

THE CHARACTERIZATION OF THE B-CELL REPERTOIRE
SPECIFIC FOR THE 2,4-DINITROPHENYL AND
2,4,6-TRINITROPHENYL DETERMINANTS IN NEONATAL
BALB/c MICE*

BY NORMAN R. KLINMAN‡ AND JOAN L. PRESS§

(From the Department of Pathology, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19174)

The process by which an individual's repertoire of unique antibody-forming cell precursor (B-cell)¹ specificities (clonotypes) is acquired has been the subject of extensive investigation and controversy over the past several years (1-3). To help clarify the mechanism by which diversification of B-cell clonotypes might occur, this laboratory has conducted an investigation of the B-cell clonotypes available during the early stages of neonatal development, i.e., at an important period in the initial development of the specificity repertoire.

Previous reports from this laboratory have demonstrated that the adoptive transfer of neonatal spleen cells to an adult, carrier-primed environment maximizes the responsiveness of neonatal B cells (4-6). The principle rationale for this approach—that neonatal B cells are indeed immunologically competent when provided with ancillary mechanisms by the adult host environment—has been verified by other investigators (7, 8). Previous analyses from this laboratory have also shown the similarity of neonatal and adult primary B cells, in terms of the kinetics and antigen dose dependence of the antibody response (4), and the relative ease of hapten inhibition of primary B-cell stimulation (6, 9, 10). Recently, an analysis of the frequency of neonatal B-cell precursors specific for the haptenic determinants 2,4-dinitrophenyl (DNP); 2,4,6-trinitrophenyl (TNP); and fluorescein (FL) has demonstrated that a disparity exists in the rate of development of FL-specific precursor cells, as compared to DNP- or TNP-specific cells (5). Thus, at an early stage in the generation of specificity, when the total number of available specificities is limited, significant disparities may exist due to potential "all or none" expressions.

This report extends these observations by an analysis of the clonotypes available in neonatal BALB/c mice which are specific for the DNP and TNP haptenic

* Supported by Research Grants A1-08778 and CA-15822 from the U. S. Public Health Service. Presented in part at the FASEB Conference on Biology of Development and Aging, April 1974.

‡ Recipient of a U. S. Public Health Service Career Development Award (1-K04-A1-33983) from the National Institutes of Allergy and Infectious Diseases.

§ Present address: Department of Medicine, Division of Immunology, Stanford University School of Medicine, Stanford, Calif. 94305.

¹ Abbreviations used in this paper: B cell, antibody-forming cell precursors; CFA, complete Freund's adjuvant; DNP, 2,4-dinitrophenyl; FL, fluorescein; Hy, *Limulus polyphemus* hemocyanin; H γ G, human gamma globulin; TNP, 2,4,6-trinitrophenyl.

determinants. The studies presented in this report demonstrate that the response of neonatal B cells is as specific as that of adult B cells, in terms of the nonoverlap stimulation of precursor cells specific for DNP and TNP (11). In addition, the neonatal clonotypes specific for DNP are demonstrably different from the neonatal clonotypes specific for TNP by the criterion of isoelectric focusing. These studies not only provide evidence for the exquisite specificity of neonatal B-cell stimulation, but also demonstrate that DNP and TNP can be used as independent determinants for an analysis of the neonatal specificity repertoire. Such analysis revealed that the neonatal specificity repertoire is indeed relatively restricted, since only three clonotypes specific for DNP and three different clonotypes specific for TNP can be identified in neonatal BALB/c spleen cell populations during the first few days of life. Since these six clonotypes are present in a high percentage of neonates, it is presumed that they reflect either an early expression of germ line genes, or an early permutation of germ line information. Further, the finding that individual neonates apparently possess several B cells of the same clonotype not only permits an analysis of clonal expansion, but also provides evidence for cellular precommitment of antibody specificity.

Materials and Methods

Antigens. The preparation of *Limulus polyphemus* hemocyanin (Hy), human gamma globulin (H γ G), dinitrophenylated hemocyanin (DNP-Hy, 10 mol of DNP per 100,000 g of Hy), trinitrophenylated hemocyanin (TNP-HY, 10 mol of TNP per 100,000 g of Hy), and dinitrophenylated-human gamma globulin (DNP-H γ G, 10 mol of DNP per 150,000 g of H γ G) has been described previously (9).

Animals. 8-10-wk-old BALB/c mice were obtained from Carworth Division, Becton-Dickinson and Co., New York, and were immunized with 0.1 mg of Hy or H γ G in complete Freund's adjuvant (CFA) 8 wks before use as irradiated, carrier-primed recipients for adoptive neonatal cell transfer. Neonatal mice were obtained from breeding pairs of BALB/c mice in our own mouse colony. Adult serum containing anti-DNP antibodies, or anti-TNP antibodies, were obtained from 10-wk-old BALB/c mice 2 wk after primary immunization with 0.1 mg of DNP-Hy in CFA, or with 0.1 mg of TNP-Hy in CFA, respectively.

Cell Transfers and Fragment Cultures. The methodology for in vitro B-cell cloning and the detection of positive fragment cultures by radioimmunoassay has been described previously (4, 9).

Isoelectric Focusing. The micro-method for sucrose density gradient isoelectric focusing of serum and monoclonal antibodies has been extensively described in a previous publication (12). Briefly, microliter amounts of serum or culture fluid were focused for 15-18 h in 1 ml discontinuous sucrose density gradient (20-50%) containing 5% Ampholine (LKB Instruments, Inc., Rockville, Md.) (70% pH 5-8, 30% pH 4-6). Single drops were then collected into 0.3 ml of 0.15 M NaCl and the pH of each drop measured. Each drop was then analyzed for antihapten antibody by the radioimmunoassay.

Results

Parameters of the Neonatal Splenic Focus Response to TNP-Hy and DNP-Hy. In Table I are presented the results of an analysis of the frequencies of DNP- and TNP-specific neonatal B cells obtained from the spleens of 1-4-day-old BALB/c donors. The frequency of TNP-specific neonatal B cells is similar to the frequency of DNP-specific neonatal B cells and does not vary significantly with donor age. This is consistent with findings reported previously (5, 6). Table I also presents the results obtained when neonatal B cells (in fragment culture) were stimulated with an equimolar mixture of DNP-Hy and TNP-Hy. The

TABLE I
The Frequency of Clones Derived from Neonatal Spleen Cells after In Vitro Stimulation with TNP-Hy, DNP-Hy, or Both TNP-Hy and DNP-Hy Together

Source of donor spleen cells	Total no. of transferred cells	Stimulating antigen(s) (10^{-6} M haptenic determinant)	No. of clones detected with:		Total detected clones	Frequency per 10^6 cells
			DNP BAC*	TNP BAC		
Day 1-2 neonates	2×10^7	TNP-Hy	38	40	40	2.0
	2×10^7	DNP-Hy	56	53	56	2.8
	2×10^7	TNP-Hy + DNP-Hy	82	79	88	4.4
Day 3-4 neonates	2×10^7	TNP-Hy	47	52	52	2.6
	2×10^7	DNP-Hy	44	41	44	2.2
	2×10^7	TNP-Hy + DNP-Hy	95	93	98	4.9

* BAC bromoacetylcellulose.

number of neonatal splenic foci obtained after stimulation with a mixture of these two antigens closely approximates the sum of neonatal clones stimulated independently by each antigen. The additivity of the neonatal splenic focus response is also independent of donor age. These findings are consistent with, and confirm, the previous findings for the nonoverlap of stimulation of primary B-cell populations (11). Thus, for neonatal B-cell populations, as well as for adult primary B-cell populations, there is a nonoverlapping set of precursor cells specific for DNP and for TNP, the stimulation of which is exquisitely specific.

Expression of DNP-Specific Clonotypes during Neonatal Development. Fig. 1 presents the isoelectric focusing spectrum of serum anti-DNP antibody obtained after primary immunization of adult mice with DNP-Hy in CFA, and the isoelectric spectra of neonatal monofocal antibodies. As has been previously reported (12), the isoelectric spectra of neonatal monofocal antibodies are markedly restricted compared to serum antibodies. When monoclonal antibodies were obtained from foci derived from donor animals in the first 3-4 days of neonatal life, the isoelectric spectra generally fell into one of three predominant patterns. Figs. 1 *b* and 1 *c* depict antibodies from two different donors which have an isoelectric maximum at 5.05. Figs. 1 *d* and 1 *e* show two neonatal monofocal antibodies from two donors with an isoelectric maximum at 5.25; and Figs. 1 *f* and 1 *g* show monofocal antibodies from two donors with a maximum at 5.55. While the vast majority of monofocal antibodies from neonates 1-4 days old gave isoelectric maxima at one of these three positions, occasional monofocal antibodies gave maxima in other positions. For example, Fig. 1 *h* presents a monofocal antibody with an isoelectric maximum at 6.55. It should be noted that this latter monofocal antibody as well as the antibodies of the predominant clonotypes are of the IgM immunoglobulin class.

The method of isoelectric focusing used in these studies discriminates antibodies on the basis of isoelectric point to within a range of approximately 0.1 pH unit (12). Since neonatal and adult IgM antibodies can be found in a pI range from approximately 4.4 to 7.6, it is possible, by this technique, to readily

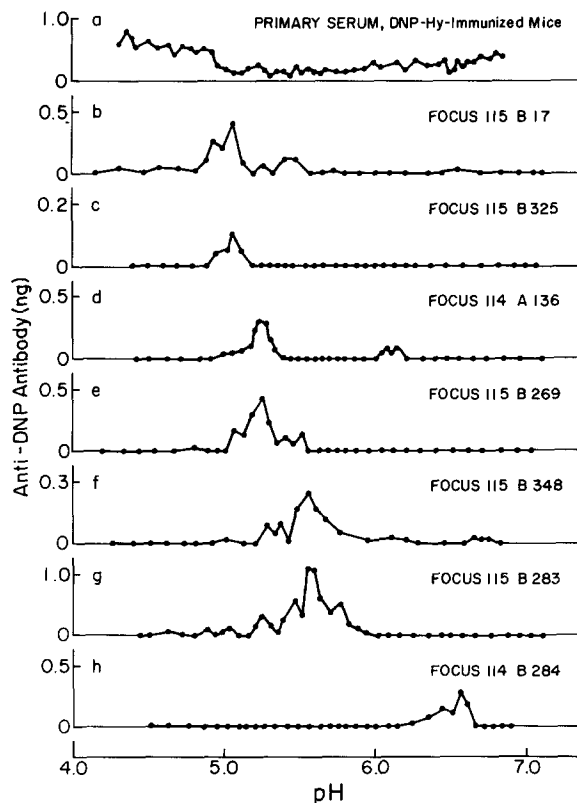


FIG. 1. The isoelectric spectra of primary serum anti-DNP antibody (1 *a*) and seven monoclonal antibodies obtained from different neonatal donors. The focusing patterns were obtained by analyzing eluted drops by the radioimmunoassay, using ^{125}I -labeled antimouse Fab antibody. The data is plotted as the nanograms of antibody per fraction vs. the pH of the fraction.

discriminate between perhaps only 30–40 isoelectric maxima. Thus, the number of distinct neonatal antibodies specific for DNP, when obtained by the methods employed here, must be considered a minimum estimate, since other clonotypes may exist which cannot be distinguished from the three major clonotypes described.

Even with the aforementioned limitation, it is nonetheless possible to categorize neonatal monofocal anti-DNP antibodies into those which appear to be predominantly expressed early in neonatal life and those which seem to occur only sporadically. Table II presents such a categorization after an isoelectric analysis of 112 monoclonal neonatal anti-DNP antibodies. On the 1st and 2nd days of neonatal life, the majority of DNP-specific B cells gave rise to clones producing antibodies of one of the three major clonotypes depicted in Fig. 1. It should be noted that at this time of development, those clonotypes with a pI of 5.05 and of 5.55 appear to predominate even over the clonotype with a pI of 5.25. By day 4 after birth, however, the frequency of clonotypes of pI 5.55 appears to be diminished relative to the clonotype of pI 5.25. The predominant clonotypes (pI 5.05, 5.25, 5.55) constitute the major population of DNP-specific precursor cells

TABLE II
The Frequency and Isoelectric-Focusing Characterization of Anti-DNP Monoclonal Responses Derived from BABL/c Splenic B Cells during Early Neonatal Development

	Age of neonatal donor (days)				
	1	2	3-4	5-6	7-9
Average no. nucleated cells/donor spleens	1×10^6	2×10^6	7×10^6	1.6×10^7	6×10^7
No. donors analyzed	8	18	8	6	8
No. monoclonal antibodies focused	10	40	20	10	32
pI	No. %	No. %	No. %	No. %	No. %
5.05	4 40	18 45	7 35	1 10	1 3
5.25	1 10	6 15	5 25	3 30	2 6
5.55	5 50	14 35	3 15	0 0	0 0
Other	0 0	2 5	5 25	6 60	29 91

until approximately the 6th day after birth. By this time, one of the clonotypes (that of pI 5.55) is very low in frequency, since it was not measured in this sampling, whereas other clonotypes, with pI's distinguishable from those of the three clonotypes which predominate early after birth, now appear to be a majority. By 9 days after birth, the "sporadic" clonotypes represent a vast majority. At this developmental stage, there is a heterogeneously dispersed population of clonotypes, and it is not clear whether those clonotypes with pI's similar to the early predominant clonotypes are in fact representative of that original clonotype.

The data presented above demonstrate the predominance of three clonotypes in the early expression of DNP-specific precursor cells. Table III presents the results obtained when several monoclonal antibodies, derived from single neonatal donors, were analyzed for clonotype expression. In general, the majority of the monoclonal antibodies produced by a single neonatal donor represent the same clonotype, and this clonotype is one of the three DNP-specific predominant clonotypes. Thus, a single individual 2-4 days old may possess many cells of a single predominant clonotype. It is possible to calculate, on the basis of the total frequency of DNP-specific B cells in neonatal spleens, that a single individual may possess as many as 200-400 precursor cells of the same clonotype (5, 10). If one assumes that these neonatal mice are not, in general, exposed to a large variety of external antigenic stimuli, then the clonal expansion of cells of a single clonotype is probably a natural mechanism reflecting the normal generative phase in the life cycle of a single clone. Furthermore, the identification of many cells of a single predominant clonotype in a given individual indicates that these cells were committed in the neonatal donor before contact with antigen *in vitro*. This point is reinforced by the fact (Table III) that two of the clones from donor 107 B (marked by an asterisk) and one of the clones from donor 107 D were obtained after cell transfer into separate recipients, yet still show the same predominant clonotype. In addition, when spleen cells from 2- to 4-day old

TABLE III
Isoelectric-Focusing Analysis of Neonatal Monoclonal Antibodies from Individual Donors

Donor	Focus no.	Stimulating antigen (10^{-6} M haptenic determinant)	pI
107 B	1	DNP-Hy	5.35
	2	DNP-Hy	5.05
	3*	DNP-Hy	5.05
	4*	DNP-Hy	5.05
109 A	1	DNP-Hy	5.05
	2	DNP-Hy	5.05
107 D	1	DNP-Hy	5.05
	2	DNP-Hy	5.05
	3*	DNP-Hy	5.05
109 D1	1	DNP-Hy	5.05
	2	DNP-Hy	5.05
	3	TNP-Hy	5.15
115 D	1	DNP-Hy	5.55
	2	DNP-Hy	5.05
	3	DNP-Hy	5.55
	4	DNP-Hy	5.25
110 A1	1	TNP-Hy	5.40
	2	TNP-Hy	5.40
	3	TNP-Hy	5.40
110 A2	22	TNP-Hy	5.15
	34	TNP-Hy	5.15
	46	TNP-Hy	5.15
110 A3	12	DNP-Hy	5.55
	24	TNP-Hy	5.00
	34	DNP-Hy	5.25
	47	TNP-Hy	5.40
110 B4	67	TNP-Hy	5.40
	71	TNP-Hy	5.00
	77	TNP-Hy	5.00
	96	TNP-Hy	5.00
110 B6	58	TNP-Hy	5.00
	60	TNP-Hy	5.00
	65	TNP-Hy	5.00
	71	TNP-Hy	5.15
	72	DNP-Hy	5.55
	77	TNP-Hy	5.80

* Clones obtained in separate recipients, with spleen cells transferred from one donor.

neonates were transferred to irradiated, H γ G-primed recipients, and the recipient spleen fragments stimulated with DNP-H γ G, the clonotypes expressed after stimulation with DNP on H γ G were the same as the predominant clonotypes obtained after stimulation with DNP on Hy. This confirms previous findings suggesting the relative carrier independence of the specificity of B cells (13, 14).

Expression of TNP-Specific Clonotypes during Neonatal Development. Fig. 2 presents the isoelectric focusing spectra of several neonatal monofocal

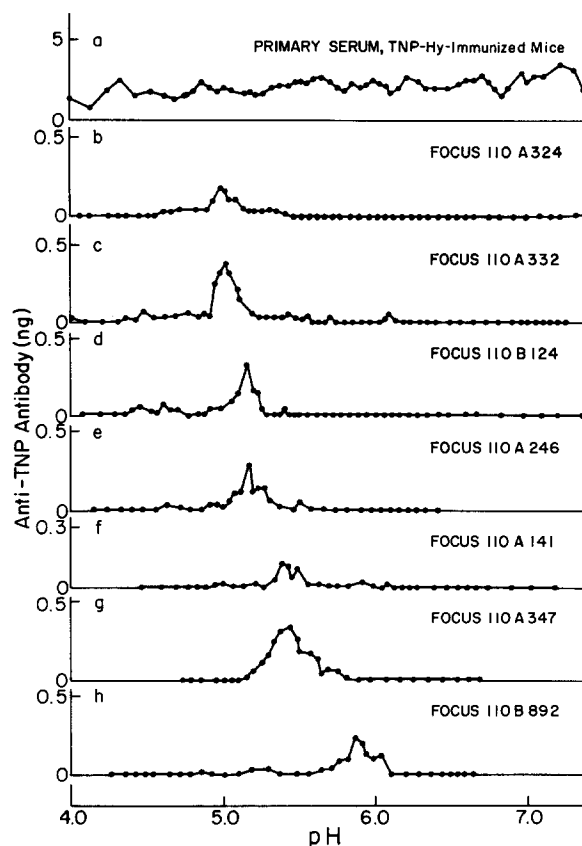


FIG. 2. The isoelectric spectra of primary serum anti-TNP antibody (2 *a*) and seven monoclonal anti-TNP antibodies. The focusing patterns were obtained by analyzing eluted drops by the radioimmunoassay, using ^{125}I -labeled antimouse Fab antibody. The data is plotted as the nanograms of antibody per fraction vs. the pH of the fraction.

antibodies obtained after fragment culture stimulation with TNP-Hy. Also presented in Fig. 2 (2 *a*) is the profile of a typically heterogeneous antibody response, i.e., the isoelectric spectrum of serum anti-TNP antibody produced 14 days after primary in vivo immunization. In contrast to Fig. 2 *a*, the isoelectric spectra of the neonatal monofocal anti-TNP antibodies (Fig. 2 *b-h*) are markedly restricted. In Fig. 2 *b* and *c*, the monofocal antibodies were derived from a single neonatal donor, and both have isoelectric maximum at pH 5.00. The isoelectric

spectra of monofocal antibodies from two different neonatal donors are presented in Fig. 2 *d* and *e*; both spectra have an isoelectric maximum at pH 5.15. In Fig. 2 *f* and *d*, the monofocal antibodies are also derived from two different donors, and both have an isoelectric maximum at pH 5.40. Monofocal antibodies with other isoelectric maxima can also be detected. For example, the isoelectric spectrum in Fig. 2 *h* has a maximum at pH 5.90; this monofocal antibody was derived from a 3-day-old donor.

An analysis of the TNP-specific clonotypes expressed during early neonatal development is presented in Table IV. It is clear that for TNP, as well as for DNP, only three predominant clonotypes are expressed very early after birth. In a fashion analogous to that observed for DNP-specific clonotypes, by 9 days after birth, the majority of TNP-specific clonotypes detected are sporadic, and hence constitute a heterogeneous array. Thus, very early after birth, the response to DNP and TNP is restricted in that three DNP-specific and three TNP-specific

TABLE IV
The Frequency and Isoelectric-Focusing Characterization of Anti-TNP Monoclonal Responses Derived from BALB/c B Cells during Early Neonatal Development

	Age of neonatal donor (days)					
	0-3		4-6		7-9	
Average no. nucleated cells/donor spleens	2×10^6		1.2×10^7		6×10^7	
No. donors analyzed	12		11		6	
No. monoclonal antibodies focused	34		30		24	
pI	No.	%	No.	%	No.	%
5.00	10	29	13	43	1	4
5.15	14	41	5	17	1	4
5.40	8	24	2	7	1	4
Other	2	6	10	33	21	88

clonotypes predominate in the neonatal population as a whole. By the end of the 1st wk after birth, the clonotypes specific for either DNP or TNP have diversified to the extent that the clonotype response appears heterogeneous instead of restricted.

It should be clear from the above studies that at least two findings demonstrate that TNP can be used as a second independent hapten to confirm the results obtained with DNP. Firstly, by isoelectric focusing, the TNP-specific clonotypes with a pI of 5.15 and 5.40 can be clearly distinguished from any of the predominant clonotypes specific for DNP (pI 5.05, 5.25, 5.55). While the TNP-specific clonotype with a pI of 5.00 is not so readily distinguished from the DNP-specific clonotype with a pI of 5.05, it is nevertheless apparent that the majority of neonatal precursor cells specific for DNP are distinct from those specific for TNP. The second way in which this is demonstrated is the finding that stimulation of neonatal B cells with a mixture of TNP-Hy and DNP-Hy

gives an additive clonal response (Table I). Data presented in Table III further elucidate the exclusiveness of the neonatal precursor pools for TNP and DNP. Although the neonatal population as a whole expresses the three predominant TNP-specific clonotypes, individual neonates tend to express only one of the three predominant clonotypes. When B cells from an individual neonatal donor are stimulated in separate fragment cultures, by DNP-Hy and TNP-Hy, often the B cells from one donor express one predominant clonotype specific for DNP and one predominant clonotype specific for TNP (Table III). Thus, individual neonatal donors have precursor cells specific for both DNP and TNP. However, in the neonatal B-cell population which contains both DNP- and TNP-specific clonotypes, DNP-Hy stimulates only those neonatal precursor cells expressing a clonotype specific for DNP, while TNP-Hy stimulates only those precursor cells specific for TNP. Hence cross-stimulation between two closely related determinants does not occur at the level of the clonotype-specific primary precursor cell.

Discussion

Although responses in mice to certain antigenic determinants may invariably include a "dominant" clonotype (15-17), a study by Kreth and Williamson has indicated that the immune response of CBA/H mice to the 4-hydroxy-5-iodo-3-nitrophenylacetyl (NIP) determinant may involve as many as 3,000-30,000 distinct clonotypes (18). Given such an enormous array of clonotypes in the adult mouse potentially available for responsiveness to determinants such as NIP and probably to DNP as well, the analysis of the generation of the specificity repertoire to these determinants is extremely hampered. The studies presented in this report have attempted, therefore, to define the specificity repertoire for both the DNP and TNP determinants which is available to the BALB/c mouse population during the period of time in ontogenic and neonatal development when the number of B cells and hence clonotypes is initially limited and undergoing rapid expansion.

The specificity repertoire in neonatal mice was examined by measuring the clonotype expression of neonatal monofocal anti-DNP and anti-TNP antibodies produced *in vitro* under conditions of maximal carrier help. The evidence for the clonal origin of neonatal monofocal antibodies produced under the conditions used in these studies has been dealt with at length in other publications (5, 9, 12) and will not be discussed here. It is important that the micro-method for isoelectric focusing employed to analyze neonatal antibodies permits only a minimum estimate of the number of distinct DNP-specific and TNP-specific clonotypes.

Independence of Neonatal Precursor Cells Specific for DNP and TNP. A previous report from this laboratory (11) demonstrated that nonimmune adult primary B cells specific for DNP and for TNP were derived from nonoverlapping sets of precursor cells. When primary adult B cells were stimulated with a mixture of DNP-Hy and TNP-Hy, the number of splenic foci obtained was approximately the sum of the number of clones obtained by stimulation with either antigen alone. Hence, the stimulation of primary B cells is exquisitely specific at the level of the precursor cell, although the antibodies elicited can be

highly cross-reactive (11). Although neonatal B cells display certain characteristics of adult primary B cells (4-6, 10), the stimulation of neonatal precursor cells specific for DNP and TNP was analyzed for its specificity, since most neonatal monoclonal anti-DNP and anti-TNP antibodies cross-react, as do antigen binding cells (19). Two findings strongly argue that the neonatal population of B cells contains two independent sets of precursor cells, one specific for DNP and the other specific for TNP. First, in experiments similar to those described above for adult B cells, neonatal B cells stimulated with a mixture of DNP-Hy and TNP-Hy yielded a number of splenic foci closely approximating the sum of the numbers of foci obtained after stimulation with each antigen alone. This finding is highly consistent with the fine specificity of primary adult B-cell stimulation and again indicates little overlap stimulation of primary or neonatal precursor cell stimulation by closely related determinants. The exclusiveness of neonatal precursor cells specific for DNP and TNP was further indicated by the finding that neonatal monofocal antibodies specific for DNP are clearly distinguishable from those antibodies elicited by TNP by the criterion of isoelectric focusing. Thus, it would appear that DNP and TNP can be used as independent haptens for stimulation, since for either neonatal or adult primary precursor cells, there is little demonstrable overlap stimulation of the precursor cell pools.

Restricted Clonotype Expression. Early in development, when only 10^5 - 10^6 B cells are present in the BALB/c neonatal mouse spleen (6), very few specificities for the DNP or TNP determinants are in fact expressed. If it is assumed that the clonotypes available at this point in ontogenic development represent the earliest expressions of the genetic code for antibody specificities present in the germ line, then it might be expected (by a germ line theory of diversification) that all mice of this inbred strain should present the same specificity repertoire at this point in development (1-3). The data presented in this paper indicate that this is essentially, though not entirely, correct.

During the first 2 days of neonatal life, only three DNP-specific clonotypes and three TNP-specific clonotypes can be identified in the population of BALB/c mice analyzed. The three DNP-specific predominant clonotypes are of the IgM immunoglobulin class and exhibit isoelectric maxima at pH 5.05, 5.25, and 5.55. Those clonotypes of pI 5.05 and 5.55 may, in fact, precede the expression of the clonotype with a pI of 5.25. The three TNP-specific predominant clonotypes are also IgM, but have different isoelectric maxima, i.e., 5.00, 5.15, and 5.40. Thus, at least the majority of TNP-specific and DNP-specific predominant clonotypes can be distinguished by isoelectric focusing. The germ line theory prediction that all mice would express the same predominant clonotype is only partially fulfilled. While the three DNP-specific and the three TNP-specific predominant clonotypes are the most frequent in the neonatal population as a whole, the expression of any one of the three DNP- or TNP-specific clonotypes in individual mice varies markedly. Nevertheless, the fact that the three DNP and the three TNP specificities are expressed so frequently in the early neonatal population is strong confirmation of the postulate that the earliest identifiable clonotype expressions are close reflections of the germ line genetic information.

The source of the variation of predominant clonotype expression in individual mice is not clear. It may reflect a regulation of germ line expression, whereby only

a portion of the predominant clonotypes encoded by the germ line are expressed at a given point in time. Alternatively the maternal environment may influence, to some degree, those clonotypes which are initially expressed (20). A further possibility is that the seeding of lymphopoietic stem cells or early clonotype precursor cells into the spleen may be random. Thus, by chance, some neonates might have one of the predominant clonotypes present in the spleen at an earlier time than other neonates. That the predominant clonotype expression in neonates as observed in this report represents the entire DNP- and TNP-specific B-cell repertoire, independent of the mechanism for carrier help, is indicated by the observation that, at least for DNP, the same three predominant clonotypes were expressed when either Hy or H γ G was used as the carrier moiety.

Clonotype Expansion and Precommitment. During the first few days of neonatal life, there exists in the spleen a large population of precursor cells of an individual clonotype. This would indicate that there is a conservative expansion of a single clonotype precursor cell which is responsible for the expression of a given clone in an individual. Since the frequency of DNP- or TNP-specific B cells in the 1- to 2-day old neonatal spleen is 1 in $3-4 \times 10^3$ B cells, an individual neonate may possess as many as 200-400 precursor cells of the same clonotype (10). Presumably, the expansion of a given clonotype is due to naturally occurring hemo- or lymphopoietic generative processes. If one assumes that the doubling time of B cells within a clonotype is on the order of 24 h, similar to that of the doubling time of the total number of splenic B cells in early development (19), then a clonal expansion yielding 200-400 precursor cells of the same clonotype by the 2nd to 3rd day of neonatal life, implies that the original clonotype precursor appeared between the 14th and 15th day of gestation. Since Ig bearing cells can be found in the spleens of 15-day-old embryos (19), it is possible that these B cells represent the original clonotype precursor cells and/or their immediate clonal progeny.

When sufficient detailed isoelectric data become available, it may be possible to follow the total life cycle of an individual clone. From the data presented in this paper, it cannot be concluded that there is considerable expansion of the early predominant clonotypes beyond the 4th-6th day of neonatal life, since their relative frequency diminishes by this time. In general, it would appear that the maximum clone size of a precursor clonotype can be at least 400 precursor cells, and one study showed that nonimmune adult BALB/c mice may have as many as 10^4 B cells of one clonotype specific for the phosphorylcholine determinant (21).

The finding that so many precursor cells of the same clonotype exist in an individual's spleen by the day of birth strongly suggests that B cells are precommitted in their specificity before contact with antigen and that B-cell clones expand as a result of normal, antigen-independent, generative processes. While it is not possible to extrapolate with certainty the data obtained for these few predominant neonatal clonotypes to all B-cell clonotypes, these findings, in conjunction with those of previous investigations (4, 5, 11), indicate that the B-cell repertoire consists of a vast array of precommitted cells derived via predetermined clonal expansion processes beginning as early as the 15th day of gestation. These findings would argue strongly against any extrinsic process, such as positive antigen selection, as an important factor in the generation of the

clonotype specificity repertoire (3). Thus, in a potentially heterogeneous system the finding that an individual neonate possesses many B cells of a single clonotype, and that these cells express the same specificity in separate recipients or when stimulated with DNP on Hy or H γ G, indicates that the specificity of these cells is a property intrinsic to the donor cell population and existed before contact with antigen.

Occurrence of Sporadic Clonotypes and Diversity. If a clonotype precursor of one specificity first appears in the fetal spleen (or liver) at day 15 of gestation and subsequently commences a clonal expansion with a B-cell doubling time of 24 h, then by the 2nd day of neonatal life, that clonotype pool size will consist of approximately 200–400 precursor cells, and thus appear as a predominant clonotype. If a second clonotype precursor cell appears somewhat later in gestation (days 16–17), then this second clonotype, after undergoing expansion, would appear predominant at a slightly later time. A process such as this might well explain why the DNP-specific clonotype with a pI of 5.55 appears to be predominant slightly earlier than the DNP-specific clonotype with a pI of 5.25.

Thus, it would appear that the fetal and neonatal spleen can be viewed as a lymphopoietic source in a high state of flux. As clonotype precursors enter this nonstatic pool, clonal expansion occurs up to a clonotype pool size of approximately 400 cells. Depending on the time in ontogenic development these clonotype precursor cells have entered the B-cell pool, they may or may not appear as predominant clones. Thus, the clonotype precursor pool will be constantly changing during early neonatal development, as clonotype precursor cells enter, expand, then become diminished in frequency as other clonotypes contribute to the expanding pool. Eventually, the adult splenic pool size will be established and the entrance and exit rates of precursor cells will presumably reach a steady state.

Such a mechanism would account not only for the appearance of predominant clonotypes very early in development, but also for the shift away from predominant clonotype expression, toward the appearance of sporadic clonotypes and specificity heterogeneity. Sporadic clonotypes could be viewed as those precursor specificities which appeared later during development (e.g., from the 18th day of gestation on). Such clonotypes will have maximally expanded only at a time when many other precursor specificities were being expressed. Thus, the population sampled for the specificity repertoire would not look very different from the heterogeneous adult clonal population and would consist of a heterogeneous array of both IgM and IgG monoclonal antibodies.

At the present time, it is not clear what mechanisms could account for the relatively late expression of some clonotype specificities and the early expression of others. As mentioned previously in the discussion, it may be assumed that the earliest expressed clonotypes directly reflect genetic information encoded in the germ line or early permutations of that information. Such permutations may take the form of chromosomal mutation (3), insertion (22), or crossing-over (2) or may simply represent different heavy- and light-chain combinations (3). The frequency of DNP- and TNP-specific B cells in neonates (10) and the finding that there are three DNP-specific and three TNP-specific clonotypes expressed early in development suggest that approximately 10^4 different clonotypes are ex-

pressed early in the neonatal B-cell repertoire. The later expression of a larger array of clonotypes may again represent the product of continuing permutations of the germ line genetic information or may represent the sequential derepression of more and more genetic information. It will be of interest to analyze the expression of frequently recurring adult clonotypes such as the major clonotypes in the response to the phosphorylcholine and arsonate determinants (15-17), to determine their time of appearance, and to ascertain whether late permutations are indeed the result of highly regulated processes.

Summary

The (B-cell) repertoire responsive to the DNP and TNP haptenic determinants in BALB/c neonates was analyzed in terms of the specificity of stimulation of neonatal B cells as well as the diversity of specificities available in neonatal populations. The results indicate that the parameters of stimulation of neonatal B cells are similar to those of nonimmune adults, particularly in the exquisitely specific stimulatory process which readily discriminates between haptens as closely related as 2,4-dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP).

The clonotypes of monoclonal anti-DNP and anti-TNP antibodies derived from isolated neonatal BALB/c splenic B cells in fragment culture were analyzed by isoelectric focusing. During the first 4 days of neonatal life almost all of the anti-DNP-specific clones were of clonotypes displaying IgM antibodies with pI's of 5.05, 5.25, or 5.55. These could be distinguished from clonotypes responding to TNP which were also predominantly of three distinct pI's, 5.00, 5.15, or 5.40. These clonotypes, which represent the vast majority of the DNP- and TNP-specific antibody capability during the first 4 days of life, represented less than half of the clones by day 6 and were a small minority by day 9. The observation that individual 1-4-day-old donors had many B cells representative of a given predominant clonotype is evidence for cellular precommitment of specificity and indicates that clones of precommitted B cells exist as the products of normal, antigen-independent, generative processes. The observation of frequently recurring clonotypes in inbred neonates attests to the "germ line" origin of these clonotypes; however, variance in the occurrence of these clonotypes from donor to donor implies a random element in their expression. The finding that several clonotypes occur repeatedly in high numbers early in neonatal development, while other clonotypes occur only sporadically at early times, has been interpreted as a reflection of a sequential ontogenic expression of clonotypes. Thus the DNP- and TNP-specific clonotypes which predominate in neonates may be seen as representative of a total of 5,000-10,000 clonotypes which are expressed as early as the 15th to 17th day of gestation while most clonotypes appear after the 18th day of gestation.

The authors wish to thank Ms. J. H. Owen and G. Ball for their excellent technical assistance in this work.

Received for publication 30 December 1974.

References

1. Smith, G., L. Hood, and W. Fitch. 1971. Antibody diversity. *Annu. Rev. Biochem.* **40**:969.

2. Gally, J., and G. Edelman. 1970. Somatic translocation of antibody genes. *Nature (Lond.)*. **227**:341.
3. Cohn, M. 1971. The take-home lesson—1971. *Ann. N. Y. Acad. Sci.* **190**:529.
4. Press, J. L., and N. R. Klinman. 1973. Enumeration and analysis of antibody-forming precursors in the neonatal mouse. *J. Immunol.* **111**:829.
5. Press, J. L., and N. R. Klinman. 1974. Frequency of hapten-specific B cells in neonatal and adult mouse spleens. *Eur. J. Immunol.* **4**:155.
6. Klinman, N. R., J. L. Press, A. R. Pickard, R. T. Woodland, and A. F. Dewey. 1974. Biography of the B cell. In *The Immune System, Genes, Receptors, Signals*. E. E. Sercarz, A. R. Williamson, and C. F. Fox, editors. Academic Press, Inc., New York. 357
7. Sherwin, W., and D. T. Rowlands, Jr. 1974. Development of humoral immunity in lethally irradiated mice reconstituted with fetal liver. *J. Immunol.* **113**:1353.
8. Goidl, E., and G. E. Siskind. 1974. Studies on the ontogeny of the B-cell response in the mouse. *J. Exp. Med.* **140**:1285.
9. Klinman, N. R. 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. *J. Exp. Med.* **136**:241.
10. Klinman, N. R., and J. L. Press. 1975. The expression of specific clones during B cell development. *Fed. Proc.* **34**:47.
11. Klinman, N. R., J. L. Press, and G. P. Segal. 1973. Overlap stimulation of primary and secondary B cells by cross-reacting determinants. *J. Exp. Med.* **138**:1276.
12. Press, J. L., and N. R. Klinman. 1973. Isoelectric analysis of neonatal monofocal antibody. *Immunochemistry*. **10**:621.
13. Wang, A. L., and A. Nisonoff. 1973. Effect of the carrier protein on idiotypic specificities of anti-hapten antibodies produced in an inbred strain of mice. *J. Immunol.* **110**:1646.
14. Klinman, N. R., and R. A. Doughty. 1973. Hapten-specific stimulation of secondary B cells independent of T cells. *J. Exp. Med.* **138**:473.
15. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1975. Heterogeneity of the BALB/c antiphosphorylcholine antibody response at the precursor cell level. *J. Exp. Med.* **141**:56.
16. Clafin, J., R. Lieberman, and J. Davie. 1974. Clonal nature of the immune response to phosphorylcholine II. Idiotypic specificity and binding characteristics of anti-phosphorylcholine antibodies. *J. Immunol.* **112**:1747.
17. Pawlak, L. L., and A. Nisonoff. 1972. Distribution of a cross-reactive idiotypic specificity in inbred strains of mice. *J. Exp. Med.* **137**:855.
18. Kreth, H. W., and A. R. Williamson. 1973. The extent of diversity of anti-hapten antibodies in inbred mice: anti-NIP (4-hydroxy-5-iodo-3-nitro-phenylacetyl) antibodies in CBA/H mice. *Eur. J. Immunol.* **3**:141.
19. Spear, P. G., A-L. Wang, U. Rutishauser, and G. M. Edelman. 1973. Characterization of splenic lymphoid cells in fetal and newborn mice. *J. Exp. Med.* **138**:557.
20. Kindred, B., and G. E. Roelants. 1974. Restricted clonal response to DNP in adult offspring of immunized mice: a maternal effect. *J. Immunol.* **113**:445.
21. Sigal, N. H., P. J. Gearhart, and N. R. Klinman. 1975. The frequency of phosphorylcholine specific B cells in conventional and germ free BALB/c mice. *J. Immunol.* In press.
22. Wu, T. T., and E. A. Kabat. 1970. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J. Exp. Med.* **132**:211.