ALTERATION OF CLONAL PROFILE

III. T15 Ontogenetic Advantages Are Not Sufficient for Establishing Idiotypic Dominance in Adoptive Transfer*

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Phosphorylcholine (PC)[1] is a component of bacterial cell walls that is highly immunogenic in mice. In fact, most murine strains have circulating natural anti-PC antibodies that share common idiotypic determinants (1, 2) and offer protection against pneumococcal infections (3). BALB/c mice offer a particularly striking example of high responsiveness to PC and restriction of idiotype expression: most BALB/c anti-PC antibodies express the T15 idiotype marker (4, 5). However, attempts to study the ontogeny of T15 idiotypic dominance in conventional adoptive transfer systems encountered unexpected difficulties (6–8). A delayed emergence of mostly $T15^-$ responsiveness to PC was observed in lethally irradiated BALB/c mice reconstituted with cells from syngeneic adult bone marrow and neonatal or fetal liver. In one instance (6), it was possible to achieve a modest, early restoration of $T15^+$ plaque-forming cell (PFC) responses by using hosts neonatally suppressed for T15 expression with anti-idiotypic antibodies. The exact significance of this finding, which was not reproduced in other studies (7), was obscure.

To clarify some of the issues raised by these experiments, we have undertaken a series of studies using *xid*-bearing (CBA/N × BALB/c)F_1 (NBF_1) hybrid male mice as recipients of immunocompetent cells (9–11). Previous studies from our laboratory using the NBF_1 transplantation model have shown that murine neonatal and fetal liver contain progenitors of PC-specific B cells (10, 11). Generation of PFC responses from transplanted neonatal liver cells follows a well-defined and highly reproducible sequence of quantal events: responses to 2,4,6-trinitrophenyl (TNP) precede those to PC and, among these, responses to PC-keyhole limpet hemocyanin (KLH) (thymus-dependent [TD]) appear before those to C-polysaccharide extract of a *Streptococcus pneumoniae* mutant (PnCs) (thymus-independent, class 2 [TI-2]). The studies in the NBF_1 model have also clearly shown that the number of cells used to reconstitute the adoptive host plays a critical role in the appearance of anti-PC PFC responses, higher numbers being needed to generate TI-2 than TD responses.

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[1] *Abbreviations used in this paper:* BA, *Brucella abortus*; HPA, hydroxyphenylacetic acid; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; *p*APPC, *p*-aminophenylphosphorycholine; PC, phosphorycholine; PFC, plaque-forming cell; PnCs, C-polysaccharide extract of a *Streptococcus pneumoniae* mutant; SRBC, sheep erythrocytes; TD, thymus-dependent TI-1, -2, thymus-independent class 1 and 2; TNP, 2,4,6,-trinitrophenyl.

In the present studies, we use the NBF_1 model to monitor the ontogeny of idiotypes in BALB/c PFC responses to TD, TI class 1 (TI-1), and TI-2 PC antigens for two reasons. First, we ensure that the idiotypic profiles that arise are generated solely by the transplanted progenitors, because the NBF_1 male cannot mount direct anti-PC PFC responses (12–14); this is a considerable advantage over the use of lethally irradiated BALB/c recipients, in which the PFC response of the donor cells can be obscured by a regenerated host contribution (11, 15). Second, the immunodeficient *xid* F_1 hybrid male provides adequate T cell help for both $T15^+$ and $T15^-$ TD anti-PC responses (14). We have corroborated these results by demonstrating that lymph node cells from carrier-primed male and female NBF_1 mice both contain saturating T cell help for T15-dominant TD PFC responses in BALB/c nude mice and non-T15 responses in C_3H nude mice (José Quintans, unpublished observations). We observe that the earliest anti-PC responses detected in NBF_1 mice reconstituted with neonatal or fetal liver cells are predominantly $T15^+$. Non-T15 clones mature later and more slowly than $T15^+$ clones, but eventually a significant proportion of anti-PC PFC responses are $T15^-$. This sequence of events, seen in lethally irradiated syngeneic recipients as well, apparently reflects a shift in the relative representation of T15 and non-T15 B cell populations themselves; host pretreatment with $T15^+$ BALB/c serum does not prevent it, and a generation of Lyt-2^+ suppressor cells has not been detected. Obviously, $T15^+$ clones have a developmental advantage over $T15^-$ clones that does not materialize in T15 dominance in this experimental model.

Materials and Methods

Mice. Adult and neonatal BALB/c mice were obtained from Cumberland View Farms, Clinton, Tenn. NBF_1 mice were raised in our animal facilities for the early studies; those used in later experiments were purchased from Laboratory Supply Co., Indianapolis, Ind. (BALB/c × CBA/N)F_1 (BNF_1) mice were bred in our facilities. Animals were used as recipients at 2–4 mo of age.

Irradiation. A ^{137}Cs source (200 rad/min) was used to irradiate the mice. Lethally irradiated animals were kept in sterile cages and given Neomycin (1.1 g/liter) in their drinking water.

Cell Transfers. Adult spleen cells suspended in Hanks' balanced salt solution were washed twice, treated two times with monoclonal antibodies plus complement as indicated in the text, and washed again before injection into recipients. Neonatal liver cells were taken from donors within 24 h of birth. Fetal liver cells were obtained from embryos taken from vaginally plugged females at the times indicated in the text. Suspensions of liver cells were prepared in Hanks' balanced salt solution and washed three times before injection.

Anti-T Cell Reagents. The high-titered IgM hybridoma antibodies AT83A, directed against the Thy-1.2 marker (16), and 3.155, directed against an Lyt-2 determinant (17), were generously provided by Dr. F. W. Fitch and D. P. Dialynas (University of Chicago, Chicago, Ill.). The antisera were used at concentrations known to be in excess of those required for complete functional depletion of T cells and/or their subsets. As a source of complement we used Low-Tox-M rabbit complement from Accurate Chemical & Scientific Corp., Westbury, N. Y.

Serum. Blood was collected retroorbitally from mice of the appropriate strain, allowed to clot, and then spun down to collect the serum fraction.

Antigens. *p*-Aminophenylphosphorylcholine (*p*-APPC; Biosearch, San Rafael, Calif.) was used to couple PC to KLH (Calbiochem-Behring Corp., San Diego, Calif.), following standard procedures with minor modifications (18, 19). PC-*Brucella abortus* (BA) was prepared according to a modification of the PC-KLH coupling procedure: heat-inactivated BA (U. S. Department of Agriculture National Veterinary Services Laboratories, Ames, Iowa) were spun down three times in borate-buffered saline, pH 9, at 10,000 *g* for 15 min. The washed bacteria was then prepared as a 10% (vol/vol) suspension in borate-buffered saline, to which was added the diazotized *p*-APPC reagent. The mixture was allowed to react overnight before being washed

three times with saline at 10,000 *g* for 15 min. PC-lipopolysaccharide (LPS) was prepared by coupling the reagent PC-hydroxyphenylacetic acid (PC-HPA; Biosearch) to LPS (Difco Laboratories, Detroit, Mich.) as follows: 10 mg PC-HPA was dissolved in 1 ml dimethylformamide (Sigma Chemical Co., St. Louis, Mo.) and mixed with 10 mg *n*-hydroxysuccinimide (Sigma Chemical Co.). 10 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma Chemical Co.) was then added. The mixture was allowed to react for 15 min at room temperature before being added to a buffered LPS solution, pH 8–9; the preparation was stirred overnight and then dialyzed extensively. The soluble PC-containing PnCs was prepared as described by Anderson and McCarty (20). TNP-conjugated *n*-(2-aminoethyl) carbamyl-methyl dextran (TNP-dextran) was prepared according to the method of Inman (21).

PFC Assays. TNP-sheep erythrocytes (SRBC) and PC-SRBC were used as indicator cells in the Cunningham-Szenberg modification (22) of the hemolytic plaque assay (23). TNP-SRBC were prepared as described by Rittenberg and Pratt (24), and C-polysaccharide was coupled to the SRBC by the chromic chloride method (25). In some experiments, PC was coupled directly to SRBC by using the diazotized *p*-APPC reagent (18) according to the following protocol: 1.25 μmol diazotized *p*-APPC was added to 5 ml of a 10% suspension of SRBC (vol/vol) in borate-buffered saline, pH 9. The reaction was stopped after 30 min at room temperature by extensive washing of the erythrocytes in phosphate-buffered saline. This coupling procedure is more readily reproduced than that using chromic chloride although both yield indicator PC-SRBC of comparable sensitivity.

Plaque Inhibitions. A/HeJ anti-S107 serum was prepared as described elsewhere (9) and used as the anti-idiotypic probe in the earliest experiments. The other assays used the hybridoma anti-HOPC 8 antibodies AB1-2 and GB4-10 prepared and characterized by Kearney et al. (26). Plaque inhibitions were performed by incorporating the appropriate dilution of either the conventional or the monoclonal antiserum into the plaquing mixture.

Results

BALB/c T15$^+$ Clones Have an Ontogenetic Advantage over T15$^-$ Clones. NBF_1 male mice were given 300 rad and then reconstituted with 2×10^7 BALB/c day 15 fetal liver cells. Recipients were immunized with PC-KLH or PnCs at 4 or 7 wk after cell transfer, and their quantal anti-PC PFC responses, described in detail elsewhere (9, 11), as well as the idiotypic profiles, were determined 5 d after challenge. In these and subsequent experiments, the PFC responses to TNP-Dextran were determined concurrently (data not shown) as an additional control for the successful engraftment of the transplanted cells (9, 11). As is shown in Table I, responses that emerged by 4 wk were T15 dominant for both antigens. By 7 wk after transfer, however, an increasing number of recipients expressed non-T15 anti-PC responses. This is particularly evident in the responses elicited by PC-KLH, where >50% of the mice exhibited non-T15 predominance. The appearance of non-T15 clones was seen to a lesser extent among the 7-wk responses to PnCs. It should be noted that at this time a considerable number of recipient mice were still unresponsive to this antigen, whereas all animals responded to PC-KLH; this is a reflection of the developmental advantage that TD anti-PC responses have over those elicited by the putative TI-2 antigen PnCs (9, 11). By 14 wk after transfer, when all recipients responded, 300 rad-irradiated NBF_1 male hosts of 2×10^7 neonatal liver cells also produced predominantly T15$^-$ responses to PnCs. That is, the sequence of idiotype shifts observed for PC-KLH was found for responses to PnCs as well.

Because there are at least two classes of TI antigens (27), we proceeded to investigate whether the sequence of ontogenetic events described above would also apply to any other classes of TI antigens. We therefore examined the emergence of responses to PC-LPS and PC-BA (Table II); responses to PC-KLH served as a temporal and

TABLE I

Ontogeny of BALB/c Anti-PC Idiotypes in the NBF_1 Transplantation Model: Early and Late Responses to PC-KLH and PnCs after Reconstitution with 2×10^7 Liver Cells

Individual responses to PC-KLH				Individual responses to PnCs					
PFC/spleen	Percent $T15^+$	PFC/spleen	Percent $T15^+$	PFC/spleen	Percent $T15^+$	PFC/spleen	Percent $T15^+$	PFC/spleen	Percent $T15^+$
4 wk		*7wk*		*4 wk*		*7 wk*		*14 wk*	
42,700	100	18,600	57	5,700	100	72,000	100	128,400	18
28,200	100	18,600	26	1,900	100	6,200	88	76,300	42
25,300	100	16,900	98			4,400	100	70,300	24
15,000	98	16,000	30			4,300	28	64,400	36
14,700	86	15,500	100			4,000	72	40,200	86
12,500	100	14,400	100			3,900	87	39,400	46
5,200	100	13,000	12			3,900	51	37,500	51
7,400	95	11,900	23			3,300	69	35,600	25
2,100	95	9,800	27			3,100	94	29,200	21
2,000	20	9,100	12			1,900	77	29,000	59
1,400	69	5,800	47			1,300	62	20,200	4
1,100	—	4,800	49			1,200	100	12,600	27
1,000	—	3,400	0			1,100	—	10,400	13
1,000	—	2,600	17						
6/20 < 1,000 PFC/spleen		0/14 < 1,000 PFC/spleen		16/18 < 1,000 PFC/spleen		11/24 < 1,000 PFC/spleen		0/13 < 1,000 PFC/spleen	

300 rad-irradiated NBF_1 male mice were reconstituted with 2×10^7 BALB/c liver cells from either day 15 fetuses taken from vaginally plugged females (4- and 7-wk assays) or neonates <24 h of age (14-wk assay). At the indicated times after transfer, groups of reconstituted mice were immunized with either PC-KLH or PnCs and anti-PC PFC responses determined 5 d after challenge. Presented are the anti-PC PFC responses, with idiotypic profiles, of individual mice and the number of mice in each group that responded with <1,000 PFC/spleen; in this experiment, recipients of fetal liver cells all responded to TNP-dextran. The percentage of $T15^+$ responses was determined by inhibition of plaque formation using an anti-idiotypic serum incorporated into the plaquing mixture. The percent of $T15^+$ of the two groups immunized with PC-KLH were compared, as were those of the groups challenged with PnCs at 7 and 14 wk, using a Wilcoxon two-sample randomization test. The null hypothesis is that the compared groups belong to the same population, i.e., that the range of the idiotypic profiles of the compared groups do not differ. In both cases, $P < 0.01$ that the null hypothesis is correct.

idiotypic reference. Sublethally irradiated NBF_1 male mice each received 1×10^7 BALB/c neonatal liver cells taken from donors within 24 h of birth. 8, 12, 15, and 55 d after cell transfer, groups of reconstituted mice were immunized with either PC-KLH, PC-BA, or PC-LPS, and assayed 5 d later for their total and $T15^+$ anti-PC PFC responses. At the time of assay, plaque inhibitions were also performed using 10^{-4} M free PC to establish the specificity of the PFC. PFC determinations were made against unconjugated SRBC as well, to account for background responses that could significantly misrepresent the idiotypic profile of animals giving low PFC responses.

At 8 d after liver cell transfer, no NBF_1 mice responsed to PC (data not shown). By 12–15 d, all three antigens elicited responses in some of the immunized animals; that is, responses to putative TI-1 antigens emerged at approximately the same early period as did responses to the TD antigen PC-KLH. Once again, we observed an early predominance of $T15^+$ responses followed by a late increase in $T15^-$ responses. Therefore, although $T15^+$ clones may appear initially in ontogeny, under the condi-

TABLE II

Ontogeny of BALB/c Anti-PC Idiotypes in the NBF_1 Transplantation Model: Early and Late Responses to PC-KLH, PC-BA, and PC-LPS after Reconstitution with 10^7 Neonatal Liver Cells

Individual responses to PC-KLH				Individual responses to PC-BA				Individual responses to PC-LPS			
PFC/ spleen	Percent $T15^+$	PFC/ spleen	Percent $T15^+$	PFC/ spleen	Percent $T15^+$	PFC/ spleen	Percent $T15^+$	PFC/ spleen	Percent $T15^+$	PFC/ spleen	Percent $T15^+$
12 d		*55 d*		*12 d*		*55 d*		*12 d*		*55 d*	
21,450	79	ND*		80,400	99	142,500	7	9,550	100	51,500	45
16,200	68			74,100	99	125,000	74	8,650	58	25,500	66
15,540	63			13,900	100	110,000	31	5,517	93	23,000	24
10,650	96			13,800	99	109,000	8	4,400	100	20,500	20
6,900	24			13,400	18	102,000	50	2,000	100	13,750	78
4,300	100			9,390	4	92,000	0	1,267	87	13,500	0
2,300	59			4,600	100	69,000	16	1,100	33	13,200	72
1,950	59			1,600	47	57,500	44			13,200	50
1,617	100					47,000	24			11,250	31
1,500	3										
10/20 < 1,000 PFC/spleen				12/20 < 1,000 PFC/spleen		0/9 < 1,000 PFC/spleen		13/20 < 1,000 PFC/spleen		0/9 < 1,000 PFC/spleen	

300 rad-irradiated NBF_1 male mice received 10^7 BALB/c neonatal liver cells from donors <24 h of age. 12–15 d after transfer, groups of mice were immunized with either PC-KLH, PC-BA, or PC-LPS and anti-PC PFC responses determined 5 d after challenge; additional groups were assayed for their responses to either PC-BA or PC-LPS at 55 d after transfer. In the earlier assays, PFC counts were corrected for responses not inhibitable by 10^{-4} M free PC. Results are presented as in Table I. For a given antigen, the comparison of percent of $T15^+$ at early and late time points by a Wilcoxon two-sample randomization test yields $P < 0.05$ (see Table I).

* Indicates not performed.

tions of adoptive transfer they cannot maintain their ontogenetic advantage: non-T15 clones will eventually emerge to supplant T15 dominance.

Since NBF_1 male mice express very low levels of circulating anti-PC antibodies (28, 29), it could be argued that some form of antibody-dependent clonal promotion might be missing due to the absence of a regulatory loop in *xid* mice (30). We accordingly investigated the effects of serum transfer using two different protocols (data not shown). The first involved the pretreatment of NBF_1 male mice with BALB/c or CBA/Ca serum to induce shifts in the normal 1:1 ratio of $T15^+$:$T15^-$ anti-PC PFC responses of transplanted female NBF_1 splenic B cells. The second method involved treament of NBF_1 recipients of BALB/c neonatal liver cells with BALB/c serum, beginning 3 wk before cell transfer and continuing for a total of 7 wk; PFC responses were analyzed 7 wk after discontinuing the serum injections, to avoid the immunosuppression reported below. In neither case did the administration of serum alter the idiotypic balance. Apparently the passive transfer of idiotype does not favor the selective expansion of idiotype-bearing clones in adoptive hosts.

Depletion of T Cells Does Not Alter the Idiotypic Profile. It is conceivable that in the experiments reported above, the emergence of $T15^+$ clones in a mouse lacking functional $T15^+$ B cells may lead to the generation of suppressor T cells with the potential to inhibit the expression of this idiotype. To investigate this possibility, the following experiment was performed: NBF_1 mice transplanted with BALB/c neonatal

cells were used as a source of spleen cells 9 wk after cell transfer, at a time when considerable expression of non-T15 clones could be expected. The spleen cells were depleted of T cells or Lyt-2^+ cells by double treatment with the appropriate hybridoma antibodies plus complement. Untreated spleen cells and Lyt-2^- cells were transferred to NBF_1 male recipients given 400 rad to eliminate the host's immunocompetence; splenic B cells were transferred to unirradiated NBF_1 male recipients, which provide T cell help. The idiotypic profiles of anti-PC PFC responses induced by PC-KLH were compared in the three groups of recipients. It can be seen in Table III that the predominantly non-T15 response was virtually identical in the three groups. We therefore conclude that suppressor T cells do not obstruct the expression of $T15^+$ antigen-reactive B cells in the NBF_1 host.

Administration of Serum Can Be Nonspecifically Immunosuppressive to Emerging Clones. The results presented above describe the patterns and kinetics of the emergence of anti-PC responses in adoptive transfer. They clarify the earlier reports of predominantly late non-T15 responses. What remain to be explained, however, are the findings of Kaplan et al. (6) that early, albeit marginal, responses could be elicited only in neonatally suppressed hosts, a result which Augustin et al. (7) did not similarly observe. One possibility is that the suppressed animals used by the two groups had regenerated their anti-PC responses to varying degrees. In particular, as it has been reported that neonatally suppressed mice initially lack detectable levels of serum anti-PC antibodies (31), and circulating antibody levels of other antigenic specificities may be indirectly depressed as well (32), it could be argued that mice progressively recover a serum-mediated immunosuppression as they overcome treatment with anti-idiotype. To test this hypothesis directly, we determined the effects of serum transfer on the development of neonatal liver cells transplanted into NBF_1 males. When adoptively transferred syngeneic BNF_1 liver cells were exposed to either the predominally $T15^+$ BALB/c serum or the largely $T15^-$ CBA/Ca serum (Table IV), it was apparent that

TABLE III

Stability of Idiotypic Profile in Secondary Adoptive Hosts

Treatment of donor cells	Recipient	Anti-PC PFC/spleen		
		Total	Non-T15	Percent $T15^+$ (range)
—	NBF_1 (400 rad)	29,512 (4.47 ± 0.10)	21,380 (4.33 ± 0.15)	(8–42)
2 × anti-Lyt-2 + C'*	NBF_1 (400 rad)	34,674 (4.54 ± 0.05)	26,303 (4.42 ± 0.06)	(14–33)
2 × anti-Thy-1.2 + anti-Lyt-2 + C'	NBF_1 (unirradiated)	28,840 (4.46 ± 0.04)	22,387 (4.35 ± 0.02)	(11–31)

Young adult NBF_1 male mice were given 300 rad and injected with 10^7 BALB/c neonatal liver cells. 2 mo after reconstitution, they were immunized with 200 μg PC-KLH and 1 wk later used as donors of spleen cells. The cells were treated as indicated in the table before transfer (2×10^7 cells/mouse) to unirradiated or sublethally irradiated secondary NBF_1 recipients. An intravenous antigenic challenge with 20 μg PC-KLH was given immediately after cell transfer and PFC determinations performed 5 d later. Presented are the geometric means of the anti-PC responses, as well as of the responses not inhibited by an anti-idiotypic serum incorporated into the plaquing mixture, for each group; the logarithm of the mean and standard error are in parentheses.

* C' indicates complement.

TABLE IV

Effect of Parental Normal Mouse Serum on the Reconstitution of PC and TNP Responses in NBF_1 Male Mice Transplanted with Neonatal BNF_1 Liver Cells

Treatment	Anti-PC PFC/spleen	Anti-TNP-PFC/spleen
None	91,205	120,226
	(4.96 ± 0.08)	(5.08 ± 0.03)
BALB/c	38,019	67,608
serum	(4.58 ± 0.09)	(4.83 ± 0.08)
CBA/Ca	15,488	48,978
serum	(4.19 ± 0.11)	(4.69 ± 0.06)

NBF_1 male mice were given 300 rad before the injection of 10^7 neonatal BNF_1 liver cells. As indicated, two groups of NBF_1 mice ($n = 6$) received intravenous injections of parental normal mouse serum according to the following protocol: 0.5 ml at the time of neonatal liver transfer and 0.25 ml weekly for the next 3 wk. Immunization with PC-KLH and TNP-dextran coincided with the last injection of serum. PFC assays were carried out 5 d later; the results are presented as the geometric means of each group, with the logarithm of the means and standard errors given in parentheses.

both parental sera had a nonspecific immunosuppressive effect on the emerging PFC responses. Thus, the seemingly contradictory results of other studies may simply have been a consequence of the extent to which the neonatally idiotype-suppressed recipients had established their circulating PC-specific antibody levels; by using relatively young hosts, which had not yet regenerated full responsiveness to PC, Kaplan et al. (6; and Zoe Quan, unpublished results) were therefore able to detect the early, low responses that would otherwise have been depressed in normal or completely recovered recipients.

Idiotype Shifts Can Be Observed in Syngeneic Recipients. To exclude the possibility that these idiotypic shifts were due to unknown factors in the environment of the immunodeficient NBF_1 male mouse, we performed reconstitution experiments using lethally irradiated BALB/c recipients of syngeneic neonatal liver cells. As is shown in Table V (see also Table VI), even in a syngeneic system the trend from an early T15 emergence to a later non-T15 appearance occurs. Hence, the progression from T15 dominance to non-T15 responses is not the result of some peculiarity of the immunodefective host.[2]

To confirm in the syngeneic system that the alteration of the $T15^+$:$T15^-$ ratio is not the outcome of active T cell regulation at the level of B cell expression, we performed the experiment reported in Table VI. When splenic cells of reconstituted BALB/c hosts were treated twice with anti-Thy-1.2 plus anti-Lyt-2 plus complement, and then transferred to the neutral NBF_1 male environment at a time when a considerable fraction of the anti-PC PFC response was non-T15, the transplanted cells maintained the idiotype profile seen *in situ*. Based upon the results obtained using both syngeneic and immunodefective recipients, we infer that the character of the emerging PC-specific B cell population itself changes with time in adoptive transfer.

[2] Hapten inhibition studies were performed on the anti-PC responses of the 96-d-reconstituted BALB/c recipients of Table VI (data not shown). Whereas the T15-dominant (>95%) normal animals produced responses of highly restricted avidity, the inhibition curves for the adoptive hosts indicated a greater number of both higher and lower avidity PFC than did the controls. That is, the transplanted anti-PC PFC responses increased in heterogeneity.

TABLE V

Ontogeny of BALB/c Anti-PC Idiotypes in Lethally Irradiated BALB/c Hosts

PC-BA		PC-LPS		PC-KLH			
PFC/spleen	Percent $T15^+$	PFC/spleen	Percent $T15^+$	PFC/spleen	Percent $T15^+$	PFC/spleen	Percent $T15^+$
16 d		*16 d*		*16 d*		*96 d*	
117,600	99	8,962	95	219,000	95	161,000	13
38,800	95	7,200	100	149,250	98	125,500	52
24,900	42	7,100	100	114,750	100	57,000	0
17,400	99	6,100	100	47,000	72	44,600	18
16,900	90	4,900	100	27,000	95	21,450	21
14,400	71	4,200	79	19,500	85		
13,700	93	4,052	85				
10,400	80	3,500	31				
7,800	62	2,000	100				
3,200	66						
0/10 <1,000 PFC/spleen		0/9 <1,000 PFC/spleen		1/7 <1,000 PFC/spleen		0/5 <1,000 PFC/spleen	

Individual responses to PC-BA, PC-LPS, and PC-KLH after reconstitution with neonatal liver cells. 800 rad-irradiated BALB/c female mice were given either 10^7 (PC-BA and PC-LPS determinations) or 1.5×10^7 (PC-KLH determinations) BALB/c neonatal liver cells taken from donors <24 h of age; animals assayed for their day 16 responses to PC-KLH had been primed intraperitoneally with 200 μg KLH in complete Freund's adjuvant at least 2 mo before cell transfer to ensure adequate T cell help after lethal irradiation. At the times indicated, groups of recipients were immunized with either PC-BA, PC-LPS, or PC-KLH, and anti-PC PFC responses were determined 5 d later. The total anti-PC and percent of $T15^+$ responses are presented as in Table I. For those mice challenged with PC-KLH, a comparison of the percent of $T15^+$ responses at 16 vs. 96 d after transfer using a Wilcoxon two-sample randomization test yields $P < 0.01$ (see Table I).

Ontogeny of $T15^-$ Clones Is Independent of the Elimination of Emerging $T15^+$ Clones. Our experiments clearly demonstrate that, once they are removed from their normal, intact environment, BALB/c fetal and neonatal liver cells will sequentially generate $T15^+$ and $T15^-$ subpopulations. Three patterns of development could yield such a later emergence of non-T15 clones: (*a*) $T15^-$ clones are derived from $T15^+$ populations, (*b*) $T15^+$ clones retard the growth of non-T15 cells, or (*c*) $T15^-$ clones have an inherently slower maturation rate. To distinguish between these possibilities, emerging $T15^+$ clones were suppressed in adoptive transfer by the administration of a highly specific, monoclonal, anti-idiotypic antibody. As can be seen in Table VII, the specific elimination of the $T15^+$ subsets does not significantly affect the expression of the non-T15 clones: untreated and suppressed recipients produced similar magnitudes of $T15^-$ anti-PC PFC responses. Thus, the removal of $T15^+$ clones neither prevents nor accelerates the appearance of non-T15 PC-specific populations. The increased anti-TNP responses found in those groups given anti-idiotypic serum may be due to some indirect effect of the treatment (32) or to the kind of cross-reactions described by Forni et al. (33), but the significance of this augmentation is unknown.

The relative ontogenetic independence of $T15^+$ and $T15^-$ clones is further illustrated in Table VIII. When spleen cells of unresponsive BALB/c mice neonatally suppressed with a hybridoma anti-idiotypic antiserum were treated twice with anti-Thy-1.2 plus complement and then transferred into the permissive environment of the unirradiated

TABLE VI

Stability of the Idiotypic Profile after the Adoptive Transfer of Reconstituted BALB/c Host Splenic B Cells to Unirradiated NBF_1 Male Recipients

	Anti-PC PFC/spleen	$T15^+$
		%
BALB/c recipients of 10^7 BALB/c neonatal liver cells	61,661 (4.79 ± 0.17)*	49 (21–69)‡
NBF_1 male recipients of 3.5×10^7 reconstituted BALB/c splenic B cells	6,866 (3.84 ± 0.05)	40 (23–60)

4-mo-old BALB/c female mice ($n = 7$) were given 800 rad preparative irradiation before the injection of 10^7 BALB/c neonatal liver cells. 2 mo later, spleen cells from three of these reconstituted animals were pooled, treated twice with monoclonal anti-Thy-1.2, monoclonal anti-Lyt-2, and complement, and transferred to unirradiated NBF_1 male hosts ($n = 7$). The remaining reconstituted BALB/c mice and the NBF_1 recipients were then immunized with PC-KLH and assayed for their PFC responses 5 d after challenge. Presented are the geometric means of the anti-PC responses for each group. The percentage of $T15^+$ was determined by inhibition of plaque formation using an anti-idiotypic serum incorporated into the plaquing mixture.

* Logarithm of mean and standard error.

‡ Range of PFC inhibition in individual animals.

TABLE VII

Effects of Anti-Idiotype Treatment of NBF_1 Recipients on the Anti-PC PFC Idiotype Profile of Transferred BALB/c Neonatal Liver Cells

		Anti-PC PFC	Non-T15 PFC	Anti-TNP PFC
Experiment 1	Control	29,925 (4.48 ± 0.10)	13,860 (4.14 ± 0.13)	73,933 (4.87 ± 0.05)
	Anti-Id treated	18,100 (4.26 ± 0.08)	18,757 (4.27 ± 0.07)	121,807 (5.09 ± 0.10)
	Significance at 1% level	−	−	−
Experiment 2	Control	41,080 (4.61 ± 0.06)	10,149 (4.01 ± 0.19)	98,473 (4.99 ± 0.03)
	Anti-Id treated	24,482 (4.39 ± 0.10)	20,950 (4.32 ± 0.11)	284,060 (5.45 ± 0.03)
	Significance at 1% level	−	−	+

Lethally irradiated NBF_1 male mice received BALB/c neonatal liver cells; one-half of the recipients additionally received two injections of a monoclonal anti-idiotypic serum, one at the time of transfer and the other 2 wk later. In experiment 1, 500 rad-irradiated males were given 1.5×10^7 liver cells; treated animals received a first injection of 12 μg and a second injection of 24 μg anti-idiotype. In experiment 2, 800 rad-irradiated recipients of 2×10^7 neonatal cells were given 16 μg of anti-idiotype at each serum injection. For both experiments, control and idiotype-suppressed mice were challenged with PC-KLH and TNP-Dextran 2 mo after cell transfer and assayed for PFC responses 5 d later. Presented are the geometric means of the responses to TNP, PC, and PC not inhibited by the monoclonal anti-idiotypic reagent incorporated into the plaquing mixture; the logarithms of the means and standard errors are given in parentheses. Groups were compared using a two-tailed Student's *t* test.

NBF_1 male, the transplanted cells continued to be unresponsive to PC at 1 mo after transfer. Evidently the PC-specific population was eliminated as transplantation of the suppressed B cells did not uncover any clones suppressed by T cells. However, by 2 mo after transfer, largely non-T-15 responsiveness to PC began to emerge. Because

TABLE VIII

Emergence of Anti-PC PFC Responses from Adoptively Transferred Splenic B Cells of Neonatally Suppressed BALB/c Donors

Time after transfer	Anti-PC PFC/spleen	$T15^+$	Anti-TNP/spleen
		%	
1 mo	444	–	23,865
	(2.65 ± 0.27)		(4.38 ± 0.15)
2 mo	33,841	9	11,902
	(4.53 ± 0.11)	(0–26)	(4.08 ± 0.07)

2 μg of a hybridoma anti-idiotypic antibody was injected into BALB/c neonates within 48 h of birth. 1 mo later, when intact animals gave on the average 1,010 anti-PC and 102,691 anti-TNP PFC/spleen, spleen cells of the neonatally suppressed mice were treated twice with monoclonal anti-Thy-1.2 plus complement, and 1.5×10^7 of these cells were then transferred into each unirradiated NBF_1 male host. At the times indicated, recipients were immunized with PC-KLH, PnCs, and TNP-Dextran; PFC determinations were performed 5 d after challenge. The results are presented as in Table VI.

intact BALB/c mice also take several weeks to completely break neonatal suppression and their subsequent anti-PC PFC responses do not include the specifically suppressed clones (26), we conclude that BALB/c $T15^-$ clones do not depend on $T15^+$ clones for their generation and, once given the opportunity to emerge, are inherently slow in their rate of growth.

Discussion

The emergence of PC-specific B cells in adoptive transfer systems follows a defined temporal sequence that reflects the developmental heterogeneity of B cell subsets with respect to their acquisition of antigen reactivity and to their expression of idiotypes. The earliest direct anti-PC PFC responses detected after transplantation of BALB/c fetal or neonatal liver cells to adoptive hosts are elicited by TD and TI-1 antigens. Responsiveness to a putative TI-2 antigen, PnCs, appears later, an observation that is in agreement with other studies on the ontogeny of B cell subpopulations (11, 27, 34), and that accounts for the delayed PC-specific responses reported previously in adoptive transfer systems (6, 7). Although it is unknown whether the same or separate populations produce the early responses inducible only by TD or TI-1 antigens, it may be that one cell type can be triggered by both antigens, as has been suggested by others (35). That is, a B cell capable of responding to a TD antigen in the presence of T cell help may also be susceptible in the absence of such help to stimulation in a TI manner by polyclonal B cell activators. Early in ontogeny, when T cell help is not yet available, such an activation would be physiologically important because the antibodies generated by a TI mechanism could then serve as an inducer of idiotype- or immunoglobulin-specific regulatory loops.

The first responses produced to TD and TI-1 PC antigens express the dominant T15 idiotype; responses of the later arising TI-2 reactive compartment(s) also initially exhibit T15 dominance. $T15^-$ clones, not expressed to any considerable extent in normal BALB/c mice, develop at an intrinsically slower rate than do $T15^+$ cells. Eventually, however, a heterogeneous non-T15 population, including clones of higher and lower avidity than T15 (data not shown), comes to constitute a major portion of

the transplanted anti-PC PFC responses. Our findings contradict those of Fung and Köhler (36) on the early emergence of $T15^-$ clones in neonatal BALB/c mice immunized with PC-LPS. Since we could not elicit early PC-specific $T15^-$ PFC responses in BALB/c neonates with either our preparation of PC-LPS or theirs (data not shown), it is likely that the low non-T15 PFC responses detected by Fung and Köhler (36) were polyclonally induced by LPS and were not PC specific.

A progression from T15 dominance to non-T15 expression in adoptive hosts can be accommodated by the interpretation that $T15^-$ clones may be somatically derived from $T15^+$ clones (37). The results of our T15-idiotype suppression studies, however, imply instead that $T15^+$ and $T15^-$ populations comprise distinct developmental compartments, the proportional representation of which changes with time. We therefore attribute the findings of Kim and Benewicz (37) to a cross-reactivity of their anti-idiotypic serum at the level of the B cell precursors, not detectable serologically.

Our observations of idiotype shifts produced by transplanted immature BALB/c cells agree with the displacement of $T15^+$ clones seen during recovery from neonatal exposure to PC tolerogen: the first clones to overcome tolerance are $T15^+$ (38), but in BALB/c mice they are gradually supplanted by non-T15 populations (José Quintáns, unpublished results). The studies described in this paper also concur with those of Kaplan and Quintáns (39) on the eventual loss of T15 dominance in sublethally irradiated BALB/c mice. The transition in all of these cases from an initial $T15^+$ expression to a later non-T15 emergence suggests that $T15^+$ clones have a developmental advantage over $T15^-$ clones, but this ontogenetic preference does not entail the establishment of clonal dominance once the normal generative environment of the intact BALB/c animal is disrupted. Hence, these results substantiate the distinction made elsewhere between the expression and the maintenance of a dominant idiotype (14).

Clearly the precedence $T15^+$ clones may exhibit in ontogeny is in itself insufficient to guarantee idiotypic dominance. Regulatory influences must additionally play a role because manipulations of the environment in which the PC-specific progenitors develop will produce alterations in the idiotypic profile. The control mechanisms may act directly to modulate idiotypic balance. Possibly in the unique formative situation of the intact BALB/c neonate, where various immunoregulatory interactions are being established, the earlier emergence of $T15^+$ clones increases the probability of the generation of self-reinforcing regulatory circuits that prevent most non-T15 progenitors from differentiating into mature B cells. Clonal dominance may then be the net outcome of multiple cellular interactions (a steady state of a network of interactions) or it may be the product of regulation by a single cell type. That we have been unable to associate the idiotype shifts reported here with a lack of idiotype-specific T cell help and/or to a generation of suppressor T cells would imply that the maintenance of idiotypic dominance does not operate primarily through conventional T cell involvement, although our failure to detect these cell types does not disprove their existence. However, active idiotype modulation may utilize other cell types, such as an idiotype-specific Thy-1^- helper cell, as recently described by Okumura et al. (40), or some other B cell capable of participation in B cell-B cell regulation (41), given the fact that congenitally athymic BALB/c mice also express T15 dominance in their responses to both TD and TI PC antigens.

Alternatively, the preponderance of $T15^+$ expression in normal BALB/c mice could

be an indirect consequence of control at a developmental level; that is, idiotypic dominance could be a passive phenomenon rather than an actively maintained state. If, for example, the selective pressures for protection against PC-bearing antigens were to stimulate the earlier arising $T15^+$ clones to expand at a rate that would quickly saturate the capacity of the developmental microenvironment to accommodate them, then the presence of these $T15^+$ progenitors would usually eliminate any opportunity for the later emerging $T15^-$ clones to proliferate and differentiate into antigen-reactive B cells. It would require a severe assault on the proliferative capabilities of the immune system, such as irradiation, tolerance induction, or adoptive transfer, to increase the demands for regeneration to an extent that would give non-T15 clones the occasion to mature. We are currently attempting to distinguish between these hypotheses.

Summary

We have examined the ontogeny of BALB/c plaque-forming cell (PFC) responses to phosphorylcholine (PC) from fetal and neonatal liver by using the (CBA/N × BALB/c)F_1 transplantation model. In this system, thymus-dependent (PC-keyhole limpet hemocyanin) and thymus-independent class 1 (PC-*Brucella abortus*, PC-lipopolysaccharide) PC antigens stimulate B cell subpopulations, which functionally emerge early after transfer. Responsiveness to a thymus-independent class 2 antigen, C-polysaccharide extract of a *Streptococcus pneumoniae* mutant, is acquired later. The response to all PC antigens tested initially exhibited T15 dominance. Non-T15 clones, which are not expressed to a great degree in normal BALB/c mice, are inherently slow in their rate of maturation; in adoptive transfer, however, they eventually comprise much of the transplanted anti-PC PFC response. Obviously, the advantages the T15 subset has in ontogeny do not result in idiotypic dominance once the immature cells are removed from the intact BALB/c environment. We discuss possible regulatory mechanisms involved in the alteration of the $T15^+$:$T15^-$ ratio.

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References

1. 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.
2. Claflin, J. L. 1976. Uniformity in the clonal repertoire for the immune response to phosphorylcholine in mice. *Eur. J. Immunol.* **6**:669.
3. Briles, D. E., M. Nahm, K. Schroer, J. Davie, P. Baker, J. Kearney, and R. Barletta. 1981. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. *J. Exp. Med.* **153**:694.
4. Cosenza, H., and H. Köhler. 1972. Specific inhibition of plaque formation to phosphorylcholine by antibody against antibody. *Science (Wash. D. C.).* **176**:1027.
5. Cosenza, H., and H. Köhler. 1972. Specific suppression of the antibody response by antibodies to receptors. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2701.
6. Kaplan, D. R., J. Quintáns, and H. Kohler. 1978. Clonal dominance: loss and restoration in adoptive transfer. *Proc. Natl. Acad. Sci. U. S. A.* **75**:1967.
7. Augustin, A., M. Julius, and H. Cosenza. 1977. Changes in the idiotypic pattern of an

immune response following syngeneic hemopoietic reconstitution of lethally irradiated mice. *In* Regulation of the Immune System: Genes and the Cells in Which They Function. E. Sercarz, L. Herzenberg, and F. Fox, editors. Academic Press, Inc., New York. 195–199.

8. Köhler, H., D. Kaplan, R. Benca-Kaplan, J. Fung, and J. Quintáns. 1978. Ontogeny of clonal dominance. *In* Physicians and Surgeons Biomedical Sciences Symposium, Columbia University, June 1978. B. Pernis and H. J. Vogel, editors. Academic Press, Inc., New York. 356–369.
9. Quintáns, J., J. P. McKearn, and D. Kaplan. 1979. B cell heterogeneity. I. A study of B cell subpopulations involved in the reconstitution of an X-linked defect of B cell differentiation. *J. Immunol.* **122:**1750.
10. Quintáns, J., and J. P. McKearn. 1979. Developmental heterogeneity of B cell subpopulations. *In* B Lymphocytes in the Immune Response. M. Cooper, D. Mosier, I. Scher, and E. Vitetta, editors. Elsevier/North-Holland Biomedical Press, New York. 131–138.
11. Quan, Z. S., R. F. Dick, B. Regueiro, and J. Quintáns. 1981. B cell heterogeneity. II. Transplantation resistance in *xid* mice which affects the ontogeny of B cell subpopulations. *Eur. J. Immunol.* In press.
12. Quintáns, J. 1977. The "patchy" immunodeficiency of CBA/N mice. *Eur. J. Immunol.* **7:** 749.
13. Mond, J. J., R. Lieberman, J. K. Inman, D. E. Mosier, and W. E. Paul. 1977. Inability of mice with a defect in B-lymphocyte maturation to respond to phosphorylcholine on immunogenic carriers. *J. Exp. Med.* **146:**1138.
14. Quintáns, J., M. R. Loken, Z. S. Quan, R. F. Dick, and B. Regueiro. 1981. Alteration of clonal profile. II. Studies on the capacity of BALB/c splenic B cells to perpetuate responsiveness to phosphorylcholine and T15 idiotypic dominance. *Eur. J. Immunol.* **11:**236.
15. Pilarski, L. M., and A. J. Cunningham. 1975. Host-derived antibody forming cells in lethally irradiated mice. *J. Immunol.* **114:**138.
16. Sarmiento, M., M. R. Loken, and F. W. Fitch. 1981. Structural differences in cell surface T25 polypeptides from thymocytes and cloned T cells. *Hybridoma.* In press.
17. Sarmiento, M., A. L. Glasebrook, and F. W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytolysis in the absence of complement. *J. Immunol.* **125:**2665.
18. Chesebro, B., and H. Metzger. 1972. Affinity labeling of a phosphorylcholine binding mouse myeloma protein. *Biochemistry.* **11:**766.
19. Quintáns, J., and H. Cosenza. 1976. Antibody response to phosphorylcholine *in vitro*. II. Analysis of T-dependent and T-independent responses. *Eur. J. Immunol.* **6:**399.
20. Anderson, H. C., and M. McCarty. 1951. The occurrence in the rabbit of an acute phase protein analogous to human C-reactive protein. *J. Exp. Med.* **93:**25.
21. Inman, J. K. 1975. Thymus-independent antigens: the preparation of covalent hapten-Ficoll conjugates. *J. Immunol.* **114:**704.
22. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology.* **14:**599.
23. Jerne, N. K., and A. A. Nordin. 1963. Plaque-formation in agar by single antibody-producing cells. *Science (Wash. D.C.).* **140:**405.
24. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenyl plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132:**575.
25. Gold, E., and H. Fudenberg. 1967. Chromic chloride: a coupling reagent for passive hemagglutination reactions. *J. Immunol.* **99:**859.
26. Kearney, J. F., R. Barletta, Z. S. Quan, and J. Quintáns. 1981. Monoclonal versus heterogeneous anti-H-8 antibodies in the analysis of the anti-phosphorylcholine response in BALB/c mice. *Eur. J. Immunol.* In press.

27. Mosier, D. E., I. A. Zitron, J. J. Mond, A. Ahmed, I. Scher, and W. E. Paul. 1977. Surface immunoglobulin D as a functional receptor for a subclass of B lymphocytes. *Immunol. Rev.* **37:**87.
28. Quintáns, J., and R. Benca-Kaplan. 1978. Failure of CBA/N mice to respond to thymus-dependent and thymus-independent phosphorylcholine antigens. *Cell. Immunol.* **38:**294.
29. Clough, E. R., D. A. Levy, and J. Cebra. 1981. CBA/N × BALB/cJ F_1 male and female mice can be primed to express quantitatively equivalent secondary antiphosphorylcholine responses. *J. Immunol.* **126:**387.
30. Bottomly, K., and D. E. Mosier. 1979. Mice whose B cells cannot produce the T15 idiotype also lack an antigen-specific helper T cell required for T15 expression. *J. Exp. Med.* **150:** 1399.
31. Cosenza, H., M. H. Julius, and A. A. Augustin. 1977. Idiotypes as variable region markers: analogies between receptors on phosphorylcholine-specific T and B lymphocytes. *Immunol. Rev.* **34:**1.
32. Bona, C., K. E. Stein, R. Lieberman, and W. E. Paul. 1979. Direct and indirect suppression induced by anti-idiotype antibody in the inulin-bacterial levan antigenic system. *Mol. Immunol.* **16:**1093.
33. Forni, L., P.-A. Cazenave, H. Cosenza, K. Forsbeck, and A. Coutinho. 1979. Expression of V-region-like determinants on Ig-negative precursors in murine fetal liver and bone marrow. *Nature (Lond.).* **280:**241.
34. Andersson, B., and H. Blomgren. 1975. T-cell response to polyvinyl pyrrolidone is linked to maturity of B-cells. *Nature (Lond.).* **253:**476.
35. Lewis, G. K., and J. W. Goodman. 1977. Carrier-directed anti-hapten responses by B-cell subsets. *J. Exp. Med.* **146:**1.
36. Fung, J., and H. Köhler. 1980. Late clonal selection and expansion of the TEPC-15 germ-line specificity. *J. Exp. Med.* **152:**1262.
37. Kim, B. S., and B. J. Benewicz. 1980. The lack of compensatory increases of cells producing anti-phosphorylcholine antibodies bearing other idiotypes in TEPC-15 idiotype-suppressed inbred and outbred mice. *Eur. J. Immunol.* **10:**171.
38. McKearn, J. P., and J. Quintáns. 1980. Idiotype-specific tolerance: preferential alteration of idiotype-expression following neonatal exposure to tolerogen. *J. Immunol.* **124:**1983.
39. Kaplan, D., and J. Quintáns. 1978. Alteration of clonal profile. I. Effects of sublethal irradiation on the responses to phosphorylcholine in BALB/c mice. *J. Exp. Med.* **148:**987.
40. Okumura, K., K. Hayakawa, and T. Tada. 1981. Immunoglobulin (Ig) gene restricted augmentation of *in vitro* secondary antibody response to novel lymphocyte (Thy-1^-, Lyt-1^+). *J. Supra. Struct. Cell. Biochem.* **5** (Suppl.):75 (Abstr.).
41. Ivanyi, J., and C. Makings. 1980. Allotype analysis of histocompatible and semiallogeneic B cell chimeric chickens. *Transplantation (Baltimore).* **29:**25.