti-DNP antibody production on days 10 and 13.

Tolerance susceptibility of B cells is assessed in this system by the addition of the tolerogen (DNP-BSA, 10^{-6} M for DNP) to cultures for 24 h before the addition of the immunogen (DNP-Hy) (8, 12).

Radioimmunoassay. The solid-phase radioimmunoassay (RIA) has been previously described (21, 34, 35). Briefly, polyvinylchloride plates (Dynatech Laboratories, Inc., Arlington, VA) were coated with DNP-BSA and blocked with 5% horse serum in buffer containing 0.2 M sodium phosphate pH 7.2 and 0.15 M sodium chloride (PBS). After washing, 20 μl of culture fluid or a dilution of hybridoma antibody or serum was added and allowed to incubate 2–4 h. After washing, the plates were incubated overnight with ^{125}I-labeled rabbit antimouse Fab or class-specific reagents (courtesy of M. P. Cancro). All ^{125}I-labeled antibodies in this study, including the rabbit anti-mouse reagents and the hybridoma antibodies used in the antiidiotype inhibition assay, were labeled by the method of Klinman and Howard (36).

Transfer Fusion. Transfer fusions were carried out by a previously described modification (37) of the Kohler and Milstein (38) procedure for preparing hybridomas. The SP2/0 Ag14 myeloma line (39) was obtained from the laboratory of R. H. Kennett, University of Pennsylvania. Lethally irradiated (800 rad) Hy-primed adult (BALB/c × A/J)F_1 mice were injected intravenously with 4×10^6 neonatal BALB/c spleen cells on day 0. On day 1, the recipient mouse was intravenously injected with 100 μg of DNP-Hy in PBS. The spleen was removed 5 d thereafter and was fused with myeloma cells at a 10:1 ratio of spleen to myeloma cells using an 8-min polyethylene glycol exposure. The cells were then distributed into microtiter dishes in hypoxanthine, aminopterin, thymidine medium and wells with growth were assayed for anti-DNP antibody. Cells of positive wells were subcloned and grown in bulk culture and in ascites form in pristane primed BALB/c mice. The H-2 haplotype of hybridoma cell lines was determined by treatment of the cells with either anti-H-2D or anti-H-2K antisera plus rabbit complement (40).

Purification and Characterization of Anti-DNP Antibodies. The anti-DNP antibodies were purified from ascites by passing clarified ascitic fluid over a DNP-BSA-Sepharose 4B column and eluting the anti-DNP antibodies with 0.5 M acetic acid (37). Association constants of purified antibodies or antibodies in ascitic fluid were determined by equilibrium dialysis by a method that has been described previously (34, 37, 41). The isoelectric point of hybridoma antibodies was

determined by a sucrose density gradient isoelectric focusing (IEF) method that was previously shown to permit analysis of small amounts of IgM antibodies (21, 42, 43).

Production of Antiidiotype Antibodies. Rabbits were injected at multiple sites subcutaneously with 3 mg purified antibody emulsified in complete Freund's adjuvant. The rabbits were ear bled 20–30 d after each of three bimonthly injections. The resulting serum was then absorbed extensively on Sepharose 4B coupled to a BALB/c anti-DNP specific hybridoma antibody of the same heavy chain and light chain class as well as Sepharose 4B coupled to normal murine immunoglobulin.

Antiidiotype RIA. The idiotypic composition of serum and monoclonal antibodies was assessed by a plate binding competition assay which has been previously described (30, 43). Serial dilutions of each rabbit antiidiotype serum in PBS were bound directly to polyvinylchloride plates at room temperature for 4 h. After blocking the wells with irrelevant protein, various dilutions of ^{125}I-labeled hybridoma protein to which the antiidiotype was raised were added and the plates were incubated overnight at 37°C. After determination of the optimal antiidiotype and labeled idiotype dilutions, unlabeled idiotype or other hybridoma or myeloma proteins were used as competitors of the antiidiotype-idiotype reaction to assess its specificity. For determination of idiotype presence in monoclonal splenic fragment culture fluids, 20 μl of culture fluid was added as the inhibitor. For determination of idiotype presence in mouse sera, 20 μl of various dilutions of mouse sera were added as the inhibitor. Culture fluids were designated positive for the idiotype only if their inhibition of binding of the ^{125}I-labeled idiotype to the antiidiotype plate was equivalent (±20%) on a weight basis to the inhibition by the homologous idiotype. Final dilutions of rabbit antiidiotypes used were $\sim 10^{-3}$. Iodinated idiotype was used at between 2 and 5 ng/well.

Results

Obtaining Hybridomas by Adoptive Transfer Fusions. Table I presents the frequencies of anti-DNP-producing hybridomas when BALB/c neonatal spleen cells were transferred into irradiated, Hy-primed (BALB/c × A/J)F_1 adults and stimulated with 0.1 mg DNP-Hy injected intravenously. The use of F_1 recipients enabled a demonstration of the donor origin of the hybridomas by virtue of the presence on their surface of H-2D haplotype alloantigens and the absence on their surface of H-2K haplotype alloantigens as determined by complement-mediated cytotoxicity. If no neonatal cells were transferred to recipients, then no growing hybrids were obtained. When neonatal cells alone were injected and antigen not given, occasional hybrids were obtained, but they did not secrete anti-DNP antibody. Injecting both neonatal cells and DNP-Hy caused a marked increase in the number of growing hybrids and several of these displayed anti-DNP antibody activity. Of the 15 anti-DNP hybridomas obtained in this manner, 2 have been subcloned and used for further study.

Characterization of Hybridoma Antibodies Derived from Neonatal B Cells. Table II presents

TABLE I

Adoptive Transfer Fusions

Number of cells* injected	DNP-HY injected	Number of hybrids	Number of anti-DNP-specific hybrids
0	100 μg	0	0
4×10^6	0	18	0
4×10^6	100 μg	107	13
4×10^6	100 μg	41	2

* 4×10^6 cells were injected intravenously into irradiated (800 rad) Hy-primed mice 24 h before antigen stimulation. Fusions were carried out 5 d thereafter and anti-DNP antibody production was assessed by RIA (35).

TABLE II

Characteristics of Two Neonatal Anti-DNP Hybridomas

Hybridoma	Ig class*	Ko (4°C)‡	pI (± 0.05)§
TF2-36	μ, κ	$2.6 \times 10^6\ M^{-1}$	5.05
TF2-76	μ, κ	$6.0 \times 10^5\ M^{-1}$	4.60

* Immunoglobulin heavy and light chain composition were determined by RIA (35).

‡ Binding constants were determined by equilibrium dialysis at 4°C (41). Binding by both antibodies extrapolated to 10 combining sites per molecule.

§ pI were determined by microsucrose gradient IEF (42).

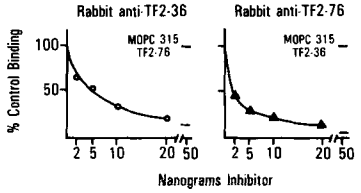

FIG. 1. Specificity of rabbit antiidiotypic antisera. The relative inhibitory patterns of both homologous and heterologous inhibitors in the competition RIA for the two antiidiotypic reagents.

the isotypes, hapten binding characteristics, and isoelectric points (pI) of the two hybridoma antibodies derived from neonatal B cell fusions. Consistent with the neonatal origin of these antibodies, both express μ heavy chains.

The hapten binding characteristics of these two monoclonal antibodies are interesting in several respects. As will be demonstrated below, the TF2-36 hybridoma antibody is indistinguishable from a large proportion of very early neonatal anti-DNP antibodies. Interestingly, this monoclonal antibody displays an association constant for the DNP haptenic determinant that is slightly higher than the average of association constants displayed by monoclonal antibodies derived from normal adult donors (34). This finding contrasts with evidence from other laboratories (44, 45) suggesting relatively low affinities of early neonatal anti-DNP antibodies. The TF2-76 monoclonal antibody displays a somewhat lower affinity than TF2-36 but is also well within the range of affinities of normal adult primary monoclonal antibodies (34).

The TF2-36 hybridoma antibody displays a pI of 5.05, which is one of the three pI characteristic of a majority of monoclonal anti-DNP antibodies derived from BALB/c mice during the first 5 d after birth (21). It would appear, therefore, that antibodies of this idiotype comprise at least a subset of early neonatal anti-DNP antibodies previously identified as displaying a pI in this range.

Antiidiotypic Analysis of Hybridoma Antibodies Derived from Neonatal B Cells. Fig. 1 depicts the reactivity of the two adsorbed rabbit antiidiotypic antisera, anti-TF2-36 and anti-TF2-76, in a solid-phase competition RIA. Each was tested against a large panel of immunoglobulins including other anti-DNP neonatal antibodies, as well as MOPC-460 and MOPC-315, two anti-DNP-reactive myeloma proteins. Both of the antiidiotypes were completely specific for the immunizing antibody.

A particularly important feature of these antiidiotype competition assays is their extreme sensitivity. Each assay is set up such that 50% inhibition of the binding of 2–5 ng of ^{125}I-labeled idiotype is achieved by 2–5 ng of unlabeled homologous

competitor. The stoichiometry and sensitivity is a critical feature of an antiidiotype assay used to screen monoclonal antibodies derived from splenic fragment cultures for idiotype since culture fluids used as competitors for the antiidiotype most often contained <10 ng of anti-DNP antibody.

Idiotypic Analysis of Monoclonal Anti-DNP Responses of B Cells from BALB/c Mice at Various Ages. A survey of anti-DNP antibodies derived from BALB/c spleen cells from mice ranging in age from 1 d after birth to 8 wk was done to determine the representation of these two idiotypes among the various B cell populations. This is depicted graphically in Fig. 2. The temporal pattern represented by TF2-36 was expected from earlier studies of the neonatal repertoire (21), in that this neonatal idiotype is found expressed by a fairly high proportion of the anti-DNP specific B cells during the first few days after birth and subsequently decreases. The idiotype represented by TF2-36 comprises 14% of the anti-DNP antibodies from spleen cells 3–4 d after birth, falls to ~2% 7–8 d after birth, and remains at this level into adulthood.

To confirm the homology of the TF2-36-positive monoclonal antibodies derived from fragment culture to the hybridoma antibody, IEF analysis was carried out. Seven IgM antibodies that displayed the TF2-36 idiotype derived from both adult and neonatal B cells were subjected to microsucrose density gradient IEF. All of the antibodies positive for the TF2-36 idiotype showed an IEF spectrum indistinguishable from TF2-36. Thus, by two criteria, idiotype analysis and IEF, antibodies bearing the TF2-36 idiotype represent a predominant anti-DNP specificity of the early neonatal BALB/c repertoire. Since the previous studies of the early BALB/c neonatal anti-DNP repertoire showed IgM monoclonal antibodies with an isoelectric point in the range of 5.05 represented ~35–45% of all anti-DNP antibodies (21), it may be assumed that at least one other anti-DNP antibody bearing a different idiotype migrated indistinguishably from TF2-36 in IEF.

The idiotype represented by TF2-76 showed quite a different pattern of temporal expression. This idiotype was present in <1% of anti-DNP antibodies secreted by spleen cells obtained during the first few days after birth. By days 7–8, the proportion of anti-DNP antibodies bearing this idiotype began to rise and reached a peak of almost 7% on days 12–13.

Confirmation of the Temporal Acquisition of Clonotypes by the Tolerance Marker. Several

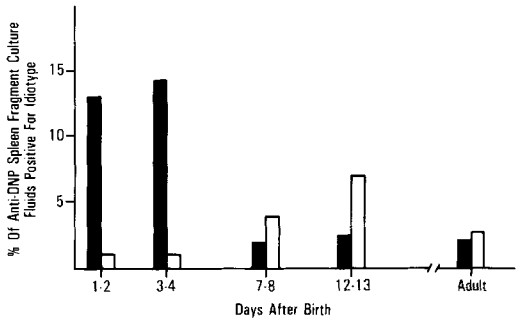

FIG. 2. The temporal expression of idiotype. The temporal expression of the TF2-36 (■) and TF2-76 (□) idiotypes among monoclonal anti-DNP antibodies derived from BALB/c spleen cells of various ages in splenic fragment cultures.

years ago, it was demonstrated that phosphorylcholine (PC)-responsive B cells appear in the splenic B cell repertoire several days later in development than do B cells responsive to DNP (23). This finding was confirmed by the fact that the majority of B cells responsive to PC are immature by the criteria of their susceptibility to in vitro tolerance induction as late as 10 d after birth, a time when the vast majority of B cells responsive to DNP are sufficiently mature to be tolerance-resistant (12). This finding not only confirmed the relatively late developmental acquisition and maturation of PC responsive B cells, but also established the principle that tolerance susceptibility is a characteristic of the developmental status of individual B cell clones, rather than the developmental status of the animal as a whole. The finding that the TF2-76 idiotype is displayed primarily by B cells that arise several days later than those bearing the TF2-36 idiotype provided an opportunity to confirm these concepts in a system in which both mature and immature B cells would be responding to the same haptenic determinant. To determine if B cells bearing the TF2-76 idiotype actually represented a B cell population that matures a week or so later in splenic development than cells bearing the TF2-36 idiotype, in vitro tolerance susceptibility of TF2-76- and TF2-36-bearing day 7 BALB/c B cells was assessed (Table III). While the majority of DNP-specific day 7 splenic B cells including those bearing the TF2-36 idiotype were unaffected by the tolerization regimen, those bearing the TF2-76 idiotype were significantly ($P = 0.01$) decreased. Thus, it appears that TF2-76 represents a relatively late-appearing and -maturing B cell clonotype.

The Distribution of BALB/c Neonatal Clonotypes in Other Murine Strains. The idiotypic reactivity of monoclonal anti-DNP antibodies derived from B cells of various murine strains in splenic fragment culture is shown in Table IV. Represented are antibodies derived from spleen cells during the 1st and 2nd wk after birth, as well as those obtained from adult mice of the BALB/c, CB.20, and (BALB/c × CB.20)F_1 strains. It can be seen that monoclonal antibodies bearing either the TF2-36 or TF2-76 idiotypes are not present in high frequency at any time during the development of mice bearing the Igh^b allotype-idiotype locus. Thus, as with the vast majority of identifiable idiotypes that have been found to predominate adult repertoires (25–31), at least the magnitude of neonatal idiotype expression within the repertoire is polymorphic, and this expression links to the Igh locus. This finding confirms the polymorphism of immunoglobulin variable region expression and (in the case of TF2-

TABLE III

Effect of In Vitro Exposure to DNP-BSA on the Frequency of Monoclonal Anti-DNP Antibody Responses and Their Idiotype Distribution

Spleen cell donor	Total number spleen cells transferred*	Tolerogen (10^{-6} M DNP-BSA)	DNP-specific cells per 10^6 splenic B cells‡	Idiotype-positive§	
				TF2-36	TF2-76
BALB/c d 7	90×10^6	–	60.6	2.2	4.4
BALB/c d 7	66×10^6	+	60.2	2.6	1.3‖

* Pooled data from four experiments.

‡ Figures given are corrected for homing and cloning efficiency of 4% for neonatal splenic B cells (8, 21).

§ A total of 137 monoclonal antibodies derived from day 7 spleen cells not exposed to tolerogen and 75 derived from day 7 spleen cells exposed to tolerogen were analyzed.

‖ Using 2 × 2 contingency table χ^2 analysis = 6.16, $P = 0.01$.

TABLE IV

Idiotypic Reactivity of Monoclonal Anti-DNP Antibodies Derived from Splenic Fragment Cultures

B cell donor	Igh locus	Age	Total number of monoclonal antibodies tested	Percent positive	
				TF2-36*	TF2-76
		d			
BALB/c	a	1–6	568	13.5	1.1
		7–13	227	1.8	4.8
		Adult	196	2.0	2.5
CB.20	b	1–6	276	1.1	0.7
		7–13	198	0.5	0.5
		Adult	108	0	0
(BALB/c x CB.20)F_1	a/b	1–6	64	10.9	1.6
		7–13	92	1.1	3.3
		Adult	108	0.9	0.9

* Idiotype analyses were carried by competitive inhibition of plate binding using individual monoclonal antibodies. Antibodies were considered positive for idiotype if inhibition of binding of ^{125}I-labeled homologous idiotype was ±20% that of unlabeled homologous idiotype on a weight basis.

36) extends it to the very earliest of expressed clonotypes. Also, consistent with previous studies of several predominant idiotypes (17, 18, 46–53), antibodies bearing idiotypes indistinguishable from the two BALB/c neonatal clonotypes can be observed at low frequency in strains in which they do not predominate. Thus clones bearing both the TF2-76 and TF2-36 idiotypes have been found in strains bearing the Igh^b heavy chain locus. This finding was confirmed by an analysis of 95 monoclonal antibodies derived from B10.D2 neonates in which we found a total of three monoclonal antibodies that expressed the TF2-36 idiotype and one that expressed the TF2-76 idiotype. Although Igh^b neonates express these clonotypes, they are exceedingly rare in adults. In an analysis of 108 monoclonal antibodies derived from CB.20 adult spleen cells and 72 monoclonal antibodies derived from B10.D2 adult spleen cells only, one antibody bore the TF2-36 idiotype, and none bore the TF2-76 idiotype. Since it must be assumed that antibodies derived from strains differing in the Igh locus would focus at different pI, the identity of monoclonal antibodies bearing the TF2-36 and TF2-76 idiotypes derived from Igh^b strains could not be confirmed by IEF.

Also shown in Table IV is the analysis of the expression of the two idiotypes in neonates and adults of F_1 hybrids constructed between BALB/c and CB.20 parents. It can be seen that both clonotypes are expressed in a temporal fashion consistent with their expression in the BALB/c parent. Similarly, in an analysis of 96 monoclonal antibodies derived from 1-6-d-old (BALB/c × B10.D2)F_1 neonates, 10 antibodies displayed the TF2-36 idiotype. Thus the expression of these two neonatal idiotypes would appear to be consistent with codominant expression of variable region specificities. While codominant expression of variable region specificities has been demonstrated for clonotypes that predominate in adult repertoires (26–29), previous reports have indicated that the temporal expression of repertoire may differ between F_1 and parental strains (51).

Idiotypic Analysis of Serum Anti-DNP Responses. The various strains of mice analyzed

TABLE V
Idiotypic Reactivity of Anti-DNP Antisera

Strain	Igh	Immunization	Anti-DNP antibody*	Inhibition‡ of	
				Anti-TF2-36	Anti-TF2-76
			mg/ml		
BALB/c	a	1° DNP-HY§	1.2	6	3
		2° DNP-HY‖	10.3	15	10
CB.20	b	1° DNP-HY	0.7	<1	<1
		2° DNP-HY	8.1	<1	<1
B10.D2	b	1° DNP-HY	0.9	<1	<1
		2° DNP-HY	9.6	<1	<1
(BALB/c x B10.D2)F_1	a/b	1° DNP-HY	1.8	3	<1
		2° DNP-HY	12.5	12	4

* Values from pooled serum of five mice determined by RIA.
‡ Percent inhibition = [(number of counts bound in well containing 1/μl normal serum) − (number of counts bound in well containing 1/μl immune serum)]/[number of counts bound in well containing 1 μl normal serum].
§ Serum taken 10 days after 100 μg DNP-HY in complete Freund's adjuvant injected intraperitoneally.
‖ Serum taken 10 d after second DNP-HY injection (30 d after 1°).

at the monoclonal level for the expression of these neonatal anti-DNP idiotypes were further examined at the level of anti-DNP serum antibody. Adult mice were immunized with DNP-Hy and serum was collected during both primary and secondary responses. These sera were then evaluated for their content of anti-DNP antibodies as well as their ability to compete in the antiidiotype RIA (Table V). Since no determination of idiotype sharing could be made on a weight basis, as was done for monoclonal antibodies, the percent inhibition of ^{125}I-labeled idiotype binding to the antiidiotype plate by 1 μl of immune serum was calculated. Only BALB/c and (BALB/c × B10.D2)F_1 anti-DNP antisera contained detectable amounts of antibodies either identical or cross-reactive with the two neonatal anti-DNP antibodies. The small amounts of inhibition obtained, even with BALB/c anti-DNP antisera, indicate a very low concentration (~1 μg/ml in secondary sera) of antibodies bearing these idiotypes in serum. Thus, while in the BALB/c and F_1 adults these clonotypes are expressed by 1–2% of DNP-specific precursor cells, antibodies derived from these precursors would appear to represent no more than 1/10,000 of the total anti-DNP serum antibody response. The inordinately low expression of these antibodies in adult immune serum is similar to disparities in serum antibody vs. precursor cell frequency for the TEPC-15 idiotype in certain strains (47) and presumably reflects the multifactorial control of serum antibody expression.

Discussion

Although mechanisms have been defined that can readily account for an extraordinarily diverse antibody repertoire (1–7), previous studies have indicated that neonatal mice have a relatively limited repertoire and that repertoire diversity is gradually acquired in a highly reproducible manner within an inbred murine strain (21–24). To investigate the characteristics of early neonatal antibodies and probe the mechanisms that may be responsible for repertoire acquisition, we have established two hybridoma cell lines derived from neonatal B cells antigenically stimulated in an

irradiated adoptive host. In this case, the adoptive host strategem was necessary to provide carrier-primed T cells, which have been shown previously to facilitate responses of neonatal B cells.

The availability of well-defined neonatal hybridoma antibodies and sensitive antiidiotypic assays for their identification has enabled us to refine and extend earlier studies of the developmental acquisition of the anti-DNP repertoire in BALB/c mice. One of the two IgM hybridoma antibodies, TF2-36, is indistinguishable by idiotypic and IEF analyses from the monoclonal antibody product of 10–15% of DNP-specific BALB/c B cells during the first few days after birth. The finding of an identifiable clonotype that is present in high frequency in the B cell population of early neonates of a given strain confirms previous indications that early neonatal repertoire expression is both restricted and reproducible. In particular, cells of the TF2-36 clonotype represent 1 in 10^4–10^5 of all BALB/c B cells during the first few days after birth. In comparison with previous findings concerning the IEF spectrum of early neonatal anti-DNP antibodies (21), clones of the TF2-36 clonotype represent approximately one-third of all clones that isofocus at a pI of ~5.05. Thus, careful analysis of repertoire by both idiotypic and IEF analyses should ultimately yield a more comprehensive overview of the exact extent of repertoire diversity than did analysis by IEF alone.

The availability of idiotypic analysis of the expression of the TF2-36 clonotype has also permitted a previously unavailable assessment of the fate of a predominant neonatal clonotype during the course of murine development. The findings indicate that the relative representation of B cells bearing this clonotype wanes rapidly towards the end of the first week of neonatal development. However, B cells of the TF2-36 clonotype continue to be expressed even in BALB/c adults. Thus, B cells of a given clonotype can be expressed continuously, and early neonatal expression neither precludes adult expression, nor does it necessarily correlate with predominant expression in the adult repertoire.

The developmental acquisition of B cells expressing the TF2-76 clonotype is strikingly different from that of B cells expressing the TF2-36 clonotype. The temporal expression of this clonotype would seem much more similar to certain anti-influenza hemagglutinin specificities, in that its proportional representation within the B cell repertoire reaches a maximum towards the end of the 2nd wk after birth (24). The identification of a clonotype that is apparently expressed in all BALB/c neonates in high frequency as late as 2 wk after birth confirms the conclusion that repertoire expression is highly patterned and reproducible within genetically identical individuals, even at a developmental stage wherein the entire repertoire includes $>10^6$ clonotypes (24). As with TF2-36, B cells of the TF2-76 clonotype continue to be expressed into adulthood in high numbers, although they represent but a small proportion of the highly diversified adult repertoire. Additionally, the relatively late acquisition of B cells of the TF2-76 clonotype has been verified by the relative susceptibility to tolerance of cells bearing this clonotype in the spleen 7 d after birth. The majority of DNP-specific B cells present at that time, including B cells of the TF2-36 clonotype, are resistant to in vitro tolerance induction.

The availability of antiidiotypic clonotype identification also provides a means to compare the expression of given clonotypes within the B cell repertoire of disparate murine strains. In the past, it has been the general case that clonotypes and families of clonotypes that can be identified in one strain are often either absent or present in

much lower frequencies in other murine strains, particularly strains that differ in the Ig heavy chain gene locus (25–33), and, where appropriate, the light chain gene locus (54). In the majority of cases, such disparities appear to be not absolute but rather quantitative (17, 18, 46–50). However, with rare exceptions (53), it remains unknown whether indistinguishable clonotypes present in two distinct strains are completely identical at the amino-acid sequence level, and whether genes expressing equivalent clonotypes in different strains are actually alleles of one another. The findings in this paper extend these comparisons to the control of clonotype expression in distinct strains at the earliest times in the neonatal development of the B cell repertoire. The findings indicate that for both the TF2-36 early clonotype and TF2-76 later clonotype, high frequency and temporal appearance associate with the Ig heavy chain locus. Both expressions occur in F_1 progeny of Igh differing parentals, thus implying codominant expression in the temporal appearance of predominant neonatal clonotypes.

It is important to note that antibodies of both clonotypes can be identified, albeit at very low frequency, in strains homozygous for the Igh^b heavy chain locus. Thus, these clones would appear to fall into the category of those for which polymorphism is not absolute but rather a function of magnitude of expression (17, 18, 32, 46–50). The infrequent but clear-cut expression of both the TF2-36 and TF2-76 clonotypes in CB.20 and B10.D2 neonates (and the early low expression of TF2-76 in BALB/c as well) may place these clonotypes in the category previously referred to as "sporadic neonatal clonotypes" (21). Such infrequently recurring clonotypes may represent the earliest expression of relatively late arising clones that would be sparsely represented in the very early neonatal repertoire, such as TF2-76 during the first 4 d of BALB/c neonatal development. Alternatively, such clonotypes may, for some reason, never achieve the clonal expansion characteristic of the more predominant clonotypes.

It is interesting to note that both TF2-76 and TF2-36 are extremely rare in the mature adult repertoire of Igh^b mice. The paucity of this specificity in the repertoire of adult Igh^b mice is consistent with the lack of any detectable TF2-36 or TF2-76 antibodies in the immune serum of mice of these strains. Thus, in the context of expression in the adult serum repertoire, these two markers may appear to represent clonotypes whose expression is absolutely polymorphic between these strains. The reproducible expression of B cells of these clonotypes in the early neonatal development of Igh^b mice, however, contradicts the absoluteness of such apparent polymorphisms. Thus, both gene cloning analysis and developmental analysis may be necessary before any conclusions can be reached about whether the potential for the expression of a given clonotype is truly missing in certain murine strains, or concluding that given clonotypes are "private" (55) to an individual and thus the likely product of random somatic events rather than "germ line" expressions. This reservation is consistent with recent findings wherein antiidiotypic selection revealed the potential for expression of clonotypes that otherwise had been assumed to be absent in given murine strains (17, 18) as well as careful analyses which have shown that even low frequency clonotypes can be expressed throughout a strain (32, 33).

Summary

Two hybridoma cell lines were established with B cells derived from neonatal BALB/c spleen cells. The anti-dinitrophenyl (DNP) antibodies derived from these

lines were characterized with respect to their isotype, affinity, and isoelectric point. Antiidiotypic reagents were prepared that permit an analysis of the representation of antibodies sharing idiotype with these two hybridomas in the developing and mature B cell pool of BALB/c mice (Igh^a) and other murine strains. One of the two antibodies, TF2-36, was found to be indistinguishable from 14% of anti-DNP monoclonal antibodies derived in fragment culture from spleen cells of 1–4-d-old BALB/c donors. B cells expressing this idiotype were found to represent ~2% of the anti-DNP-specific repertoire after the 1st wk of neonatal development and into adulthood. The second hybridoma antibody, TF2-76, was found to be expressed at very low levels during the first several days of neonatal development; however, B cells expressing this idiotype increased in frequency during the 2nd wk of neonatal development representing 7% of all DNP-responsive B cells 12–13 d after birth. The proportion of B cells expressing this idiotype also decreased to ~2% in adults. The relatively late appearance of B cells bearing this idiotype was confirmed by their susceptibility to tolerance induction after the 1st wk of neonatal development. Both the early neonatal clonotype, TF2-36, and the late neonatal antibody clonotype, TF2-76, were found to be expressed in a similar fashion in F_1 mice constructed between Igh^a and Igh^b parentals, but both were expressed at very low levels during the development of Igh^b mice. Thus, the control of the magnitude of expression of these neonatal clonotypes appears to be associated with the Igh locus.

We wish to express our gratitude to Dr. Roger Kennett and Dr. Michael Cancro at the University of Pennsylvania for their assistance and advice during the course of this research.

Received for publication 22 November 1982.

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