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Immunology

Clonal dominance: Loss and restoration in adoptive transfer

(clonotypic analysis/B cell ontogeny/regulation of clonal profile)

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ABSTRACT **An adoptive transfer system was used to study the mechanism responsible for clonal dominance of the anti-phosphorylcholine response in BALB/c mice. The adult spleen contains phosphorylcholine-specific precursor cells that are capable of developing into antibody-producing cells after transfer into lethally irradiated animals. The neonatal liver of the BALB/c mouse lacks precursor cells specific for phosphorylcholine but contains immature cells that differentiate into specific precursors during the normal course of ontogeny. The transfer of fetal or neonatal liver cells into lethally irradiated recipients prevents the appearance of the dominant H8 clone which constitutes the majority of the clones responding to phosphorylcholine in adult BALB/c mice. However, if these cells are transferred into neonatally suppressed recipients that lack the H8 idiotype, dominance of the H8 clone can develop. The conversion of the committed immature progenitor cell into a responsive B lymphocyte precursor is a regulated event. Regulation at the level of progenitor cells determines the eventual clonal profile of the immune response to phosphorylcholine. It is suggested that selection of the dominant clone occurs at this level.**

The murine fetal or neonatal liver is a significant site of lymphocyte maturation. Cells from this organ have been found to synthesize Ig, incorporate the Ig into their membranes, and bind various antigens to their surfaces (1–3). Functionally, the young liver cells are capable of reconstituting the entire spectrum of blood cell elements in lethally irradiated hosts. The development of transferred B lymphocytes has been monitored by immunizing the recipients with several different antigens. In such adoptive transfers it has been shown that the murine fetal or neonatal liver contains cells that can mature into specific antibody-secreting cells on stimulation by a diverse array of antigens, T-dependent or T-independent, particulate or soluble: sheep erythrocytes (4, 5); dinitrophenol coupled to *Salmonella* flagella (6), bovine serum albumin (7), or bovine gamma globulin (8); fluorescein coupled to bovine serum albumin (7); the bacteriophages F2, T4, and ϕX-74 (7); hen egg lysozyme; sperm whale myoglobin; and bovine pancreatic ribonuclease (7).

In the response to phosphorylcholine (*P*Cho) of the BALB/c mouse which is characterized by dominance of the HOPC-8 (H8) idiotype (9–13), it has been shown that liver, spleen, and bone marrow of the neonate do not contain *P*Cho-specific precursor cells. At age 1 week, the H8 clone appears in the spleen and bone marrow (14). This clone can be chronically suppressed by injecting neonatal mice with antiserum directed against the H8 idiotype (15). Thus, it seems that in the neonatal period the clonal pattern of the anti-*P*Cho response is susceptible to specific immunologic manipulation (16).

Rosenberg and Cunningham (17) have used anti-sheep erythrocyte responses in adoptive transfer experiments to show that the immature B lymphocytes of the fetal liver have to undergo an additional differentiation step to reach the level of the precursors in the adult spleen. In the present study we investigated the differentiation of the noncommitted stem cell into a responsive B cell precursor of defined idiotypic specificity. We have demonstrated that the selection of the dominant clones takes place prior to the acquisition of responsiveness by the precursors.

MATERIALS AND METHODS

Mice. Adult BALB/c mice were obtained from Cumberland Farms, Clinton, TN. These animals were used as recipients in adoptive transfer experiments at age 8–20 weeks. Neonatal and fetal mice were obtained from our breeding colony. The age of the fetal mice was determined by the appearance of a vaginal plug. Adult A/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, ME.

Antigens and Immunization. Formalin-killed *Streptococcus pneumoniae* strain R36a (R36a) was used in these experiments. For immunization, 5×10^8 R36a cells suspended in 0.2 ml of saline were injected intravenously. Trinitrophenol-modified R36a (TNP-R36a) was synthesized by incubating the cells with 2,4,6-trinitrobenzenesulfonic acid (Sigma), 20 mg/ml in cacodylate buffer (pH 9.0) for 3 hr at room temperature. For immunization, 2×10^9 TNP-R36a cells were injected intravenously. TNP-Ficoll was a gift from H. Kiefer (Basel Institute of Immunology); each mouse was immunized by injection of 10 μg into the retro-orbital venous sinus. Animals receiving both *P*Cho and TNP antigens were given them in a single injection.

Anti-Idiotypic Antiserum. Antiserum to the H8 idiotype (anti-H8 id) was prepared as described (18). The antiserum produced was absorbed on a column containing normal mouse serum conjugated to Sepharose 4B; the normal serum had been previously absorbed with *P*Cho coupled to Sepharose 4B to remove all anti-*P*Cho antibodies. This absorbed antiserum inhibited more than 90% of BALB/c anti-*P*Cho plaque-forming cells (PFCs), only 40–60% of A/He anti-*P*Cho PFCs, and none of the CBA/J anti-*p*cho PFCs. The antiserum did not inhibit anti-TNP PFCs of these strains.

Adoptive Transfer. BALB/c mice to be used as recipients were maintained in sterile conditions on water containing Terramycin (Pfizer Laboratories). The experimental mice were given 800 rads (8 J/kg) of total body irradiation from a ^{137}Cs source. Fetal or neonatal liver and adult spleen were dissected under sterile conditions and gently teased through a fine screen to obtain a suspension of single cells in Hanks' balanced salt solution. Within 3 hr of irradiation, cells were injected via the lateral tail vein.

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Abbreviations: *P*Cho, phosphorylcholine; R36a, *Streptococcus pneumoniae* strain R36a; anti-H8 id, antiserum directed against the idiotype of the HOPC-8 plasmacytoma; TNP, trinitrophenol; PFC, plaque-forming cell.

Table 1. Anti-TNP and anti-*P*Cho responses of lethally irradiated BALB/c mice reconstituted with syngeneic cells

Exp.	Cells* transferred	Recipient mice, no.	Antigens	Log_{10} (direct PFC)† Anti-TNP	Anti-*P*Cho
1	5×10^6 neonatal liver	6	R36a TNP-Ficoll	4.37 ± 0.04 (23,442)	<3.00 (<1,000)
2‡	1×10^7 neonatal liver	11	R36a TNP-Ficoll	4.21 ± 0.07 (16,218)	<3.00 (<1,000)
3‡	3×10^7 neonatal liver	17	R36a TNP-Ficoll	4.35 ± 0.08 (22,387)	<3.00 (<1,000)
4	6×10^7 neonatal liver	3	R36a TNP-Ficoll	4.40 ± 0.24 (25,119)	3.37 ± 0.11 (2,344)
5	3×10^7 adult spleen	6	R36a TNP-Ficoll	4.36 ± 0.10 (22,909)	4.55 ± 0.15 (35,481)
6	1×10^7 neonatal liver	4	TNP-R36a	4.13 ± 0.13 (13,490)	2.77 ± 0.21 (589)
7	1×10^7 adult spleen	10	TNP-R36a	3.75 ± 0.08 (5,623)	3.71 ± 0.07 (5,129)

* All neonatal cells transferred were pools of cells from mice less than 48 hr old.

† Lethally irradiated and reconstituted mice were immunized during the 3rd week after transfer, and direct PFCs were enumerated 5 days later. The logarithm of the geometric mean and standard error are given with the geometric mean in parentheses. Most experiments were performed on different days.

‡ Cumulative results from several experiments are presented.

Hemolytic Plaque Assay. A modification of the hemolytic plaque technique was used (19, 20). R36a extract (21) and TNP (22) coupled to sheep erythrocytes were used as target cells.

RESULTS

Specific Low Responsiveness to *P*Cho in Recipients Reconstituted with Neonatal Liver Cells. The stability of mature and immature cells to respond to *P*Cho and TNP antigens in an adoptive transfer system was tested. Lethally irradiated BALB/c mice were reconstituted with either adult spleen or neonatal liver cells. These animals were immunized during the 3rd week after transfer and direct PFCs were measured 5 days later. Recipients of adult spleen cells responded both to TNP and *P*Cho antigens. The transfer of neonatal liver cells from mice less than 48 hr old restored the anti-TNP response to levels of adult spleen reconstitution, but the anti-*P*Cho response was very low compared to the transfer of adult spleen cells (Table 1). This response pattern was independent of cell dose. Even transferring 6×10^7 cells, the cellular equivalent of two neonatal livers, we were unable to restore the anti-*P*Cho response.

TNP-R36a is an antigen with both the *P*Cho and TNP epitopes incorporated in a single antigenic species. This antigen was used to show that the difference in responsiveness was not a consequence of antigen processing but represented a difference in B cell expression. BALB/c mice were lethally irradiated and reconstituted with either 1×10^7 neonatal liver cells or 1×10^7 adult spleen cells. Eighteen days after the transfer, both groups were immunized with TNP-R36a (last two entries, Table 1). Direct PFCs in the spleen were counted 5 days later. Mice reconstituted with adult spleen responded equally well to both haptens. The irradiated animals given neonatal liver cells produced more anti-TNP PFCs than did the animals given adult spleen cells, but again the anti-*P*Cho response was low.

To exclude the possibility that the low response to *P*Cho was due to a shift in the time of peak response, the direct PFCs in the spleen were counted at various times after challenge (Fig. 1). The peak of the response to both antigens by mice reconstituted with either tissue was at 5 days after immunization. Normal BALB/c mice also exhibit a maximum of direct PFCs after the same interval. Therefore, in this study, all responses were measured 5 days after immunization.

Adult thymocytes have been used to provide T cell help in adoptive transfer experiments (8). To ascertain that a source of adult T cells would not alter the low responsiveness to the *P*Cho antigen, we gave one group of animals thymocytes as well as neonatal liver cells. Five irradiated BALB/c mice received 1×10^7 neonatal liver cells. These animals gave good anti-TNP responses (4.30 ± 0.05) but their anti-*P*Cho response was low (2.81 ± 0.28). Five other recipient mice given 1×10^7 neonatal

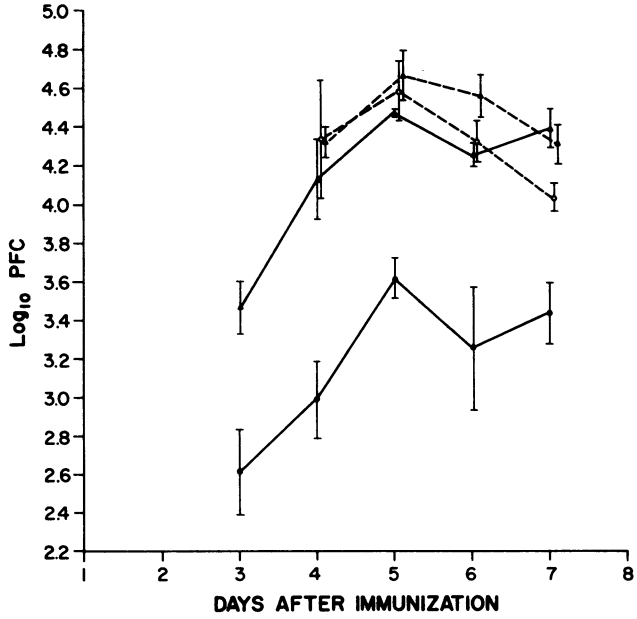

FIG. 1. Kinetics of the response to R36a and TNP-Ficoll in the adoptive transfer experiment. Lethally irradiated BALB/c mice were reconstituted with either syngeneic adult spleen cells or liver cells pooled from mice less than 72 hr old. Animals given 1×10^7 neonatal liver cells were immunized 17 days after transfer and direct PFC to *P*Cho-sheep erythrocytes (●—●) and to TNP-sheep erythrocytes (▲—▲) were enumerated at various times after challenge. Animals given 3×10^7 adult spleen cells were immunized 13 days after transfer and direct PFC to *P*Cho-sheep erythrocytes (○- - -○) and to TNP-sheep erythrocytes (△- - -△) were enumerated at various times after challenge. Vertical bars represent the standard error of the geometric mean.

Table 2. Clonotypic analysis of lethally irradiated BALB/c mice reconstituted with syngeneic cells

Cells transferred	Recipient mice, no.	Time of assay*	Log_{10} (direct PFC) Anti-TNP	Anti-*P*Cho	Non-H8 id anti-*P*Cho†	% non-H8 id PFCs
1×10^7 neonatal liver	6	10	4.83 ± 0.16 (64,608)	3.99 ± 0.17 (9772)	3.86 ± 0.21 (7244)	74
1×10^7 adult spleen	5	3	4.18 ± 0.09 (15,276)	3.94 ± 0.14 (8630)	2.51 ± 0.17 (324)	4
1×10^7 adult spleen	5	10	4.35 ± 0.18 (22,387)	3.74 ± 0.05 (5470)	2.30 ± 0.20 (200)	4

Lethally irradiated BALB/c mice were reconstituted with neonatal liver cells (<48 hr old) or with adult spleen cells. Mice were immunized with R36a and TNP-Ficoll either 3 weeks or 10 weeks after transfer. Direct PFCs were counted 5 days after challenge. The log of the geometric mean and standard error are given with the geometric mean in parentheses.

* Weeks after transfer.

† Clonotypic analysis was carried out by incorporating a 1:500 dilution of anti-H8 id into the plaquing mixture. Anti-*P*Cho PFCs not inhibited by anti-H8 id were 100% inhibited by 10 μM *P*Cho. (For the specificity of the plaque inhibition, see *Materials and Methods.*)

liver cells from the same pool were also given 5×10^7 adult thymocytes. Again the anti-TNP response was restored (4.20 ± 0.21) but the anti-*P*Cho response (2.94 ± 0.21) could not be increased by adding adult T lymphocytes.

The inability to reconstitute the anti-*P*Cho response in lethally irradiated mice was not restricted to neonatal liver cells. Fetal liver showed the same pattern. In a representative experiment we transferred 4×10^6 fetal liver cells (13 days gestation) to irradiated BALB/c mice. Three weeks later the animals were immunized with R36a and TNP-Ficoll. The anti-TNP response was high (4.46 ± 0.06) and the anti-*P*Cho response was very low (2.68 ± 0.20).

Loss of Clonal Dominance in Mice Reconstituted with Neonatal Liver. Although there was a low responsiveness to the *P*Cho antigen 20 or 30 days after the transfer of neonatal liver cells, by day 60 the anti-*P*Cho responses were substantially increased. The clonotype of these responses was analyzed by inhibition of plaque formation with anti-H8 id (9, 10) (Table 2). It was found that the pattern of clonal dominance characteristic of BALB/c mice was lost. The majority of PFCs appearing after 10 weeks lacked the HOPC-8 idiotype. The results in Table 2 for neonatal liver reconstitution are derived from two experiments. We have analyzed the clonotype of 20 additional mice from three other similar experiments and found the same pattern. Clonotypic analysis of mice reconstituted with adult spleen cells and assayed 3 weeks or 10 weeks later showed that more than 90% of the direct PFCs were inhibited with anti-H8 id (Table 2).

Restoration of Clonal Dominance by the Transfer of Neonatal Liver Cells into Neonatally Suppressed Hosts. When BALB/c mice treated as neonates with anti-H8 were used as recipients for neonatal liver cells, different results were obtained (Table 3). In the three experiments shown in Table 3, the response to *P*Cho was increased. Furthermore, clonotypic analysis of the anti-*P*Cho responses showed that a majority of the anti-*P*Cho response was inhibited by anti-H8 id. Although irradiated normal BALB/c mice were unable to support the development of the H8 clone from neonatal liver cells, with the use of neonatally suppressed mice as hosts in adoptive transfer, the H8 idiotype was dominantly expressed.

DISCUSSION

The H8 idiotype is dominant in the response of BALB/c mice to *P*Cho (9–13). Even unimmunized conventional BALB/c mice have high levels of circulating H8 idiotype (23). On the other hand, the development of this clonal dominance is easily interrupted by anti-idiotypic antiserum given at birth (15). In this study we transferred neonatal BALB/c liver cells into lethally irradiated adults and found that the development of clonal dominance depended on the status of the recipient.

Neonatal liver cells reconstitute responses in adoptive transfer to many antigens (4–8); however, lethally irradiated normal BALB/c mice reconstituted with syngeneic neonatal liver cells exhibited a specific low responsiveness to the *P*Cho epitope that cannot be attributed to antigen dynamics. TNP-R36a stimulates anti-TNP and anti-*P*Cho responses in hosts reconstituted with adult spleen cells, but only anti-TNP responses are observed if neonatal liver cells are used. Also, the kinetics of the response were determined to ensure that the maximal response was counted. Finally, using thymocytes as a source of adult T cells, we were unable to show any change in the pattern of responsiveness.

When neonatal liver cells were used to reconstitute lethally irradiated, neonatally suppressed BALB/c mice, we observed a response to the *P*Cho antigen, and the majority of these direct PFCs were H8 idiotype. A point to consider is the origin of the B cells producing the H8 response. It is most likely that the clonally dominant response originated from the transferred cells because neonatally suppressed mice are unable to respond to *P*Cho with H8 id, either before or after suppression has been broken (ref. 24; unpublished data). Moreover, preliminary experiments show that anti-idiotypic suppression of neonatal cells before transfer completely abrogates the response in neonatally suppressed hosts.

It is known that the neonatal liver and spleen do not contain the *P*Cho-specific precursors found in adult spleen, and that the ability to respond to *P*Cho appears late in ontogeny (14).

Table 3. Clonotypic analysis of neonatally suppressed, lethally irradiated BALB/c mice reconstituted with syngeneic neonatal liver cells

Exp.	Mice no.	Log_{10} (direct PFC) Anti-TNP	Anti-*P*Cho	Non-H8 id anti-*P*Cho
1	5	4.29 ± 0.08 (19,498)	3.79 ± 0.13 (6166)	2.99 ± 0.05 (16%) (977)
2	6	4.38 ± 0.11 (23,988)	3.89 ± 0.16 (7762)	2.58 ± 0.18 (5%) (380)
3	8	4.24 ± 0.07 (17,378)	3.66 ± 0.17 (4571)	2.47 ± 0.23 (6%) (294)

Experimental details as in Table 1 except that the recipients were neonatally suppressed with 0.05 ml of a 1:5 dilution of anti-H8 id given within 48 hr of birth. Recipient mice were at least 2 months old. Inhibition of anti-PFCs as in Table 2.

Because there are no *P*Cho-specific precursor cells in the neonatal liver, certain immature cells must be present which can differentiate into *P*Cho-specific clones in neonatally suppressed mice. We have called these cells "progenitor cells." The precursor cell is separate and distinct from the progenitor cell. Precursors are committed cells that are ready to respond to stimulation by antigen (14). In contrast, the progenitors are immature B lymphocytes that require further differentiation to become precursor cells. Without this differentiation, progenitor cells themselves are incapable of mounting an immune response.

The progenitor and precursor cell stages described here for anti-*P*Cho responses are similar to those studied by Phillips and Melchers (4) in anti-sheep erythrocyte responses. By using an idiotype specificity as a marker, we could analyze the anti-*P*Cho response of the reconstituted recipient and determine whether the transferred progenitor cells give rise to the dominant H8 idiotype. Furthermore, we have been able to modulate the status of the dominant idiotype in the recipient and thereby observe that the transition of the H8 progenitor to precursor is dependent on the environment of the host. The development of the H8 clone in neonatally suppressed recipients and the loss of this dominant clone in normal BALB/c hosts suggest that the selection of progenitors is regulated. Inasmuch as this regulation is idiotypically specific, progenitor cells must be committed cells with idiotypically defined cell surface markers. Because this regulation determines the eventual clonal profile in adoptive transfer, selection of the dominant clone and the phenotypic expression of antibody diversity could originate at this level *in vivo*.

The expression of non-H8 clones late after transfer of neonatal liver cells is similar to the delayed appearance of non-H8 idiotypes after neonatal suppression by anti-H8 id (24). This late expression of non-H8 progenitors indicates a time-dependent regulatory event; however, we do not know the factors responsible for the delay.

As mentioned above, the *P*Cho-specific precursor cells are manifested late in ontogeny, at 1 week of age. Although this finding has been interpreted as the late acquisition of a germline specificity (14), regulatory influences at the level of the progenitor cell could also contribute to this delayed appearance.

Note Added in Proof. After submission of this manuscript we received a report from Augustin, Julius, and Cosenza (25). These investigators have also found that fetal liver cells or bone marrow cells given to lethally irradiated adult BALB/c recipients failed to restore the response to phosphorylcholine with H8 clonal dominance. Herein we confirm these findings, but our results differ from theirs in that we could restore clonal dominance by using neonatally suppressed mice as recipients.

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