EXPRESSION OF PHOSPHORYLCHOLINE-SPECIFIC B CELLS DURING MURINE DEVELOPMENT*

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In recent years significant progress has been made in defining the adult and neonatal B-cell repertoires. After Kreth and Williamson's isoelectric focusing analysis of the B-cell repertoire specific for 4-hydroxy-5-ido-3-nitrophenacetyl in CBA mice (1), similar studies have investigated the responses to a large variety of haptenic, protein, and carbohydrate determinants in mice, rabbits, and guinea pigs (2-6). In toto, these studies, together with analyses of monoclonal antibodies and assessments of the frequency of B cells which represent identifiable specificities, indicate that the adult B-cell repertoire is extremely heterogeneous, and probably consists of greater than $10^7$ unique antibody specificities (clonotypes) (6-8).

The acquisition of the specificity repertoire during ontogenetic development poses problems and raises questions which are somewhat distinct from a similar analysis of the adult repertoire. Since the fetus and neonate are dynamic systems, one must define the B-cell repertoire at various points in ontogeny. Does the neonate have fewer specificities than the adult, since the individual perforce must start with fewer cells? How does the acquisition of the repertoire vary from individual to individual? Does antigen play a role in this process? These questions bear directly on the mechanism by which the B-cell repertoire is generated.

The results of the splenic focus technique have provided significant insights into the ontogeny of the B-cell repertoire. These studies have demonstrated that neonatal B cells can be stimulated in an environment which provides adult antigen-specific T cells (9) and that neonatal B cells are unipotential, giving rise to clonal progeny which produce homogeneous monoclonal antibody (10, 11). The frequency of neonatal B cells which are specific for dinitrophenyl (DNP) and trinitrophenyl (TNP) is approximately the same as the adult frequency, but the frequency of neonatal B cells which are specific for fluorescein (FL) is significantly lower than the adult (12). During the first few days after birth, three DNP and three TNP-specific clonotypes can be identified by isoelectric focusing pattern in the BALB/c splenic B-cell population (11). Since the neonatal spleen cell population, as a whole, repeatedly expresses all three TNP and all three DNP clonotypes,
these predominant clonotypes fulfill the classical definition of germline expressions. Each of the predominant clonotypes represents one-third of the total DNP or TNP-specific neonatal repertoire, or 1 per 10,000 B cells, and by 2–3 days of life, each clonotype is represented by 50–200 cells. If the precursor doubling time is 24 h (13–15), then the original clonotype precursor cell must have arisen before the 15th day of fetal life. Thus, at least 10^4 different clonotypes may be expressed early in development and can expand in the absence of antigenic contact (in utero) to reach a clone size of up to 200 cells (7, 11). By 6–7 days after birth, the predominant clonotypes are replaced by a more heterogeneous array of specificities (11). These sporadic clonotypes could be viewed as arising later in development than the early predominant ones.

It was of considerable interest to identify within the neonatal repertoire phosphorylcholine (PC)-specific clonal precursor cells which bear the same idiotype as the TEPC 15 (T15) plasmacytoma protein, since the T15 clonotype is present in high frequency in all individuals of the BALB/c strain (16–20) and, therefore, is presumably encoded by germline variable region genes (4, 21). The time of its appearance in the repertoire may have implications for the generation of antibody diversity since the acquisition of a well-defined clonotype can be mapped. Precursor cell analyses have indicated, however, that PC-specific B cells, and the T15 clonotype in particular, do not appear until approximately 1 wk of age (22), at a time when the DNP repertoire is already quite diverse. In addition, by the criterion of susceptibility to tolerance induction, PC-specific B cells arising during the 2nd-wk of life are, in fact, immature cells which have been recently generated from the stem cell pool (23).

In this report these findings are extended and confirmed by the demonstration that (a) the late arising of the T15 clonotype is a highly predictable process which is not altered by antigenic environment, maternal influences, or genetic background; (b) cells capable of binding ^125I-labeled PC-BSA accurately reflect the frequency of PC-specific cells in both neonatal and adult spleen; and (c) the T15 clonotype is not expressed in fetal liver or neonatal bone marrow before its appearance in the spleen. These results are discussed in terms of their implications on the mechanism by which antibody diversity is generated.

Materials and Methods

Animals and Antigens. 6-to-8-wk-old BALB/cAnN and A/He mice were obtained from the Institute for Cancer Research, Philadelphia, Pa., and BALB/cJ mice from Carworth Division, Becton Dickinson and Co., New York. Neonatal mice were obtained from breeding pairs of BALB/cAnN mice in our own mouse colony as well as pregnant BALB/cAnN females purchased from the Institute for Cancer Research, Philadelphia, Pa. and Ace Breeding Laboratory, Philadelphia, Pa. (C57BL/6j × BALB/c) F1 and (BALB/c × C57BL/6j) F1 neonates were bred in our mouse colony by using C57BL/6j mice from The Jackson Laboratory, Bar Harbor, Maine. Germfree BALB/c neonates were purchased from Charles Rivers Breeding Laboratories, Wilmington, Mass. or raised in our own germfree mouse colony from germfree BALB/c breeding pairs obtained from the National Institutes of Health, Bethesda, Md. The preparation of Limulus polyphemus hemocyanin (Hy), phosphorylcholine-hovine serum albumin (PC-BSA) and 3-(p-azophenylphosphorylcholine)-N-acetyl-l-tyrosylglycylglycine Boc hydrazide-Hy (PPC-TGG-Hy) have been described previously (19, 24).

Plasmacytoma Proteins. The plasma cell tumors T15, MOPC 167, and MOPC 460 were obtained from Dr. M. Potter, National Cancer Institute, National Institutes of Health, Bethesda, Md., maintained by serial passage in BALB/c mice, and purified as previously described (24). T15 and MOPC 460 were conjugated to Sepharose 4B by the procedure of Cuatrecasas (25).

Immunizations. BALB/c mice were immunized intraperitoneally with 0.1 mg of Hy in complete Freund's adjuvant 6-10 wk before use as recipients in spleen cell transfer experiments. A/He
mice were immunized with purified T15 protein according to the protocol of Lieberman and Humphrey (26). A rabbit was immunized with 1 mg of T15 protein in complete Freund's adjuvant in eight subcutaneous sites and 1 mo later with 1 mg of T15 in incomplete Freund's adjuvant in the same sites. The rabbit was bled weekly and the injection and bleeding schedule was repeated 2 mo later.

**Purification of Anti-Idiotype Sera.** Anti-T15 sera from A/He mice was diluted 1:1 with 0.02 M phosphate-0.15 M NaCl buffer solution, pH 7.4 (PBS) and passed over a MOPC 460-Sepharose column. 20 ml of rabbit anti-T15 serum was dialyzed into 0.05 M sodium borate-0.15 M NaCl buffer, pH 8.3 (BBS), and 19 mg of MOPC 167 myeloma protein in BBS was added. The mixture was allowed to incubate at 4°C overnight and the resulting precipitate was removed by centrifugation. Purified T15 protein was added to the supernate slowly until a precipitate formed, and after incubation at 4°C overnight, the precipitate was removed, and added to 30 ml of BBS containing 10^{-4} M PC. This mixture was incubated at 4°C overnight, the precipitate removed, and the supernate extensively dialyzed against BBS. This purified anti-T15 antibody showed the same pattern of hapten inhibition of T15 binding as antibody previously described (27).

**Splenic Focus Technique.** Spleens from neonatal or adult donor mice and BALB/c fetal livers were homogenized in Dulbecco's modified Eagles medium. Bone marrow cells were obtained by the ejection of marrow plugs from adult femoral shafts or all four limbs in the neonates with Dulbecco's modified Eagles medium or RPMI 1640, plus 25 mM HEPES and 10% fetal calf serum, by using a syringe and 26 gauge needle. Cell clumps were removed by passage through a nylon filter, with 75-90% viability of the remaining cells. Donor cells were injected intravenously into syngeneic, Hy-primed, adult recipients that had been irradiated with 1,300 rads 4 h earlier. Fragment cultures were prepared from spleens of recipient mice 16 h after cell transfer, as previously described (28). The fragments were individually stimulated in culture with PPC-TGG-Hy (5 \times 10^{-7} M hapten), and the culture fluids were changed every 2-3 days. Fluids collected 9-13 days after stimulation were assayed for anti-hapten antibody, heavy chain isotype, and idiotype.

**Radioimmunoassays for Antibody and Idiotype.** 20 µl of culture fluids were added to PC-BSA immobilized on bromoacetyl cellulose (28) or polystyrene plates (29) (Cooke Laboratory Products Div., Alexandria, Va.), as previously described. Bound anti-hapten antibody was detected by the subsequent binding of \(^{125}\text{I}\)-labeled purified rabbit anti-mouse Fab antibody or \(^{125}\text{I}\)-labeled purified goat antibody against mouse μ- or γ-heavy-chain constant region determinants (11). The amount of antibody was quantitated in the anti-Fab assay by using T15 protein as a standard.

Idiotypic assays were performed by a modification of a previously described solid phase inhibition radioimmunoassay (24). 0.1 ml of the appropriate dilution of anti-idiotype in PBS was added to wells of polystyrene plates (Cooke Laboratory Products Div.) and incubated overnight at 4°C. The antibody was withdrawn and the wells washed with PBS. Then, 0.2 ml of PBS containing 1% BSA was added, and the wells incubated for 60 min at room temperature. The solution was removed and 20 µl of various concentrations of unlabeled inhibitor proteins or culture fluids were added, followed by addition of 0.1 ml of 1% BSA in PBS containing 2-4 ng of \(^{125}\text{I}\)-labeled purified T15 protein, with a specific activity of 15-20 µCi/µg protein. After incubation at 37°C for 18 h, the wells were washed three times with PBS and counted in a gamma counter.

**Antigen and Anti-Immunoglobulin Binding Cells and Autoradiography.** Antigen and anti-immunoglobulin binding cells were enumerated by methods described by Davie and Paul (30) and Raff (31), respectively. Spleen cells were suspended in 0.2 ml of Dulbecco's modified Eagles medium containing 10% gamma horse serum (North American Biologicals, Inc., Miami, Fla.) and 1.0 mg/ml of sodium azide. Either \(^{125}\text{I}\)-labeled PC-BSA (100 µg \(^{125}\text{I}\)-PC-BSA/ml in 1% BSA) was added to a final concentration of 3 \times 10^{-7} M PC or \(^{125}\text{I}\)-labeled anti-immunoglobulin (100 µg \(^{125}\text{I}\)-anti-Fab/ml in 1% BSA) was added to a final concentration of 0.1 µg/ml. After a 30-min incubation at 0°C, the cell suspensions were diluted to 1.0 ml with fresh medium, layered onto 2 ml of gamma horse serum, and the cells were sedimented by centrifugation at 900 g for 10 min. The cells were sedimented through serum twice more, re suspended in 50 µl of medium, and smeared on gelatin-coated microscope slides. Slides were fixed in 1% glutaraldehyde and dipped in NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, N. Y.). After a 7-14 day exposure, slides were developed in D-19 developer (Eastman Kodak Co.), stained with buffered Giemsa and examined for silver grains at 400 and 1,000 times magnification, by using a Zeiss microscope equipped with a 4x Dynascope head (Vision Systems, Inc., Ivyland, Pa.).
TABLE I
Number of PC-Specific Foci and Percentages of Those Foci Positive for the T15 Idiotype in Conventionally-Reared and Germfree Mice of Various Ages

| Source of donor spleen cells | Age | Total number of neonates | Total number of cells analyzed | Number of foci | Percent foci with T15 idiotype |
|-----------------------------|-----|--------------------------|-------------------------------|----------------|------------------------------|
| Conventionally-reared BALB/c mice | 0-3 | 61 | $171 \times 10^6$ | 0 | - |
|                             | 4-5 | 25 | $146 \times 10^6$ | 1 | 0 |
|                             | 6-7 | 52 | $144 \times 10^6$ | 93 | 63 |
|                             | 8-9 | 34 | $602 \times 10^6$ | 34 | 68 |
|                             | 14  | 31 | $911 \times 10^6$ | 65 | 75 |
|                             | 21  | 6  | $231 \times 10^6$ | 24 | 57 |
|                             | 28  | 3  | $199 \times 10^6$ | 25 | 72 |
| Adult*                      |     |    | $614 \times 10^8$ | 197 | 71 |
| Germfree BALB/c mice        | 0-3 | 13 | $62 \times 10^6$ | 0 | - |
|                             | 4-5 | 17 | $133 \times 10^6$ | 5 | 0 |
|                             | 6-8 | 14 | $393 \times 10^6$ | 39 | 56 |
| Adult*                      |     |    | $308 \times 10^8$ | 131 | 95 |

* Number of adult donors analyzed: 11 conventionally-reared BALB/c mice and 7 germfree BALB/c mice.

Results

Acquisition of the T15 Clonotype in Conventionally-Reared Mice. Table I shows that of the 61 neonates examined from birth to 3 days of age, no foci-producing anti-PC antibody were detected. One 4-day old neonate of the 25 analyzed had a PC-specific precursor cell, but this clone was not of the T15 idiotype. The vast majority of neonates acquire their first PC-specific B cells at approximately 1 wk after birth, and the majority of these are of the T15 idiotype, as is the case in the BALB/c adult (24). Fig. 1 plots the frequency of DNP and PC-specific precursor cells in adults and neonates during the first several weeks of life. As discussed in the Introduction, DNP-specific cells are present in high frequency at birth and remain relatively constant into adulthood (12). In contrast, PC-specific B cells do not appear in significant numbers until 6-7 days after birth and remain in relatively low frequency for several weeks. Assuming that 25% of the lymphocytes in a 1-wk old spleen of a BALB/c mouse are B cells (13, 32), the average frequency of the T15 clonotype at that point in development is approximately 1/250,000 B cells. Although, as Fig. 1 demonstrates, both conventionally-reared neonates and adults show wide variations in PC-specific precursor frequency, the average neonatal frequency at 1 wk of age is five times lower than the average adult precursor frequency.

Acquisition of the T15 Clonotype in Germfree Mice. Conventionally-reared
Fig. 1. The frequency of clonal precursor cells specific for PC or DNP at various ages. Each small circle represents the PC-specific precursor cell frequency derived from 1 to 10 donor spleens injected into 1 to 8 Hy-primed recipients; the bars indicate the average frequency at each age. Since no PC-specific foci were detected in 0- to 2-day-old donors, the bar at that age denotes the maximum precursor frequency if one focus had been detected. The triangles indicate the average PC-specific frequency in germfree donors at various ages. The open circles represent the average DNP-specific precursor frequency at various ages, derived from previously published data for neonates (11, 12) and for adults (28). The closed boxes indicate the average frequency and standard deviation of single DNP predominant clonotypes, assuming that each of the three predominant clonotypes is represented equally in the early neonatal period.

BALB/c adult mice have high titers of T15 in their serum (18, 24) and are constantly being antigenically stimulated with PC in the form of Lactobacillus acidophilus and intestinal helminths (33). Therefore, the role of maternal influences or the presence of antigen in the acquisition of PC-specific precursor cells was investigated by analyzing PC precursor frequencies in germfree neonates. The studies presented in Table I and Fig. 1 indicate that the kinetics of appearance of PC-specific precursors in germfree neonates is, in general, similar to that seen in conventionally-reared mice. Most importantly, no PC-specific precursor cells were found in 13 germfree neonates analyzed between birth and 3 days of age. While the precursor frequency of germfree neonates at 4 days of age
is higher than the conventionally-reared average, none of the foci from the 4-day old mice were of the T15 idiotype, and the 1-wk germfree frequency falls within the range seen for individual conventionally-reared mice. Thus, although the presence of antigen or maternal antibodies cannot be completely ruled out as the reason for the late acquisition of the T15 clonotype during the neonatal period, the data obtained from germfree neonates nursed by germfree mothers, who have no T15 in their serum (18, 24), suggest that such influences are not likely to be significant.

**Acquisition of the T15 Clonotype in Individual Mice.** Fig. 2 presents an analysis of the clones from individual 1 wk old conventionally-reared and germfree neonates. While, on the average, two-thirds of all foci from 1-wk old neonates are T15 positive, the representation of the T15 clonotype varies markedly from individual to individual. The majority of neonates have both T16-positive and T15-negative or cross-reactive foci, but others are composed solely of one group or the other. In many cases, this observation may be due to the small number of clones available for analysis from an individual neonate, making an all or none sampling phenomenon likely. However, in other cases, e.g. mice in conventionally-reared litter 2 or germfree litter 1, a sufficient number of clones have been analyzed to make the observation valid.

Fig. 2 also illustrates that the PC precursor frequencies of individual neonates within a single litter vary less from one another than the variance among the strain as a whole. Both germfree and conventionally-reared neonates demonstrate this correlation, although conventionally-reared 1 wk old mice fluctuate over a 20-fold range and germfree individuals vary only over a 8-fold range. The difference between germfree and conventionally-reared neonates mirrors the finding with adult donors, with adult germfree mice varying 4-fold and conventionally-reared adults fluctuating over a 30-fold range (19). The relative constancy of PC-specific precursor frequencies within a litter suggests that maternal factors may play a minor, but undefined, role in the expression of antibody specificities.

**Acquisition of the T15 Clonotype in CBFl Neonates.** One potential source of maternal influence on the level of PC-specific precursor cells is the transfer of T15, or perhaps auto-anti-T15, across the placenta. Although there is no correlation between the neonatal PC-specific precursor frequency and maternal anti-PC antibody level among litters tested (data not shown), the maternal-fetal interactions may be more complex than such a simple analysis allows. Previous work had indicated that a small portion of the anti-PC antibody present in nonimmune conventionally-reared adult mice is IgG1 (24). Table II demonstrates that the small amount of T15 present in BALB/c neonatal serum is probably of maternal origin, since IgM antibody does not appear in detectable quantities until the 2nd or 3rd wk of life. Since all four subclasses of mouse IgG can cross the placenta (34), other subclasses may be present in neonatal serum in addition to IgG1. Thus, since C57BL/6 mice do not have T15 in their serum, CBFl neonates raised by C57BL/6 mothers can be used to examine whether the presence of maternal T15 (and perhaps anti-T15) influences the expression of the T15 clonotype.

Table III presents the PC-specific precursor frequencies of CBFl neonates of
FIG. 2. The frequency of PC-specific clonal precursor cells in individual 7 day old mice. Spleen cell suspensions from 1 wk old germfree or conventionally-reared neonates were individually transferred to two Hy-primed syngeneic recipients. Solid bars indicate the number of T15-positive precursors, the clear bars represent the T15-negative precursor frequency, and the striped areas depict the frequency of clonotypes which cross-react with the murine anti-T15 serum.

TABLE II
Detection of Anti-PC Antibody and T15 Idiotype in Neonatal Sera

| Serum source* | Age | Anti-PC antibody | Heavy chain class | T15 idiotype |
|---------------|-----|------------------|-------------------|--------------|
|               | days| µg/ml            | µ     | γ1 | µg/ml |
| BALB/c        | 1‡ | 5.0              | -     | +  | 0.6   |
|               | 7  | 4.5              | -     | -  | 0     |
|               | 14 | 6.0              | +     | -  | 1.4   |
| Adult§        |    | 64.0             | +     | -  | 60.0  |
| (C57BL/6 ♀ × BALB/c ♂)F₁ | 1  | 1.1              | -     | -  | 0     |
|               | 7  | 1.7              | -     | -  | 1.5   |
|               | 14 | 9.0              | +     | -  | 1.0   |
| (BALB/c ♀ × C57BL/6 ♂)F₁ | 1  | 1.3              | -     | +  | 0     |
|               | 1  | 1.2              | -     | -  | 0     |
|               | 7  | 7.5              | ±     | -  | 0     |
|               | 14 | 10.0             | +     | -  | 1.0   |

* Serum dilutions containing 0.04-2 µl of serum were run in quadruplicate in the radioimmunoassays for anti-PC antibody and T15 idiotype as described in the Materials and Methods. Serum antibody was quantitated by using purified T15 protein. The specificity of the radioimmunoassay for T15 idiotype has been described previously (25).
‡ Sera from 8-10 1 day, 6-10 7 day, and 4-6 2-wk-old mice were pooled.
§ Values for BALB/c adult sera represent the average of five individual donors.
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TABLE III
Comparison of (C57BL/6 × BALB/c × F1 and (BALB/c × C57BL/6 × F1 Neonates in the Frequency of PC-Specific and T15-Positive Clones

| Mother | Age  | Total number of neonates | Total number of cells analyzed | Number of foci | Number of precursors per 10⁶ spleen cells | Number of precursors with T15 idiotype per 10⁶ spleen cells |
|--------|------|-------------------------|-------------------------------|---------------|------------------------------------------|-------------------------------------------------|
|        | days |                         |                               |               |                                          |                                                 |
| BALB/c | 1    | 12                      | 16 × 10⁶                      | 0             | -                                        | -                                               |
|        | 4-5  | 27                      | 316 × 10⁶                     | 15            | 1.19                                     | 0                                               |
|        | 6-8  | 21                      | 415 × 10⁶                     | 85            | 5.12                                     | 0.42                                           |
|        | 13   | 5                       | 127 × 10⁶                     | 16            | 3.15                                     | 1.18                                           |
| C57BL/6 | 1    | 14                      | 18 × 10⁶                      | 0             | -                                        | -                                               |
|        | 4-5  | 13                      | 86 × 10⁶                      | 3             | 0.87                                     | 0                                               |
|        | 6-8  | 8                       | 256 × 10⁶                     | 48            | 4.68                                     | 0.95                                           |

various ages born to BALB/c or C57BL/6 mothers. The data indicate that the acquisition of the T15 clonotype is unaffected by the maternal environment of the C57BL/6 strain. It is of interest that the PC-specific precursor frequency of 4–5 day old CBF₁ neonates, irrespective of the maternal parentage, is higher than in BALB/c neonates at the same point in development (approximately one per 10⁶ versus one per 6 × 10⁶ spleen cells, respectively). However, none of these earlier arising clones are of the T15 idiotype. These B cells may represent specificities arising from the C57BL/6 variable region gene pool, which may be programmed to arise earlier in development.

Antigen-Binding Cell Analysis of PC-Specific B Cells. One explanation for the late appearance of the T15 clonotype is that although the T15 clonotype is generated at the same point in development as cells committed to the predominant DNP and TNP-specific clonotypes, the B cells specific for PC require more time to acquire the ability to collaborate with T cells or, in some other way, are masked from detection in the splenic focus assay. Alternatively, neonatal PC-specific B cells may display different homing properties than adult or neonatal DNP and TNP-specific cells and thus may be obscured from analysis. If these arguments were valid, it would be predicted that PC-binding cells could be detected in neonatal spleen cell suspensions before their appearance in the splenic focus system. Table IV presents the data from several representative experiments comparing PC-BSA-binding cells to the number of cells responsive to PC in the splenic focus system. The frequency of PC-binding cells in suspensions of adult spleen cells is consistent with previous reports and represents the upper and lower range of precursor frequencies observed in the conventionally-reared adult. The cell suspension from a pool of 4- to 5-day-old BALB/c neonates demonstrates the same correlation between PC antigen-binding cells and stimulated PC-specific precursors in the splenic focus assay as that seen in the adult. Since previous reports (9) have shown that 5% of both neonatal and adult spleen cells lodge in recipient fragments, the cloning efficiency of 4% indicates that 80% of resident precursor cells are stimulated to detectable antibody-forming cell
TABLE IV
Frequency of PC-Specific Antigen-Binding Cells and Clonal Precursor Cells

| Donor                | Antigen-binding cells per $10^8$ spleen cells* | Total cells counted | Foci per $10^8$ transferred cells | Total cells transferred | Efficiency†% |
|----------------------|-----------------------------------------------|--------------------|----------------------------------|------------------------|--------------|
| Adult 1              | 24.0                                          | $5 \times 10^8$    | 0.96                             | $12.5 \times 10^8$     | 4.0          |
| Adult 2              | 5.8                                           | $5 \times 10^8$    | 0.25                             | $6 \times 10^8$        | 4.3          |
| Neonate (4–5 days)   | 0.35                                          | $20 \times 10^8$   | 0.013                            | $7.6 \times 10^8$      | 3.7          |

* Number of $^{125}$I-PC-BSA-binding cells per $10^8$ spleen cells.
† Foci per $10^8$ transferred cells $\times 100$/Antigen-binding cells per $10^8$ spleen cells.

TABLE V
Frequency of PC-Specific B Cells in the Fetal Liver and Bone Marrow of Conventionally-Reared BALB/c Mice of Various Ages

| Source of cells | Age | Total number of donors | Total number of cells analyzed | Number of precursors per $10^8$ injected cells |
|-----------------|-----|------------------------|-------------------------------|-----------------------------------------------|
| 19 day fetal liver | –   | 10                     | –                             | 0                                             |
| Bone marrow     | 5   | 9                      | $13 \times 10^8$              | 0                                             |
|                 | 7   | 41                     | $112 \times 10^8$             | 1.3                                           |
|                 | 10  | 10                     | $4 \times 10^8$               | 3.1                                           |
|                 | 14  | 10                     | $40 \times 10^8$              | 2.5                                           |
|                 | Adult | 5                       | $145 \times 10^8$            | 12.6                                          |

clones. Therefore, it is unlikely that major B-cell subsets are being ignored in this analysis. In addition, the antigen-binding cell studies suggest that neonatal B cells behave similarly to adult B cells when provided with adult T-cell help in the splenic focus system and confirm the finding that PC-specific B cells are extremely rare in early neonatal spleen cell populations.

Ontogeny of PC-Specific Precursor Cells in Neonatal Bone Marrow and Fetal Liver. Since recent evidence suggests that B cells develop independently in both the fetal liver and fetal spleen (35, 36), it is possible that PC-specific B cells arise exclusively in the fetal liver, travel to the neonatal bone marrow, and subsequently seed to the spleen. As seen in Table V, no PC-specific B cells are found in 19-day fetal liver, in contrast to the ability to detect DNP-specific B cells at this point in ontogeny (9). Table V also demonstrates that there are no precursor cells of the T15 clonotype in the bone marrow until day 7 of life, coincident to their appearance in the spleen.

Discussion

To distinguish among the many theories of antibody diversity generation, the processes involved in the acquisition of the B-cell specificity repertoire during
ontogeny must be clearly understood. Indeed, the fundamental distinction between whether all clonotypes are encoded in the germline or whether a few germline specificities are diversified by random somatic events is most easily evaluated during the neonatal period. If random somatic events, such as somatic mutation or chromosomal recombination, are involved in diversification, different specificities should arise in each individual during ontogeny, so that the repertoire of syngeneic neonates should diverge early in development and would reflect the individual's antigenic experience far more than its genetic background.

Previous studies have led to conflicting views concerning the development of the B-cell repertoire. The pioneering work of Silverstein et al. (37) demonstrated a sequential hierarchy of antibody responsiveness to a variety of antigens in the fetal sheep. However, the interpretation of these experiments was complicated by the fact that the animals were genetically diverse and that other cell types involved in antibody production (T cells and macrophages) are functionally immature at birth (38-42). Nevertheless, Sherwin and Rowlands (43) still observed a sequential hierarchy of antibody responsiveness when neonatal B cells were provided with mature T cells and macrophages (43). This sequential pattern was invariant among individuals within a mouse strain and the sequence of expression could not be altered by the presence of antigen. These studies, as well as those of Goidl and Sikind (44), which demonstrated a marked restriction of the neonatal repertoire, suggest a genetically predetermined acquisition and expansion of the B-cell repertoire during ontogeny.

Several antigen-binding cell analyses of developing cell populations (13, 45, 46) have indicated that cells binding many different antigens appear simultaneously, increase in parallel with the ontogeny of immunoglobulin-bearing cells, and express the full range of antigen-binding avidities on development. Because of the rapid expression of a heterogeneous array of specificities, these studies are inconsistent with classical somatic mutation theories as well as theories postulating sequential acquisition of the repertoire. However, in a recent study by D'Eustachio et al. (47), the frequency of cells binding TNP and sheep erythrocytes in 18-day fetal livers was found to be under genetic control in two inbred mouse strains. This finding may be explained by the genetically-programmed expression of clonotypes in the two strains. Consistent with this interpretation are the experiments of Lydyard et al. (48), who studied the anatomical distribution of B-cell clones generated within the bursa of Fabricius of chickens. Small foci of antigen-binding cells appeared in multiple bursal follicles, and this development occurred in an invariant, antigen-independent, sequential pattern.

Since antigen-binding cell analyses are often relatively nonspecific and do not have the capacity to delineate developing specificities at the clonotype level, we have investigated the developing B-cell repertoire at the level of stimulatable clonal precursor cells. The major findings have provided evidence of a patterned process of repertoire acquisition within neonates of a single strain. Our early studies demonstrated an unusual reproducibility in the early expression of BALB/c clones responsive to DNP and TNP (9, 11, 12); however, the most striking observation and the finding which most clearly establishes the patterned expression of clonotypes throughout the 1st-wk of postnatal development
is the evidence that PC-responsive B cells, particularly the T15 clonotype, are reproducibly found only after the 1st-wk of life.

The ability to tolerize PC-specific splenic B cells as late as day 10 after birth (23) is the best evidence that the T15 clonotype arises from the generative cell pool at a much later point in development than the DNP predominant clonotypes, which are expressed and tolerizable only during the first few days of neonatal life. Since this assay appears to be an unambiguous marker of immature cells, the finding can only be accounted for if the T15 clonotype in a 7-day old spleen bears the same relationship to the stem cell pool as the DNP predominant clonotypes in a newborn spleen. These findings, along with the evidence presented in this report, confirm the invariant, late occurrence of PC-specific B cells and support a highly-ordered, rigorously predetermined mechanism for the acquisition of the B-cell repertoire.

Previous reports have shown that the frequency of any one of the DNP or TNP-specific predominant clonotypes represents 1/10,000 B cells at birth (11). If the clonotypes which dominate the repertoire at birth represent the earliest expression of the germline genetic information for antibody specificity, the data presented here indicate that, early in development, the T15 clonotype is at least 100 times less frequent than the previously identified germline clonotypes. Thus, although the number of PC-specific B cells in neonates varies among individuals, few PC-specific B cells are observed before day 6. It is of interest that the variance among individuals was less among littermates, a finding similar to that seen for DNP and TNP predominant clonotypes (11). Although BALB/c neonates expressed the three DNP and the three TNP clonotypes in approximately equal proportions among the population as a whole, an individual neonate expressed a preponderance of one of the three DNP and one of the three TNP predominant clonotypes, and these specificities were also expressed preferentially by the individual's littermates.

The appearance of PC-specific precursor cells—and the T15 clonotype in particular—would fit into the category of sporadic clonotypes, i.e., a late arising specificity, in the terminology used to describe the development of the DNP, TNP, and FL repertoires (11), and as such is not significantly different than the vast majority of specificities. The importance of this observation for the T15 clonotype is that: (a) it fulfills many of the criteria of a germline antibody specificity (16–20); (b) in the adult it is expressed by an unusually large B-cell clone (19); and (c) the highly ordered nature of this process has been demonstrated. The designation of the nonpredominant DNP and TNP clonotypes as sporadic may, in fact, have been a misnomer. The acquisition of these specificities may be just as predictable as the T15 clonotype, but because of the heterogeneity of the DNP and TNP repertoires and the relative insensitivity of sucrose gradient isoelectric focusing, the ordered nature of these events was obscured. Thus, the generation of not only the T15 clonotype but perhaps all specificities may be the result of precisely programmed events.

To evaluate possible maternal influences in the kinetics of expression of PC-specific B cells, two types of experiments were carried out. First, germfree neonates were shown to acquire PC-specific B cells with similar kinetics as litters from conventional mothers. Second, to insure that the BALB/c maternal
environment was not, in some unknown way, suppressing the early appearance of the T15 clonotypes, the arisal of B cells bearing the T15 idiotype was examined in both (C57BL/6 \( \times \) BALB/c) \( \times \) F\(_1\) and (BALB/c \( \times \) C57BL/6 \( \times \) C) \( \times \) F\(_1\) mice. Although germfree mice do not have T15 in their serum (18, 24), they still may have other serum factors (perhaps auto-anti-idiotype) responsible for both the predominance of this clonotype in in vivo antibody responses and the alteration of expression of the T15 clonotype during the neonatal period. The results show that CBF\(_1\) mice from both BALB/c and C57BL/6 mothers demonstrate similar kinetics of appearance of the T15 clonotype as seen in BALB/c neonates, indicating that the BALB/c maternal environment does not suppress the early expression of this specificity. Furthermore, the presence of the C57BL/6 gene pool does not alter the time of appearance or the highly ordered nature of the T15 clonotype's expression. This observation suggests that the acquisition of the B-cell specificity repertoire is rigorously programmed; the variable region genes are read out in a sequence intrinsic to the information contained on that chromosome and operates independently of any information, such as enzymes or episomes, carried in another variable region gene pool. The exception to this notion may be the generation of novel heavy-light chain pairs, but since recent evidence suggests that many mouse strains have T15-like light chains (49), this may not affect the expression of the T15 clonotype.

The late appearance of PC-specific B cells in BALB/c mice appears to be due to the absence of these cells, since antigen-binding cell analysis of neonatal spleen and examination of fetal liver and neonatal bone marrow as early as day 5 of life fail to detect unexpected numbers of PC-specific precursor cells. Comparison of adult and neonatal PC-binding cell frequencies confirms that both populations home to the spleen and are stimulated with the same efficiency. Since B cells responsive to PC appear to arise simultaneously in both the spleen and bone marrow but remain immature for a longer period of time in the latter pool (23), it is unlikely that the T15 clonotype was initially generated in the bone marrow during fetal or early neonatal life and subsequently migrated to the spleen. The T15 clonotype may arise independently in the bone marrow and spleen from the generative cell pools or, alternatively, may first be generated in the spleen.

We have focused a great deal of attention on the T15 clonotype since it fulfills many prerequisites of a germline expression. However, late appearance in the repertoire is characteristic of most or all other PC-specific clonotypes as well as most precursor cells responsive to DNP, TNP, and FL. It should also be noted that there is no apparent correlation between the time in ontogeny of expression of a clonotype and its eventual representation in the adult B-cell pool. While the T15 clonotype is acquired at approximately 1 wk of age and can reach a clone size of as many as 3,000 cells in the adult (19), T15-negative clonotypes which are expressed at the same point in development, constitute a minority of the PC-specific repertoire, and may be represented by, on the average, fewer than 10 cells in the adult mouse (27).

The relatively late appearance of the T15 clonotype has profound implications for the acquisition of the B-cell specificity repertoire. Although this specificity is present in high frequency in every adult BALB/c mouse and serves as the prototype of a germline antibody specificity, it displays none of the characteris-
tics of the six DNP and TNP predominant clonotypes. In addition to its late acquisition, the appearance of the T15 clonotype is a highly predictable process in that between the 5th and 8th day of postnatal life, essentially every individual BALB/c neonate expresses the T15 clonotype. The results presented in this report argue strongly against random, antigen-driven events playing a role in this highly patterned process of diversity acquisition and suggest a reliance on evolutionary rather than environmental selective forces. Nucleic acid hybridization experiments (50), now clearly indicate that very few inherited variable region genes are responsible for the large number of specificities present in the adult repertoire. If this is so, repertoire acquisition may best be accounted for by the predetermined permutation of the genetic information inherent in the germline (8). While such a theory does not define a molecular model for the generation of the repertoire, the postulates provide a conceptual framework within which any more detailed model must be constrained. It is critical to the predetermined permutation theory that all potential specificities which arise from heavy-light chain associations be expressed at least once during the lifetime of the individual, so that evolutionary selection can apply to the species as a whole. Somatic expansion of the heavy and light chain variable region gene pools would occur in a programmed fashion and may involve the sequential expression of multiple hypervariable regions within a single variable region framework. A model which is consistent with the predetermined permutation theory involves recombinational events among a limited number of germline genes with subsequent correction of base pair mismatches to produce new, but predictable, sequences (M. Gefter, personal communication). To distinguish between this and other mechanistic models such as hypervariable region insertion (51), future experiments must assess the developing neonatal and adult repertoires at the level of amino acid or nucleotide sequence analysis, which may provide information concerning the relationship between variable region genes.

Summary

The TEPC 15 (T15) clonotype, a putatively germline antibody specificity, does not appear in the neonatal B-cell repertoire until approximately 1 wk of age. This report extends this observation by the demonstration that (a) the T15 clonotype follows similar kinetics of appearance in germfree as well as conventionally-reared mice; (b) maternal influences and genetic background play a minor role in the development of the T15 clonotype since CBFI neonates raised by C57BL/6 or BALB/c mothers acquire the T15 clonotype at the same time in ontogeny as BALB/c neonates; (c) the lack of phosphorylcholine (PC)-specific B cells shortly after birth is reflected in a dearth of PC-binding cells in the neonate as well; and (d) no PC-specific B cells are found in 19-day fetal liver or in bone marrow until 7 days of life, coincident with their appearance in the spleen. These findings, along with a previous report that PC-specific splenic B cells are tolerizable as late as day 10 after birth, confirm the invariant, late occurrence of the T15 clonotype and support a highly-ordered, rigorously predetermined mechanism for the acquisition of the B-cell repertoire. The results are discussed in light of other studies on the ontogeny of B-cell specificity, and in
terms of the implications on the mechanism by which antibody diversity is generated.

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References

1. Kreth, H. W., and A. R. Williamson. 1973. The extent of diversity of anti-hapten antibodies in inbred mice: anti-NIP (4-hydroxy-5-ido-3-nitro-phenacetyl) antibodies in CBA/H mice. Eur. J. Immunol. 3:141.
2. Pink, J., and B. Askonas. 1974. Diversity of antibodies to crossreacting nitrophenyl haptens in inbred mice. Eur. J. Immunol. 4:426.
3. Braun, D. G., J. Quintáns, A. L. Luzzati, I. Lejkovits, and S. E. Read. 1976. Antibody response of rabbit blood lymphocytes in vitro: kinetics, clone size, and clonotype analysis in response to streptococcal group polysaccharide antigens. J. Exp. Med. 143:360.
4. Mäkelä, O., and K. Karjalainen. 1977. Inherited immunoglobulin idiotypes of the mouse. Transplant. Rev. 34:119.
5. Ju, S-T., A. Gray, and A. Nisonoff. 1977. Frequency of occurrence of idiotypes associated with anti-p-azophenyl-arsonate antibodies arising in mice immunologically suppressed with respect to a cross-reactive idiotype. J. Exp. Med. 145:540.
6. Köhler, G. 1976. Frequency of precursor cells against the enzyme β-galactosidase. An estimate of the BALB/c strain antibody repertoire. Eur. J. Immunol. 6:340.
7. Klinman, N. R., and J. L. Press. 1975. The B-cell specificity repertoire: its relationship to definable subpopulations. Transplant. Rev. 24:17.
8. Klinman, N. R., and J. L. Press, N. H. Sigal, and P. J. Gearhart. 1976. The acquisition of the B-cell specificity repertoire: the germline theory of predetermined permutation of genetic information. In The Generation of Diversity: A New Look. A. J. Cunningham, editor. Academic Press, Inc., New York. 127.
9. Press, J. L., and N. R. Klinman. 1973. Enumeration and analysis of antibody-forming cell precursors in the neonatal mouse. J. Immunol. 111:829.
10. Press, J. L., and N. R. Klinman. 1973. Isoelectric analysis of neonatal monofocal antibody. Immunochemistry. 10:621.
11. Klinman, N. R., and J. L. Press. 1975. The characterization of the B-cell repertoire specific for the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl determinants in neonatal BALB/c mice. J. Exp. Med. 141:1133.
12. Press, J. L., and N. R. Klinman. 1974. Frequency of hapten-specific B cells in neonatal and adult mouse spleen. Eur. J. Immunol. 4:155.
13. 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.
14. Gelfand, M. C., G. J. Elfenbein, M. M. Frank, and W. E. Paul. 1974. Ontogeny of B lymphocytes. II. Relative rates of appearance of lymphocytes bearing surface immunoglobulin and complement receptors. J. Exp. Med. 139:1125.
15. Nossal, G., and B. Pike. 1973. Studies on the differentiation of B lymphocytes in the mouse. Immunology. 25:33.
16. Sher, A., and M. Cohn. 1972. Inheritance of an idiotype associated with the immune response of inbred mice to phosphorylcholine. Eur. J. Immunol. 2:319.
17. Claflin, J. L., R. Lieberman, and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine. I. Specificity, class, and idiotype of phosphorylcholine-binding receptors on lymphoid cells. J. Exp. Med. 139:58.
18. Lieberman, R., M. Potter, E. B. Mushinski, W. Humphrey, Jr., and S. Rudikoff. 1974. Genetics of a new IgVH (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. J. Exp. Med. 139:983.

19. Sigal, N. H., P. J. Gearhart, and N. R. Klinman. 1975. The frequency of phosphorylcholine-specific B cells in conventional and germfree BALB/c mice. J. Immunol. 68:1354.

20. Cosenza, H., J. Quintans, and I. Lefkovits. 1975. Antibody response to phosphorylcholine in vitro. I. Studies on the frequency of precursor cells, average clone size and cellular cooperation. Eur. J. Immunol. 5:343.

21. Eichmann, K. 1975. Genetic control of antibody specificity in the mouse. Immunogenetics. 2:491.

22. Sigal, N. H., P. J. Gearhart, J. L. Press, and N. R. Klinman. 1976. The late acquisition of a "germline" antibody specificity. Nature (Lond.). 259:51.

23. Metcalf, E. S., N. H. Sigal, and N. R. Klinman. 1977. In vitro tolerance induction of neonatal murine B cells as a probe for the study of B-cell diversification. J. Exp. Med. 145:1382.

24. 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.

25. Cuatrecasas, P. 1970. Protein purification by affinity chromatography: derivatizations of agarose and poly-acrylamide beads. J. Biol. Chem. 245:3059.

26. Lieberman, R., and W. Humphrey. 1971. Association of H-2 types with genetic control of immune responsiveness to IgA allotypes in the mouse. Proc. Natl. Acad. Sci. U. S. A. 68:2510.

27. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1977. The monoclonal antiphosphorylcholine antibody response in several murine strains: genetic implications of a diverse repertoire. J. Exp. Med. 145:876.

28. Klinman, N. R. 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. J. Exp. Med. 136:241.

29. Klinman, N. R., A. Pickard, N. H. Sigal, P. J. Gearhart, E. S. Metcalf, and S. K. Pierce. 1976. Assessing B-cell diversification by antigen receptor and precursor cell analysis. Ann. Immunol. 127C:489.

30. Davie, J. M., and W. E. Paul. 1974. Antigen binding receptors on lymphocytes. Contemp. Top. Immunobiol. 3:171.

31. Raff, M. C. 1973. T and B lymphocytes and immune responses. Nature (Lond.). 242:19.

32. Sidman, C. L., and E. R. Unanue. 1975. Development of B lymphocytes. I. Cell populations and a critical event during ontogeny., J. Immunol. 114:1730.

33. Potter, M., and R. Lieberman. 1970. Common individual antigenic determinants in five of eight BALB/c IgA myeloma proteins that bind phosphoryl choline. J. Exp. Med. 132:737.

34. Nisonoff, A., J. Hopper, and S. Spring. 1975. In The Antibody Molecule. Academic Press, Inc., New York. 336.

35. Owen, J. J. T., M. Raff, and M. Cooper. 1975. Studies in the generation of B lymphocytes in the mouse embryo. Eur. J. Immunol. 5:468.

36. Owen, J. J. T., R. K. Jordan, J. H. Robinson, U. Singh, and H. N. A. Willcox. 1976. In vitro studies on the generation of lymphocyte diversity. Cold Spring Harbor Symp. Quant. Biol. 41:29.

37. Silverstein, A. M., J. Uhr, K. L. Kraner, and R. Lukes. 1963. Fetal responses to antigenic stimulus. II. Antibody production by the fetal lamb. J. Exp. Med. 117:799.

38. Argyris, B. F. 1968. Role of macrophages in immunological maturation. J. Exp. Med. 128:459.
39. Landahl, C. 1976. Ontogeny of adherent cells. I. Distribution and ontogeny of B cells participating in the response to sheep erythrocytes in vitro. *Eur. J. Immunol.* 6:130.

40. Chison, M., and E. Golub. 1970. Functional development of the interacting cells in the immune response. I. Development of T cell and B cell function. *J. Immunol.* 108:1379.

41. Arrenbrecht, S. 1973. Normal development of the thymus-dependent limb of humoral immune responses in mice. *Eur. J. Immunol.* 3:506.

42. Spear, P. G., and G. M. Edelman. 1974. Maturation of the humoral immune response in mice. *J. Exp. Med.* 139:249.

43. Sherwin, W. K., and D. T. Rowlands, Jr. 1975. Determinants of the hierarchy of humoral immune responsiveness during ontogeny. *J. Immunol.* 115:1549.

44. Goidl, E. A., and G. W. Siskind. 1974. Ontogeny of B-lymphocyte function. I. Restricted heterogeneity of the antibody response of B lymphocytes from neonatal and fetal mice. *J. Exp. Med.* 140:1285.

45. Decker, J., J. Clarke, L. Bradley, A. Miller, and E. Serarz. 1974. Presence of antigen-binding cells for five diverse antigens at the onset of lymphoid development: lack of evidence for somatic diversification during ontogeny. *J. Immunol.* 113:1823.

46. D'Eustachio, P., and G. M. Edelman. 1975. Frequency and avidity of specific antigen-binding cells in developing mice. *J. Exp. Med.* 142:1078.

47. D'Eustachio, P., J. E. Cohen, and G. M. Edelman. 1976. Variation and control of specific antigen-binding cell populations in individual fetal mice. *J. Exp. Med.* 144:259.

48. Lydyard, P. M., C. E. Grossi, and M. D. Cooper, 1976. Ontogeny of B cells in the chicken. I. Sequential development of clonal diversity in the bursa. *J. Exp. Med.* 144:79.

49. Claflin, J. L., and S. Rudikoff. 1976. Uniformity in a clonal repertoire: a case for a germline basis of antibody diversity. *Cold Spring Harbor Symp. Quant. Biol.* 41:725.

50. Tonegawa, S., N. Hozumi, G. Matthyssens, and R. Schuller. 1976. Somatic changes in content and context of immunoglobulin genes. *Cold Spring Harbor Symp. Quant. Biol.* 41:877.

51. Capra, J. D., and T. J. Kindt. 1975. Antibody diversity: can more than one gene encode each variable region? *Immunogenetics.* 1:417.