Brief Definitive Report

IN VITRO TOLERANCE INDUCTION OF NEONATAL MURINE B CELLS AS A PROBE FOR THE STUDY OF B-CELL DIVERSIFICATION*

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The mechanism by which the generation of the B-cell specificity repertoire proceeds, and the role of antigen-driven events in the generation of the repertoire, have been the subjects of intensive theoretical controversy (1–3). Results in both murine and avian experimental systems have supported the postulate that the acquisition of the B-cell repertoire is a highly ordered, specifically predetermined process (4, 5). Although a major tenet of this theory states that antigen-driven events do not play a major role in the generation of the specificity repertoire, it has yet to be proven that the interaction of neonatal (immature) B cells and antigen does not participate in the generation of new specificities. This laboratory has recently examined the capacity of immature, developing B cells to interact with antigen, either in the presence or absence of antigen-specific T cells (6).[1] Whereas neonatal B cells and developing B cells in the adult bone marrow can be specifically stimulated in the presence of antigen-specific T cells, the interaction of immature B cells and antigen in the absence of antigen-specific T cells results in the specific functional inactivation of these cells. Susceptibility to tolerance induction in this system is presumed to be a characteristic of B cells early in their development since: (*a*) dinitrophenyl(DNP)- and trinitrophenyl(TNP)-reactive B cells (which arise early in development) are tolerizable only during the first few days after birth (6); (*b*) tolerance susceptibility persists longer in neonatal bone marrow B cells;[1] and (*c*) 25% of adult bone marrow B cells are tolerizable.[1]

Previous studies have established that several clonotypes specific for DNP and TNP are present in BALB/c mice at birth, whereas B cells responsive to phosphorylcholine (PC) are not detected until approximately 1 wk after birth (3, 4, 7, 8). The results of the current investigation demonstrate that at 7–10 days after birth, when over 90% of the DNP-specific splenic B cells are resistant to tolerance induction, the majority of PC-specific B cells are tolerizable. These results re-emphasize tolerance susceptibility as a characteristic of developing clones, confirm the late acquisition of PC-specific B cells even those of the T15 clonotype, a germline antibody specificity, and support the contention that the acquisition of the specificity repertoire is a highly ordered, specifically predetermined process which is independent of the antigenic stimulation of B cells.

Materials and Methods

Hapten-Carrier Conjugates. The preparation of *Limulus polyphemus* hemocyanin (Hy), DNP-

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[1] Metcalf, E. S., and N. R. Klinman. 1976. In vitro tolerance induction of bone marrow cells: a marker for B-cell maturation. *J. Immunol.* In press.

TABLE I

The Susceptibility of Day 7–10 PC- and DNP-Specific Spleen and Bone Marrow B cells to Tolerance Induction

Donor	Number of cells analyzed $\times 10^{-6}$	Tolerogen	Stimulating antigen	Number of clones per 10^6 cells transferred*	Percent of control response
Spleen, 8–10 wk	124	–	DNP-Hy	1.92	
	128	DNP-MγG	DNP-Hy	1.77	92.1
	120	–	PPC-TGG-Hy	0.30	
	160	PPC-TGG-MγG	PPC-TGG-Hy	0.28	93.3
Spleen, 7–10 days	28	–	DNP-Hy	1.97	
	28	DNP-MγG	DNP-Hy	2.00	101.5
	330	–	PPC-TGG-Hy	0.14	
	330	PPC-TGG-MγG	PPC-TGG-Hy	0.06	43.3
Bone marrow, 7–10 days	68	–	DNP-Hy	0.39	
	68	DNP-MγG	DNP-Hy	0.14	35.9
	108	–	PPC-TGG-Hy	0.09	
	108	PPC-TGG-MγG	PPC-TGG-Hy	0.02	22.2

* 4–20 × 10^6 donor spleen or bone marrow cells were transferred to each recipient mouse. Recipient fragment cultures were incubated in the presence or absence of DNP-MγG at 10^{-6} M DNP or PPC-TGG-MγG at 5×10^{-7} M PC for 24 h, washed, and stimulated with earlier DNP-Hy at 10^{-6} M DNP, or PPC-TGG-Hy at 5×10^{-7} M PC, respectively. Clones were detected by radioimmunoassay of culture fluids with ^{125}I-labeled anti-mouse Fab, IgM, or IgG_1.

Hy, and DNP-mouse gamma globulin (DNP-MγG), has been described previously (6, 9). 3(*p*-azophenyl-phosphorylcholine)-N-acetyl-L-tyrosylglycylglycine t-butyloxycarbonyl (Boc) hydrazide (PPC-TGG) was conjugated to either Hy (PPC-TGG-Hy) or MγG (PPC-TGG-MγG) by a modification of the procedure by Inman et al. (10), as described elsewhere (11).

Animals. 6- to 8-wk old BALB/c mice (Institute for Cancer Research, Philadelphia, Pa.) received intraperitoneal injections of 0.2 ml containing 100 μg of Hy in complete Freund's adjuvant. 6- to 12-wk after carrier-priming these mice were used as recipients in adoptive transfers of adult or neonatal nonimmune cells. 4- to 6-h before cell transfer, the recipients received 1300 R of total body irradiation from a cesium source.

Cell Transfers and In Vitro Tolerance Induction. Suspensions of neonatal or adult BALB/c spleen and bone marrow cells were prepared as described previously (6).[1] 4-20 × 10^6 viable bone marrow or spleen cells were injected intravenously into Hy primed, irradiated, syngeneic, adult recipients. Recipient fragment cultures were individually incubated with Dulbecco's modified Eagles medium or with either PPC-TGG-MγG (5×10^{-7} M PC) or DNP-MγG (10^{-6} M DNP) for 24 h, washed, and stimulated with PPC-TGG-Hy (5×10^{-7} M PC) or DNP-Hy (10^{-6} M DNP), respectively (6).

Radioimmunoassay. 20 μl of culture fluids which were collected 10 or 13 days after stimulation were quantitatively assayed for anti-hapten antibody using a solid phase radioimmunoassay (12). Bound mouse anti-hapten antibody was detected by the addition of ^{125}I-labeled purified goat anti-mouse Fab, IgM (μ), or IgG_1 (γ_1) (7), removal of excess iodinated antibody, and the determination of radioactivity of each sample in a gamma counter.

Results

Table I compares the susceptibility of DNP-and PC-specific splenic B cells to tolerance induction in the adult and in neonates 7–10 days after birth. Pre-incubation of fragment cultures containing either adult or day 7–10 splenic B cells with 10^{-6} M DNP-MγG did not significantly diminish the subsequent response to DNP-Hy. In addition, pre-incubation with PPC-TGG-MγG did not affect adult B cells, whereas the response of the majority of day 7–10 splenic B cells to PPC-TGG-Hy was eliminated.

An analysis of isotype distribution for fragment cultures derived from day 3

neonatal spleens previously demonstrated that both IgM and IgG_1-producing DNP-specific clones were markedly reduced after pre-incubation with DNP-MγG (6). A similar analysis of isotype distribution of day 7–10 PC-specific splenic B cells after pre-incubation with PPC-TGG-MγG also demonstrated that both IgM and IgG_1 responses were reduced by 46 and 65%, respectively. Furthermore, B cells of the T15 clonotype were proportionately diminished by pre-incubation of fragment cultures with PPC-TGG-MγG, representing 61% of the PC-responsive B cells before and 63% after tolerance induction. Table I also compares the susceptibility of DNP- and PC-specific bone marrow B cells to tolerance induction at 7–10 days after birth. PC-specific bone marrow B cells were more susceptible to tolerance induction than either age matched splenic PC-specific B cells or DNP-specific bone marrow B cells.

Discussion

Previous studies have indicated that DNP- and TNP-specific B-cell clones arise during BALB/c intrauterine development, whereas B cells responsive to PC are not detected until approximately 1 wk post-parturition (7, 8). Furthermore, in vitro tolerance studies have demonstrated that DNP-reactive B cells are tolerizable only during the first few days after their expression in the population (6). If the acquisition of the B-cell specificity repertoire is a highly ordered, predetermined process and developing B cells are uniquely susceptible to tolerance induction, then it would be predicted that PC-specific cells at 7–10 days, which represent newly expressed clones, should also be susceptible to tolerance induction. The results of the present study confirm these predictions by the demonstration that although DNP-specific splenic B cells are resistant to tolerance induction 7–10 days after birth, the majority of age-matched donor PC-specific B cells are tolerizable.

The suggestion that the susceptibility of B cells to tolerance induction reflects their state of maturation derives primarily from the finding that DNP- and TNP-specific splenic B cells are susceptible to tolerance induction for only a few days post-parturition and that only a proportion of the cells in the bone marrow retain this susceptibility thereafter. The demonstration that 7–10 day PC-specific but not DNP-specific B cells are tolerizable indicates that tolerance susceptibility reflects the maturational status of a given B cell rather than the developmental status of the animal or B-cell population as a whole. This contention is supported by the finding that 7–10 day bone marrow B cells which are specific for PC are more susceptible to tolerance induction than either 7–10 day DNP-specific bone marrow or 7–10 day PC-specific splenic B cells. These data suggest that B cells arising from the generative cell pool in fetal and early neonatal life are initially expressed in the spleen but by the end of the 1st-wk of life the generative cell population resides primarily in the bone marrow.

The results which demonstrate that the majority of PC-specific B cells in 7–10 day old BALB/c mice are tolerizable confirm not only the unique susceptibility of developing B cells to tolerance induction but also verify the relatively late appearance of B cells of this specificity. Thus, by several independent criteria, including the late appearance of PC binding cells (12), the late appearance of PC-specific precursor cells (8), and the maturational status of newly arising PC-specific B cells as determined by the criterion of tolerance susceptibility, PC-

specific B cells appear to be generated relatively late in neonatal development. Since the T15 clonotype, which is present in high frequency in all adult BALB/c mice, is among the PC-specific clonotypes which display these characteristics, these findings support the contention that repertoire expression is highly ordered and predetermined.

Theories which postulate random somatic events as the mode of B-cell diversification are generally dependent on antigen-driven events and positive selection of specific clonotypes for divergence from germline antibody specificities (1). However, the present study and earlier analyses of the parameters of tolerance induction of developing DNP- and TNP-specific B cells demonstrate that tolerance is highly specific and that B cells, early in their development, are already committed to a unique specificity. Thus, at the earliest time in a B cell's development when receptor expression can be evidenced functionally, that cell is committed to a unique antibody specificity; moreover, antigen-receptor contact is predominantly tolerogenic, not stimulatory. Although these results may imply a role for a tolerance mechanism during diversification and indicate that tolerance can be circumvented by antigen-specific T cells, they argue strongly against a positive role for antigen-driven selective events in B-cell diversification.

Summary

The susceptibility to in vitro tolerance induction has been implicated as a characteristic of B cells early in their development, since DNP-reactive B cells are tolerizable only during the first days after birth, and 25% of adult bone marrow cells are tolerizable. In the present study, a modification of the in vitro splenic focus technique was utilized to determine if PC-specific B cells, by virtue of their late expression (approximately 1 wk post-parturition), also display susceptibility to tolerance induction. The results demonstrate that at 7–10 days after birth, when over 90% of the DNP-specific splenic B cells are resistant to tolerance induction, the majority of PC-specific B cells are tolerizable. These results re-emphasize tolerance susceptibility as a characteristic of developing clones, confirm the late acquisition of PC-specific B cells, and support the contention that the acquisition of the specificity repertoire is a highly ordered, specifically predetermined process which is independent of antigen-driven events.

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