LATE CLONAL SELECTION AND EXPANSION OF THE TEPC-15 GERM-LINE SPECIFICITY*

By JOHN FUNG AND HEINZ KÖHLER

From La Rabida-University of Chicago Research Institute and the Departments of Pathology and Biochemistry, The University of Chicago, Chicago, Illinois 60649

The BALB/c mouse possesses the potential to express different idiotypes which bind to phosphorylcholine (PC).[1] Analysis of the murine repertoire for PC-containing antigens has shown that the total number of PC-specific clones is quite large, on the order of several hundred clones or more (1). This clonotype diversity can be seen within the family of numerous PC-binding myeloma proteins, derived in BALB/c mice which differ in idiotype (2, 3), and also within the PC-specific B cell repertoire expressed in the splenic focus assay system (4–6). Yet, the BALB/c mouse, if immunized with T cell-dependent (TD) or T cell-independent (TI) PC antigens, responds with the predominant expression of a single idiotype, that of the TEPC-15 idiotype (7–10). The clonal dominance of the TEPC-15 idiotype in the response to PC (11, 12) or of other dominant idiotypes in responses to bacterial antigens, such as dextran (13) or streptococcal group A carbohydrates (14, 15), has been considered a hallmark for germ-line gene responses. The highly predictable appearance of PC-specific/TEPC-15-bearing precursors during ontogeny added further support to the germ-line "closeness" of the TEPC-15 idiotype. Sigal and co-workers (11, 12) have described the late acquisition of the TEPC-15 clonotype and emphasized the importance of this finding for the germ-line character of TEPC-15 coding gene(s). Thus, the late and predictable expression of the TEPC-15-predominant idiotype was thought to have significant implications for the mechanisms by which the B cell repertoire is acquired.

In the present report we have reinvestigated the maturation of PC-specific clones in BALB/c mice using a novel PC antigen which stimulates immature B cells effectively. We also used the splenic fragment culture technique described by Klinman and co-workers (11, 16), which maximizes T help and the conditions for expression and development of maturing B cells, to search for the earliest detectable precursors for PC in the neonatal liver. By these approaches, PC-specific responses or precursors were detected as early as 1 d after birth. These early responding PC clones, however, were not restricted to the predominant expression of the TEPC-15 idiotype.

¹ *Abbreviations used in this paper:* ATS, anti-thymocyte serum; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DMEM, Dulbecco's modified Eagle's medium; DPPC, *p*-diazonium phenyl phosphorylcholine; Hy, Limulus polyphemus hemocyanin; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide from *E. coli*; PBS, phosphate-buffered saline; PC, phosphorylcholine; PFC, plaque-forming cells; PnC, C-polysaccharide from R36A; PPC-TGG, 3-(*p*-azophenyl phosphorylcholine)-*N*-acetyl-L-tyrosylglycylglycine; RGG, rabbit gamma globulin; SRBC, sheep erythrocytes; TD, T cell dependent; TI, T cell independent; TNP, 2,4,6-trinitrophenyl.

Materials and Methods

Mice. BALB/c females, 6–8 wk of age, were purchased from Cumberland View Farms, Cumberland, Tenn. Mice used as recipients in splenic focus assays were primed by intraperitoneal injection of 100 μg limulus polyphemus hemocyanin (Hy) in complete Freund's adjuvant (CFA). A/He females were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c neonates were raised in our breeding colony.

Antigens. TEPC-15, the major anti-PC idiotype in BALB/c mice (7, 17), was obtained from mice carrying the myeloma tumor in ascites form (tumors were originally received from Dr. M. Potter). The purified protein was obtained by hapten elution from a PC-tyrosyl-glycine-Sephadex 4B column according to the procedure described by Cheseboro and Metzger (18). MOPC-315, a 2,4,6-trinitrophenyl (TNP)-binding myeloma protein, was obtained from Litton Bionetics, Kensington, Md.; Hy was obtained from Worthington Biochemical Corp., Freehold, N. J.; bovine serum albumin (BSA) and rabbit gamma globulin (RGG) were obtained from Miles Laboratories, Inc., Elkhart, Ind.; *E. coli* 0111:B4 lipopolysaccharide (LPS) was purchased from Difco Laboratories, Detroit, Mich.

$TNP_{25}Hy$ and $TNP_{44}BSA$ were prepared according to Klinman (19) using 2,4,6-trinitrobenzene sulfonic acid (Sigma Chemical Co., St. Louis, Mo.) as the conjugating agent. TNP_{15}-LPS was synthesized by the procedure described by Fidler (20). PC_{30} keyhole limpet hemocyanin (KLH), $PC_{21}BSA$, 3-(*p*-azophenyl phosphorylcholine)-*N*-acetyl-L-tyrosylglycylglycine (PPC-$TGG)_8$-Hy, and (PPC-$TGG)_6$-RGG are PC-containing proteins prepared by reacting *p*-diazonium phenyl phosphorylcholine (DPPC) (18) to proteins according to the procedure described by Lee et al. (7) with appropriate modifications described by Inman et al. (21, 22) and Sigal et al. (12). (PPC)$_8$-TGG-LPS was synthesized according to the procedure described by Inman et al. (21, 22) with the following modifications. TGG Boc hydrazide (compound G) was first conjugated to LPS which had been previously dialyzed against 10^{-3} M NaOH in saline. This TGG-LPS compound was dialyzed against borate-buffered saline, pH 8.5, and DPPC was added and allowed to react overnight at 4°C. Unreacted DPPC was removed by dialysis against saline. All conjugation ratios are expressed as moles PC per 10^5 dalton carrier.

R36A, the rough strain of *Streptococcus pneumoniae*, was grown in Columbia broth, harvested, and the soluble extract, the C-polysaccharide (PnC), was prepared by the method of Liu and Gotschlich (23). Mouse F(ab')$_2$ was prepared by pepsin digestion of mouse IgG followed by Sephacryl S-200 filtration.

Antisera. A/He anti-TEPC-15 antisera was prepared according to Potter and Lieberman (24). The antisera was absorbed on insolubilized mouse immunoglobulin which had been passed over PC-Sepharose. Idiotype binding capacity and specificity of the antisera were determined by radioimmunoassays. Typically, the antisera had a binding capacity of 100 μg/ml and did not cross-react with other non-TEPC-15 PC-binding myeloma proteins. Rabbit anti-mouse thymocyte serum was purchased from Microbiological Associates, Walkersville, Md. Rabbit anti-mouse F(ab')$_2$ antisera was prepared by repeated injections of rabbits with mouse F(ab')$_2$ in CFA. Goat anti-rabbit IgG was purchased from Litton Bionetics, absorbed on a rabbit IgG immunoabsorbant and iodinated by the chloramine T method.

Hemolytic Plaque Assay. Enumeration of cells secreting anti-PC IgM antibodies were determined by a slide modification of the Jerne-Nordin hemolytic plaque assay (25). Adult animals (ages day 25 and over) received either 50 μg PC-KLH, 1 μg PnC, or 10 μg PC-LPS intravenously. Neonatal animals, ages day 0–10 received either 5 μg PC-KLH, 0.5 μg PnC, or 1 μg PC-LPS intraperitoneally; ages day 11–25 received either 20 μg PC-KLH, 1 μg PnC, or 5 μg PC-LPS intraperitoneally. Doses were determined from the dose that would give maximal responses from a representative group of animals in a given age group (data not shown). Spleens were analyzed for plaque-forming cells 5 d after immunization. PC-specific plaque-forming cells were detected using PnC-coated sheep erythrocytes (SRBC) as target cells (26). Clonotype analysis of anti-PC-secreting antibody-forming cells was performed according to the procedure of Cosenza and Köhler (27) incorporating A/He anti-TEPC-15 antisera at a final dilution of 1:750 in the plaquing mixture.

Splenic Focus Assay. Spleens from 6- to 8-wk-old BALB/c mice, and neonatal spleens and livers from day 1 or 6 neonates were first gently pressed through a fine mesh screen to obtain a single cell suspension. The cells were washed two times in Dulbecco's modified Eagle's

medium (DMEM) and passed through a glass wool column to remove debris. 5–20 × 10^6 cells were injected intravenously via the lateral tail vein into primed mice, 6–8 h after 1,400-rad total body irradiation. The conditions of in vitro culture of splenic fragments are briefly described (6, 11, 28). The spleens of the recipient mice were removed 20–24 h after transfer and sliced into 1-mm fragments with a McIlwain tissue chopper (Brinkmann Instruments, Inc., Westbury, N.Y.). The fragments were cultured in sterile 96-well culture plates (Costar, Data Packaging, Cambridge, Mass.), containing DMEM-high glucose (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% fetal calf serum (KC Biological, Inc., Lexena, Kan.) containing nonessential amino acids, penicillin, streptomycin, gentamicin, and L-glutamine. Fragments were then stimulated with 5 × 10^{-7} M hapten (either TNP-Hy or PPC-TGG-Hy). The fragments were incubated at 37.5°C in 92% O_2 and 8% CO_2. On day 4, the antigen was removed and fresh medium was added. Supernates were collected and changed on days 7, 10, and 13 and analyzed for anti-hapten idiotype by radioimmunoassay.

Radioimmunoassay. Anti-hapten radioimmunoassays (6, 12) were modified for use in 96-well, polyvinyl microtiter plates (Cooke Engineering Co., Alexandria, Va.). PC-BSA or TNP-BSA was added at 1 μg/well and allowed to incubate overnight at 4°C. The plates were washed with phosphate-buffered saline (PBS) and 1% BSA in PBS was added and allowed to sit at room temperature for 1 h. The plates were then washed with PBS and 25 μl of culture supernates or varying amounts of serum antibody was added. Standard anti-hapten antibodies, TEPC-15 for PC and MOPC-315 for TNP, were used to quantify anti-hapten antibodies. After overnight incubation at 4°C, the wells were washed five times with PBS and rabbit anti-mouse F(ab')$_2$ antisera added at a 1:500 dilution in 1% BSA. The plates were washed five times with PBS after 4 h at room temperature and 20,000 cpm/well of I^{125}-labeled goat anti-rabbit IgG in 1% BSA was added and allowed to incubate overnight at 4°C. Wells were washed six times with tap water, dried, cut, and counted in a Micromedic 4/600 gamma counter (Micromedic Systems, Hozsham, Pa.). Data was analyzed on a Wang 2200 computer (Wang Laboratories, Inc., Lowell, Mass.)

Idiotype Determination. Idiotype analysis was done by an inhibition of binding of labeled TEPC-15 to immobilized anti-idiotype. Anti-idiotypic antiserum was added to 96-well microtiter plates and incubated overnight at 4°C. Samples were diluted in BSA and added to plates along with 20,000 cpm of labeled TEPC-15 F(ab')$_2$ and allowed to incubate at 4°C overnight. Wells were then washed and counted.

Statistical Analysis. Foci secreting anti-hapten-specific antibodies were scored positive on the basis of three criteria (29): (a) continued secretion during two consecutive supernatant collections; (b) correlation, on a weight basis, of anti-hapten antibody with total idiotype; and (c) statistical significance of samples over control backgrounds, determined by 99% confidence intervals of mean backgrounds from fragment cultures of mice not reconstituted with spleen cells. Manipulation and calculation of precursor frequencies were determined by Poisson distribution-based formulation.

A significance level of $P < 0.05$ was considered statistically significant by Student's t analysis of variance of independent groups.

Results

Characterization of the Response to PC-LPS. It has been shown that hapten-LPS conjugates, which are TI class 1 antigens (30, 31), can stimulate immature B cells. These antigens can induce an anti-hapten response in adult and neonatal animals (30, 32, 33), and in mice bearing the immune-deficient X-linked defect (33–36), that are otherwise refractile to antigenic stimulation with other antigens, either TI class 2 or TD. Lipopolysaccharide is a cell wall component of gram-negative bacteria and has been shown to act as a mitogen for B cells (37–39). The presence of various primary amine-containing carbohydrate moieties within the polysaccharide portion of the molecule allows the conjugation reaction to proceed by conversion of amine to a substituted amide. In this manner the synthesis of TNP (20), 4-hydroxy-3-iodo-5-nitrophenylacetic acid (30), and fluorescein isothiocyanate (39)-containing LPS con-

jugates have been described. Using a modification of the procedure of hapten conjugation via an activated tripeptide linkage to primary amine groups described by Inman et al. (21, 22), we conjugated PC to LPS and examined the immunogenicity of the resultant compound in vivo. Athymic nude BALB/c mice and their heterozygous littermates were immunized with three different PC-containing antigens (Table I). Whereas heterozygous BALB/c respond to all PC-containing antigens, their nude littermates respond only to PnC and PC-LPS and not to PC-KLH. Treating BALB/c nu/+ with ATS before immunization reduces the response to PC-KLH, whereas the responses to PnC, PC-LPS, and TNP-LPS are not affected. Thus, PC-LPS can stimulate PC-specific precursors in the absence of T cells.

Onset and Idiotype Analysis of the Neonatal PC-LPS Response In Vivo. PC-LPS was used to investigate the in vivo response by mice of different ages. The appearance of a response to PC-LPS was compared to the time when the neonates begin to respond to the TI class 1 antigen, PnC, and the TD antigen PC-KLH. As shown in Fig. 1, the response to PnC and PC-KLH cannot be induced before day 5. In contrast, PC-LPS stimulates PC-specific responses as early as in day 1 neonates. The antibodies, induced by PC-LPS, are specific for PC because >90% of the plaques are inhibited by 10^{-3} M-free hapten (data not shown). Thus PC-LPS was capable of stimulating PC-specific precursors in neonates that were 5 d younger than those neonates that respond to PC-KLH or PnC.

The idiotype composition of the PC–LPS-induced responses in neonatal mice was determined using inhibition of plaque formation by anti-TEPC-15 antibodies and idiotype analysis on sera from PC–LPS-immunized mice. As seen in Table II, the anti-PC-plaque-forming cell (PFC) response from early neonatal animals is largely resistant to plaque inhibition by anti-idiotypic antibody indicating that these animals produce anti-PC antibodies which are predominantly TEPC-15 negative. In Table III the amount of total anti-PC and TEPC-15 idiotype in sera measured by radioim-

TABLE I
In Vivo Responsiveness to PC-containing Antigens

Strain	Antigen	ATS treatment*	PC-PFC/spleen (log 10)‡	TNP-PFC/spleen (log 10)‡
BALB/c Nu/+	PC-KLH	−	4.42 ± 0.08	ND
		+	3.12 ± 0.04	ND
	PnC	−	4.52 ± 0.07	ND
		+	4.60 ± 0.07	ND
	PPC-TGG-LPS	−	4.85 ± 0.10	<3.00
		+	4.65 ± 0.12	<3.00
	TNP-LPS	−	<3.00	4.80 ± 0.03
		+	<3.00	4.72 ± 0.04
BALB/c nu/nu	PC-KLH	−	<3.00	ND
	PnC	−	4.40 ± 0.07	ND
	PPC-TGG-LPS	−	4.32 ± 0.05	<3.00
	TNP-LPS	−	<3.00	4.65 ± 0.03

* Mice were given 0.15 ml ATS 24 h before injection of antigen.
  Antigen doses given for: PC-KLH, 50 µg/mouse; PnC, 1 µg/mouse; PC-LPS, 10 µg/mouse; TNP-LPS, 10 µg/mouse.
‡ PC- and TNP-specific plaques were detected 5 d after immunizations using PnC- and TNP-coated SRBC as indicator target cells.
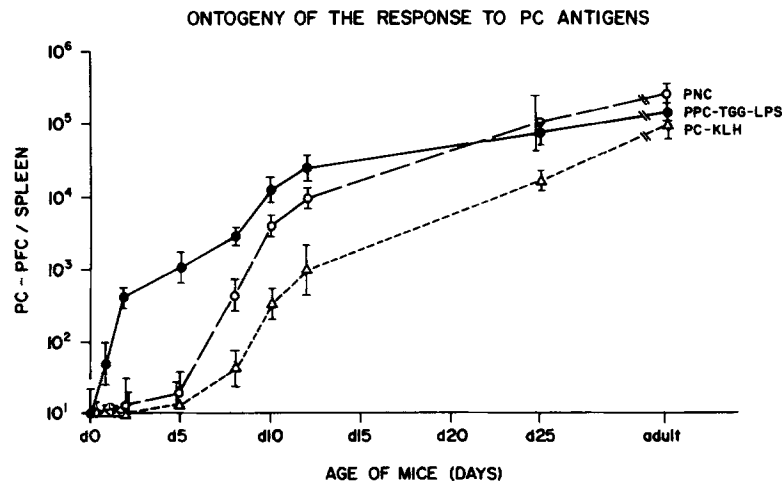
ONTOGENY OF THE RESPONSE TO PC ANTIGENS

FIG. 1.   Age of mice in days, when immunized with PC antigens, is plotted against the number of direct PC-specific PFC determined 5 d after injection, using PnC-coated SRBC as target cells in a modified Jerne-Nordin plaque assay. Dosages of antigens given are: neonates, ages day 0–10 received either 5 µg PC-KLH, 0.5 µg PnC, or 1 µg PC-LPS intraperitoneally; ages day 11–25 received either 20 µg PC-KLH, 1 µg PnC, or 5 µg PC-LPS intraperitoneally; and adults, ages day 25 and over received either 50 µg PC-KLH, 1 µg PnC, or 10 µg PC-LPS intravenously.

TABLE II

Idiotype Analysis of the In Vivo Response to PPC-TGG-LPS

Age at time of immunization*	PC-PFC/spleen‡	PC-PFC/spleen (log 10) with anti-idiotype§	Range of inhibition
d	log 10		
0	0.94 ± 0.37	0.90 ± 0.37	0–30
1	1.64 ± 0.27	1.61 ± 0.29	0–33
2	2.67 ± 0.10	2.60 ± 0.13	7–21
5	2.97 ± 0.13	2.89 ± 0.21	8–24
6	3.14 ± 0.14	3.04 ± 0.08	11–29
8	3.20 ± 0.10	3.10 ± 0.12	19–35
10	4.08 ± 0.11	3.55 ± 0.19	57–75
12	4.24 ± 0.14	3.62 ± 0.20	61–81
25	4.72 ± 0.10	3.81 ± 0.17	76–90
Adult	5.05 ± 0.13	4.15 ± 0.13	80–97

* Age of mice in days when immunized with PC-LPS. Dosages given were: ages day 0–10: 1 µg PC-LPS, intraperitoneally; day 11–25: 5 µg PC-LPS, intraperitoneally; day 25 and over: 10 µg PC-LPS, intravenously.

‡ PC-PFC were enumerated using PnC-coated SRBC as target cells in hemolytic plaque assay.

§ Clonotype analysis was accomplished using A/He anti-TEPC-15 antisera at a final dilution of 1:750 in the plaquing mixture.

munoassay from groups of PC–LPS-immunized neonates are shown. Only in animals older than 10 d is the TEPC-15 idiotype clone dominantly expressed. The appearance of dominant expression of the TEPC-15 idiotype after PC-LPS immunization parallels the TEPC-15 expression induced by PnC or PC-KLH immunization. As observed earlier (40, 41) and demonstrated here (Fig. 1), the response to PC-KLH or PnC does

TABLE III

Anti-PC and TEPC-15 Levels in PC-LPS-immunized Neonates

Age	Anti-PC	TEPC-15*	TEPC-15‡
d		%	
2	4.9 ± 0.6	1.0 ± 0.4	20.4
5	29.7 ± 4.9	5.4 ± 2.7	18.2
10	49.9 ± 3.7	9.6 ± 5.2	19.2
15	56.6 ± 8.0	50.3 ± 17.5	88.9

* Groups of neonatal BALB/c mice were immunized with 10 μg PPC-TGG-LPS on the indicated days and bled 5 d later. The amounts of anti-PC antibodies and TEPC-15 idiotype were determined by RIA and are expressed in micrograms per mililiter serum.
‡ The percentage of TEPC-15 idiotype was calculated from the average amounts of anti-PC and TEPC-15.

TABLE IV

Analysis of PC and TNP-precursors in Adult and Neonatal BALB/c Mice

Donor cells from:*	Cells analyzed ($\times 10^{-7}$)	PC precursors per 10^6 B cells‡	PC clones per 10^6 cells transferred	TEPC-15 idiotype‡	TNP precursors per 10^6 B cells‡	TNP clones per 10^6 cells transferred
				%		
Spleen cells						
Adult animals	62	32.4 ± 13.7	0.54	79	215.7 ± 28.3	4.70
Day 1 animals	24	0	0	—	ND	
Day 6 animals	18	6.67 ± 2.39	0.11	65	74.0 ± 17.3	1.56
Liver cells						
Day 1 animals	36	—§	0.07	23	—§	1.10
Day 6 animals	12	—	0.01	ND	—	0.20

* BALB/c mice were primed with hemocyanin 6–8 wk before use. 20 × 10^6 syngeneic donor cells were given to these mice 4–6 h after x-irradiation (1,400 rad). 18 h later, spleen fragments were cultured and immunized with PPC-TGG-H or TNP-HY.
‡ Culture supernates from splenic fragments were assayed for anti-PC, TEPC-15 idiotype, and anti-TNP antibodies by radioimmunoassays.
§ An absolute precursor frequency for liver cells was not established because the homing efficiency of liver cells is not known in this system.

not appear until 5–6 d after birth, and the responses to PC-KLH and PnC are always characterized by TEPC-15 dominance.

Analysis of PC-specific Precursors in Neonatal BALB/c Mice. Next we searched for PC precursors in neonatal animals using the splenic fragment culture technique described by Klinman and co-workers (5, 11, 16, 19) and modified by us (6). This technique provides optimal conditions for the expression of B cell precursors which is important for detecting responses by immature cells. Cells are injected into an environment which provides maximal T cell help, and in which B cells receive very efficient stimulation by the hapten carrier PPC-TGG-Hy (5,12). This precursor assay has been shown to allow expression of a wide range of maturing B cells and their precursors (42, 43) responding to TD antigens. We therefore used the TD antigen PPC-TGG-HY to stimulate precursors from neonatal livers and spleens whereas cells taken from adult animals served as controls. As shown in Table IV, normal adult BALB/c spleen cells contain PC-specific precursors at a frequency of 32.4 ± 13.7/10^6 B cells. This is

in excellent agreement with the PC precursor frequency given by Sigal et al. (12). Of these PC-specific precursors, 79% are of the TEPC-15 idiotype. We found that day 1 neonatal spleens do not contain PC-specific precursors or cells able to give rise to these precursors, whereas the frequency of PC-specific precursors of day 6 spleen cells is about five times less than that of adults. Sigal and co-workers (12) conclude that the appearance of PC responsiveness and of the TEPC-15 idiotype clones is a predictable occurrence around 5–6 d after birth. We also examined the ability of neonatal liver cells to give rise to PC-specific precursors. As shown in Table IV, PC-specific precursors can be detected in the inoculum from neonatal livers. These PC-positive clones, when analyzed for the TEPC-15 idiotype, show a marked difference from the idiotype profile seen in adult mice. Approximately 80% of day 1 neonatal PC-specific clones are TEPC-15 idiotype negative.

In the course of neonatal development, hemopoietic stem cells and their progeny are known to migrate from the liver to the spleen and bone marrow (44–46). Table IV shows that in the first 5 d after birth, there is a shift of PC and TNP precursors from the neonatal liver to the neonatal spleen. During this period, PC-specific precursors of the TEPC-15 idiotype establish "clonal dominance", because the percentage of PC-specific precursors in day 6 spleens bearing the TEPC-15 idiotype approximate that of the adult spleen.

These results from in vivo and in vitro analysis of PC-specific responses by cells from neonatal animals confirm observations of the definite and predictable late acquisition of the TEPC-15 clonal dominance (11, 12). But more importantly, our data demonstrate that PC responses and PC precursors mature before the onset of TEPC-15 dominance and that among those earliest precursors are clones which carry the TEPC-15 specificity.

Discussion

In the BALB/c strain, the response to PC-containing antigens has been shown, by many criteria (6–9, 47), to be dominated by antibodies which bear variable region determinants of the TEPC-15 myeloma protein. Whereas the normal adult response is characterized by a restricted heterogeneity in the antibody population, the precursor repertoire is diverse and several hundred clonotypes have been identified in the precursor pool of potentially responsive B cells (1, 4). Thus, the establishment of dominance of the TEPC-15 clone is not caused by a restriction of the available pool of precursor idiotypes. Sigal et al. (11, 12) have reported that both PC responsiveness and TEPC-15 clonal dominance appear in neonatal spleen ~1 wk after birth. They failed to detect PC-specific precursors in day 19 fetal liver cells; however, several lines of evidence point to the presence of PC-committed cells in fetal and neonatal liver cells. Kaplan et al. (48) and Augustin et al. (49) have shown that adoptive transfer of fetal or neonatal liver cells into a syngeneic recipient leads to acquired responsiveness to TNP and PC. The PC response in these liver cell-recipient animals was mainly TEPC-15 negative. Quintans et al. (50) used neonatal liver cells from BALB/c mice to reconstitute the genetic unresponsiveness of (CBA/N × BALB/c)F$_1$ males with both TEPC-15 and non-TEPC-15 responses. However, none of these observations demonstrated a rigorous cell dose dependence which is essential for precursor analysis. Using the splenic fragment culture, as described by Klinman and co-workers (5, 12, 16, 19), we were able to study the development and maturation of immature B cell

precursors in an environment promoting maximal T cell recognition and antigen stimulation which otherwise is insufficient in neonatal animals. Thus, we found that the neonatal liver cell contains PC-specific precursors and that the majority of these PC-specific precursors bore non-TEPC-15.

In an attempt to study the relation of TEPC-15 and non-TEPC-15-positive clones in vivo, we have synthesized a PC-conjugated LPS compound. Like other hapten-LPS conjugates, we found that this antigen can induce expression of relatively immature B cells and their precursors at an early maturational age where other PC antigens are not effective stimulators. The response of PC precursors in neonatal animals using PC-LPS as antigen corroborates the observations in splenic fragment cultures. Comparison of neonates immunized with various PC-containing antigens indicates that BALB/c neonates develop and acquire sequential responsiveness to various classes of PC antigens similar to that described for TNP antigens (32, 33). Responsiveness to PC-LPS is noted earliest in ontogeny, and within days after birth, a substantial anti-PC response can be observed. The response to PC-LPS, when analyzed by anti-idiotypic antibodies, indicates that the earliest anti-PC response is comprised of largely non-TEPC-15 idiotypes, whereas later in ontogeny, an increase of the TEPC-15 clone shifts the equilibrium of precursors toward TEPC-15 clonal dominance. On the other hand, immunization of neonates <6 d of age with PnC or PC-KLH does not result in detectable anti-PC responses. When these responses do arise, we find that the TEPC-15 idiotype constitutes the large majority of antibodies produced. Interestingly, whereas in other responses an increase in the heterogeneity of antibodies during maturation was observed, we find that the response to PC becomes apparently more restricted during neonatal development. This restriction, however, is caused by the relative expansion and expression of the TEPC-15-bearing clones, because by precursor analysis, non-TEPC-15 clones exist in significant numbers in normal neonatal and adult BALB/c mice.

Thus, it appears that the expansion of TEPC-15-bearing precursors to clonal dominance and the maturation of B cell precursors responding to different classes of antigens are independent maturational events. Accordingly, three maturational phases in the development of a given response can be envisioned. In the first phase the genetic repertoire of V-genes is expressed. These precursors are functionally immature because only strongly mitogenic antigens (i.e. LPS conjugates) can stimulate and triggering occurs only in an environment of maximized T help (reviewed in reference 51). For responses to certain antigens, which are characterized by clonal dominance, a cellular mechanism selects certain clones and induces their expansion. For most antigens, however, clonal selection and expansion evidently does not happen and these responses appear therefore to be polyclonal. The third step in clonal maturation entails the maturation of clones to full responsiveness to all forms of antigens, i.e., TD and TI antigens. Within this framework of maturation clonal restriction and timing of the emergence of clonal dominance has no bearing on the definition of germ-line gene responses; rather, clonal restriction and late acquisition of dominant responses are merely reflections of regulatory processes which occur during maturation and which determine the clonal profile in the adult response (48, 52, 53).

Administration of anti-idiotypic antibodies affects the expression of idiotype bearing B cells differentially depending on the amount administered. High doses of anti-idiotypic antibodies has been shown to lead to suppressor T cell induction (54–56), or

clonal deletion (57–59), as postulated by Jerne (60). On the other hand, low dose anti-idiotype administration has been shown to prime a helper T cell population able to allow maximal expression of B cells (61) and, in addition, in some reports B cells bearing the idiotype have been shown to be directly stimulated by low dose priming (55). Our laboratory reported that immunization of neonatally suppressed BALB/c mice with anti-idiotype results in the induction of the idiotype (62). We (47) and others (10) have demonstrated the presence of anti-idiotypic antibodies in neonates immediately after birth which is not detectable after 5–6 d of age. Using a sensitive radioimmunoassay to detect anti-idiotype in the neonate, we have reported that the total idiotype binding capacity of neonatal serum corresponds to 10^{-3} μg TEPC-15. Correcting for weight differences, the amount of anti-idiotype in these neonates is on the same order of magnitude as that needed for optimal priming of T helper cells, as proposed by Julius et al. (63). In addition, this level of anti-idiotype found in neonates is roughly 1,000–5,000 times less anti-idiotype than is used to induce suppression. We proposed earlier a mechanism for the establishment of clonal dominance (47) which involves idiotype-specific regulation of preexisting clones. TEPC-15-bearing cells are stimulated by anti-idiotypic factors or cells to expand and become dominant over non-TEPC-15 clones. The late acquisition of the TEPC-15 idiotype clone may result from the time requirement for its positive selection. Although the exact mechanism of establishment of clonal dominance is not known, it is tempting to speculate that the early auto-anti-idiotypic antibody helps to select and to promote the TEPC-15-positive clones to attain clonal dominance (47).

Summary

The maturation of the antibody response to phosphorylcholine (PC) in neonatal BALB/c mice was studied. A T cell-independent class 1 PC-antigen, 3-(p-azophenyl phosphorylcholine)-N-acetyl-L-tyrosylglycylglycine lipopolysaccharide, was synthesized and used to immunize neonatal mice of different ages. The earliest anti-PC hemolytic plaque-forming response could be induced in 1-d-old neonates. Idiotype analysis on these early anti-PC antibodies showed that the response was not TEPC-15 dominant although TEPC-15-positive plaque-forming cells were detected. However, idiotype analysis of the anti-PC-LPS response in 7 d or older animals indicated that clonal dominance had been established. Similar results were obtained in splenic fragment culture with cells from neonatal livers and spleens. PC-specific precursors were detected in the liver of 1-d-old neonates, whereas the spleen of those animals contained no precursors for PC. Precursors for PC residing in the neonatal liver are not TEPC-15 dominant, whereas the splenic PC precursors of 5- to 6-day-old animals express the TEPC-15 idiotype dominantly. These findings demonstrate that during ontogeny PC-specific B cells appear before the TEPC-15 clone becomes dominant. Thus clonal dominance in the adult anti-PC response and late acquisition of the TEPC-15 specificity during ontogeny do not signify a particularly unique or direct relationship to the expression of genes encoded in the germline.

References

1. Owen, J., and N. Sigal. 1980. Clonotype heterogeneity of Balb/c anti-phosphorylcholine antibody response. *Immunogenetics.* In press.

2. Potter, M. 1971. Antigen-binding myeloma proteins in mice. *Ann. N. Y. Acad. Sci.* **190**:306.

3. Leon, M. A., and N. M. Young. 1971. Specificity for phosphorylcholine of six murine myeloma proteins reactive with pneumococcus C polysaccharide and beta-lipoprotein. *Biochemistry* **10**:1424.

4. Sigal, N. H., and N. R. Klinman. 1978. The B-cell clonotype repertoire. *Adv. Immunol.* **26**: 255.

5. 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.

6. Fung, J. J., and H. Köhler. 1980. Immune response to phosphorylcholine: VII. Functional evidence for three separate B-cell subpopulations responding to TI and TD PC antigens. *J. Immunol.* In press.

7. Lee, W., H. Cosenza, and H. Köhler. 1974. Clonal restriction of the immune response to phosphorylcholine. *Nature (Lond.).* **247**:55.

8. Quintans, J., and H. Cosenza. 1976. Antibody response to phosphorylcholine in vitro. II. Analysis of T-dependent and T-independent responses. *Eur. J. Immunol.* **6**:359.

9. Claflin, J. L., and M. Cubberley. 1978. Clonal nature of the immune response to phosphorylcholine. VI. Molecular uniformity of a single idiotype among Balb/c mice. *J. Immunol.* **121**:1410.

10. Cosenza, H., M. Julius, and A. Augustin. 1977. Idiotypes as variable region markers: Analogies between receptors on phosphorylcholine-specific T and B lymphocytes. *Immunol. Rev.* **34**:3.

11. Sigal, N. H., P. J. Gearhart, J. L. Press, and N. R. Klinman. 1976. Late acquisition of a germline antibody specificity. *Nature (Lond.).* **259**:51.

12. Sigal, N. H., A. R. Pickard, E. S. Metcalf, P. J. Gearhart, and N. R. Klinman. 1977. Expression of phosphorylcholine-specific B cells during murine development. *J. Exp. Med.* **146**:933.

13. Schilling, J., B. Clevinger, J. M. Davie, and L. Hood. 1980. Amino acid sequence of homogeneous antibody to dextran and DNA rearrangements in heavy chain V-region gene segments. *Nature (Lond.).* **283**:35.

14. Eichmann, K. 1973. Idiotype expression and the inheritance of mouse antibody clones. *J. Exp. Med.* **137**:603.

15. Eichman, K., and C. Berek. 1973. Mendelian segregation of a mouse antibody idiotype. *Eur. J. Immunol.* **3**:599.

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

17. Lieberman, R., M. Potter, E. B. Mushinski, W. Humphrey, Jr., and S. Rudikof. 1974. Genetics of a new IgV$_H$ (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *J. Exp. Med.* **139**:983.

18. Chesebro, B., and H. Metzger. 1972. Affinity labeling of a phosphorylcholine binding mouse myeloma protein. *Biochemistry.* **11**:766.

19. Klinman, N. R. 1971. The secondary immune response to a hapten in vitro. *J. Exp. Med.* **133**:963.

20. Fidler, J. M. 1975. In vivo immune response to TNP hapten coupled to thymus-independent carrier lipopolysaccharide. *Cell. Immunol.* **16**:223.

21. Inman, J. K., B. Merchant, and S. E. Tacey. 1973. Synthesis of large haptenic compounds having a common functional group that permits covalent linkage to proteins, cell surfaces and adsorbants. *Immunochemistry.* **10**:153.

22. Inman, J. K., B. Merchant, L. Claflin, and S. E. Tacey. 1973. Coupling of large hapten to proteins and cell surfaces: preparation of stable, optimally sensitized erythrocytes for hapten-specific, hemolytic plaque assays. *Immunochemistry.* **10**:165.

23. Liu, T. Y., and E. Gotschlich. 1968. The chemical composition of pneumococcal C polysaccharide. *J. Biol. Chem.* **238:**1928.

24. Potter, M., and R. Lieberman. 1970. Common individual antigenic determinants in five of eight Balb/c IgA myeloma proteins that bind phosphorylcholine. *J. Exp. Med.* **132:**737.

25. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science (Wash. D.C.).* **140:**405.

26. Gold, E. R., and H. H. Fudenberg. 1967. Chromic chloride: a coupling reagent for passive hemagglutination reactions. *J. Immunol.* **99:**859.

27. Cosenza, H., and H. Kohler. 1972. Specific inhibition of plaque formation to phosphorylcholine by antibody against antibody. *Science (Wash. D.C.).* **176:**1027.

28. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1975. Heterogeneity of the Balb/c anti-PC antibody response at the precursor cell level. *J. Exp. Med.* **141:**56.

29. Klinman, N. R., and J. L. Press. 1975. The B cell specificity repertoire: its relationship to definable subpopulations. *Transplant. Rev.* **24:**41.

30. Fidler, J. M. 1979. The induction of hapten-specific immune tolerance and immunity in B lymphocytes. VI. Differential tolerance susceptibility in adult spleen as a function of B cell maturation level. *J. Exp. Med.* **150:**491.

31. McKearn, J. P., and J. Quintans. 1980. Delineation of tolerance-sensitive and tolerance-insensitive B cells in normal and immune defective mice. *J. Immunol.* **124:**77.

32. McKearn, J. P., and J. Quintans. 1979. Ontogeny of murine B cell responses to thymus-independent trinitrophenyl antigens. *Cell. Immunol.* **44:**367.

33. Mosier, D. E., J. J. Mond, and E. A. Goldings. 1977. The ontogeny of thymic-independent antibody responses in vitro in normal mice and mice with an X-linked B cell defect. *J. Immunol.* **119:**1874.

34. Mosier, D. E., J. J. Mond, I. M. Zitron, I. Scher, and W. E. Paul. 1977. Functional correlates of surface Ig expression for T-independent antigen triggering. *In* The Immune System: Genes and the Cell in which they Function. E. Sercarz, L. A. Herzenberg, and C. F. Fox, editors. Academic Press, Inc., New York. 699.

35. Merchant, B., H. Snippe, E. F. Lizzio, and J. K. Inman. 1978. X-linked genetic control of hapten-polysaccharide-mediated specific immune unresponsiveness in CBA/N mice. *J. Immunol.* **120:**1362.

36. Slack, J., G. P. Der-Balian, M. Nahm, and J. M. Davie. 1980. Subclass restriction of murine antibody. II. The IgG plaque-forming cell response to thymus-independent type 1 and type 2 antigens in normal mice and mice expressing an X-linked immunodeficiency. *J. Exp. Med.* **151:**853.

37. Andersson, J., O. Sjoberg, and G. Moller. 1972. Induction of immunoglobulin and antibody synthesis in vitro by lipopolysaccharide. *Eur. J. Immunol.* **2:**349.

38. Skidmore, B. J., J. M. Chiller, D. C. Morrison, and W. O. Weigle. 1975. Immunologic properties of bacterial lipopolysaccharide to its function in mitogenesis and adjuvanticity. *Proc. Natl. Acad. Sci. U.S.A.* **70:**2129.

39. Skelly, R. R., P. Munkerbeck, and D. C. Morrison. 1980. Immune responses to hapten-lipopolysaccharide conjugates: 1. Modulation of in vivo antibody responses to the polysaccharide. *Cell. Immunol.* **49:**99.

40. Quintans, J., and J. P. McKearn. 1979. Developmental heterogeneity of B cell subpopulations. *In* B Lymphocytes in the Immune Response. M. Cooper, D. E. Mosier, I. Scher, and E. S. Vitetta, editors. Elsevier North-Holland, Inc., New York. 131.

41. Mosier, D. E., E. A. Goldings, and K. Bottomly. 1979. Activation requirements of neonatal B lymphocyte in vitro and in vivo. *In* B Lymphocytes in the Immune Response. M. Cooper, D. E. Mosier, I. Scher, and E. S. Vitetta, editors. Elsevier North-Holland, Inc., New York. 91.

42. Teale, J. M., M. C. Howard, E. Falzon, and G. J. V. Nossal. 1978. B lymphocyte

subpopulations separated by velocity sedimentation. I. Characterization of immune function in an in vitro spleen focus assay. *J. Immunol.* **121:**2554.

43. Teale, J. M., M. C. Howard, and G. J. V. Nossal. 1978. B lymphocyte subpopulations separated by velocity sedimentation. II. Characterization of tolerance susceptibility. *J. Immunol.* **121:**2561.
44. Teale, J. M., and T. E. Mandel. 1980. Ontogenetic development of B-lymphocyte function and tolerance susceptibility in vivo and in an in vitro fetal organ culture system. *J. Exp. Med.* **151:**429.
45. Owen, J. J. T., M. C. Raff, and M. D. Cooper. 1975. Studies on the generation of B lymphocytes in the mouse embryo. *Eur. J. Immunol.* **5:**460.
46. Cooper, M. D., and A. R. Lawton. 1974. The development of the immune system. *Sci. Am.* **231:**58.
47. Köhler, H., D. Kaplan, R. Kaplan, J. Fung, and J. Quintans. 1978. Ontogeny of clonal dominance. *In* Cells of Immunoglobulin Synthesis. Academic Press, Inc., New York. 357.
48. Kaplan, D. R., J. Quintans, and H. Kohler. 1978. Clonal dominance: loss and restoration in adoptive transfer. *Proc. Natl. Acad. Sci. U.S.A.* **75:**1967.
49. Augustin, A. A., M. H. Julius, and H. Cosenza. 1977. Changes in the idiotypic pattern of an immune response, following syngeneic hemapoietic reconstitution of lethally irradiated mice. *In* Immune System: Genetic and Regulation. E. E. Sercarz, L. A. Herzenberg, and C. F. Fox, editors. Academic Press, Inc., New York. 195.
50. Quintans, J., J. P. McKearn, and D. Kaplan. 1979. B cell heterogeneity. I. A study of B cell subpopulation involved in the reconstitution of an X-linked immune defect of B cell differentiation. *J. Immunol.* **122:**1750.
51. McKenzie, I. F. C., and T. Potter. 1979. Murine lymphocyte surface antigens. *Adv. Immunol.* **27:**179.
52. Kaplan, D., and J. Quintans. 1978. Alterations of clonal profile. I. Effect of sublethal irradiation on the response to phosphorylcholine in Balb/c phosphorylcholine in adult Balb/c mice. *J. Exp. Med.* **148:**1216.
53. Accolla, R., P. Gearhart, N. Sigal, M. Cancro, and N. Klinman. 1977. Idiotype specific neonatal suppression of phosphorylcholine-responsive B cells. *Eur. J. Immunol.* **7:**876.
54. Eichmann, K. 1974. Idiotype suppression. I. Influence of the dose and of the effector function of anti-idiotype antibody on the production of an idiotype. *Eur. J. Immunol.* **4:**296.
55. Eichmann, K., and K. Rajewsky. 1975. Induction of T and B cell immunity by anti-idiotypic antibody. *Eur. J. Immunol.* **5:**661.
56. DuClos, T. W., and B. S. Kim. 1977. Suppressor T cells: presence in mice rendered tolerant by neonatal treatment with anti-receptor antibody or antigen. *J. Immunol.* **119:**1769.
57. Strayer, D., W. Lee, D. A. Rowley, and H. Kohler. 1975. Anti-receptor antibody. II. Induction of long-term unresponsiveness in neonatal mice. *J. Immunol.* **114:**728.
58. Köhler, H., D. Kaplan, and D. Strayer. 1974. Clonal depletion in neonatal tolerance. *Science (Wash. D.C.).* **186:**643.
59. Etlinger, H. M., and J. M. Chiller. 1979. Maturation of the lymphoid system. II. Characterization of the cellular levels of unresponsiveness induced in neonates by a T-dependent antigen that is an obligate immunogen in adults. *J. Immunol.* **122:**2564.
60. Jerne, N. K. 1974. *In* Cellular Selection and Regulation of the Immune Response. G. M. Edelman, editor. Raven Press, New York. 39.
61. Bottomly, K., B. J. Mathieson, and D. E. Mosier. 1978. Anti-idiotype induced regulation of helper cell function for the response to phosphorylcholine in adult Balb/c mice. *J. Exp. Med.* **148:**1216.
62. Köhler, H., J. Fung, and S. Smyk. 1980. Induced recovery of a suppressed idiotype by immunization with anti-idiotype. *Cell. Immunol.* **52:**458.
63. Julius, M. H., H. Cosenza, and A. A. Augustin. 1978. Evidence for the endogenous production of T cell receptors bearing idiotypic determinants. *Eur. J. Immunol.* **8:**484.