

SUSCEPTIBILITY TO IN VITRO TOLERANCE INDUCTION
OF ADULT B CELLS FROM
MICE WITH AN X-LINKED B-CELL DEFECT*

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CBA/N mice, a mutant subline of the CBA/Ca strain, have an X-linked B lymphocyte-immune defect. These mice and F₁ male progeny derived from CBA/N females fail to produce specific antibody after immunization with certain thymic-independent (TI) antigens (1-4). In addition, these mice fail to produce IgM or IgG responses to either the TI or T-dependent derivatives of the hapten phosphorylcholine (PC) (5, 6). Moreover, analysis of surface membrane characteristics indicates that B cells derived from immune defective mice fail to express determinants that are present on a population of B lymphocytes that appears late in ontogeny. These include minor lymphocyte-stimulating determinants (7), Lyb5 (8), and Lyb3 (9). Furthermore, the B cells of CBA/N mice have unusually high amounts of surface IgM (sIgM) and exhibit a low ratio of surface $\delta:\mu$ heavy chains when compared with the B cells of normal mice (10, 11). These surface characteristics are similar to those that are exhibited by neonatal B cells (10). Taken together, these findings are consistent with the hypothesis that the immune defect(s) in CBA/N mice arise from a maturational arrest either in the development of CBA/N B cells or of a subpopulation of these B cells.

Previous studies, which used a modification of the in vitro splenic focus technique (12), have suggested that susceptibility to in vitro tolerance induction may serve as a functional marker for developing (immature) B cells and B-cell maturation (12-14). This conclusion is based primarily on the following observations. (a) 2,4-dinitrophenyl (DNP)- and PC-reactive splenic B cells are tolerizable only for the first few days after their initial expression in the murine neonate (12, 13). (b) Neonatal bone marrow B cells can be rendered unresponsive to antigen longer in development than neonatal splenic B cells (13, 14). (c) Whereas adult splenic B cells are not susceptible to in vitro tolerance induction, 25% of adult bone marrow B cells are tolerizable (13, 14).

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Therefore, if the immune defects of the adult CBA/N mouse reflect a maturational arrest in B-cell development, then it might be expected that adult immune defective CBA/N splenic B cells may be significantly more susceptible to in vitro tolerance induction than normal adult splenic B cells.

The results of this investigation demonstrate that in the murine adult (8–10 wk after birth), >90% of the DNP-specific splenic B cells in normal mice are resistant to tolerance induction, whereas >50% of the DNP-specific splenic B cells in adult immune-defective CBA/N or F₁ males are tolerizable. These findings are consistent with the hypothesis that the immune defect(s) in CBA/N mice may arise, in part, from a maturational arrest in the development of a given B-cell subpopulation. In addition, these studies define at least two subpopulations in CBA/N mice, only one of which is tolerizable.

Materials and Methods

Hapten-Carrier Conjugates. *Limulus polyphemus* hemocyanin (Hy) was purchased from Worthington Biochemical Corp., Freehold, N. J. Chicken (ovalbumin [OVA], Fraction V, Pentex Biochemical, Inc., Kankakee, Ill.) was used without further purification. The preparation of dinitrophenylated Hy (DNP-Hy), trinitrophenylated (TNP) Hy (TNP-Hy), DNP-mouse gamma globulin (DNP-M γ G), and TNP-OVA was described previously (12).

Animals. (CBA/N \times DBA/2)F₁ females and males (10–12 wk of age) were obtained from the Division of Research Services of the National Institutes of Health, Bethesda, Md. (CBA/N \times DBA/2)F₁ females received intraperitoneal injections of 0.2 ml that contained 100 μ g of Hy in complete Freund's adjuvant. 6–12 wk after carrier priming, these mice were irradiated (1,300 R from a cesium source) and used as recipients in adoptive transfers of age-matched adult F₁ male or F₁ female nonimmune spleen cells.

Cell Transfers and In Vitro Tolerance Induction. The modification of the in vitro splenic focus assay for tolerance susceptibility has been described previously (12, 13). Briefly, 4–6 \times 10⁶ viable spleen cells from (CBA/N \times DBA/2)F₁ females or males were injected intravenously into Hy-primed, irradiated, adult F₁ female recipients. Recipient fragment cultures were individually incubated with Dulbecco's modified Eagle's medium with either DNP-M γ G or TNP-OVA for 24 h, washed, and stimulated with DNP-Hy or TNP-Hy, respectively.

Radioimmunoassay. 25 μ l of culture fluids that were collected 10 or 13 d after stimulation were quantitatively assayed for anti-hapten antibody by a solid-phase radioimmunoassay (12). Bound mouse anti-hapten antibody was detected by the addition of ¹²⁵I-labeled affinity-purified rabbit or goat anti-mouse Fab, IgM, or IgG₁.

Results

Previous studies that compared the susceptibility of neonatal (immature) and adult (mature) splenic B cells to in vitro tolerance induction demonstrated that immature B cells could be rendered unresponsive by low concentrations of hapten on a wide array of carriers, whereas the vast majority of adult splenic B cells remained unaffected (12, 13). In Table I, the susceptibility to tolerance induction of splenic B cells from adult F₁ males was analyzed and compared with the response of adult F₁ female B cells. It is evident that the response of DNP-specific splenic B cells from F₁ females is not significantly reduced by preincubation of fragment cultures with 10⁻⁶ M DNP-M γ G. In contrast, preincubation of fragment cultures that contained adult F₁ male splenic B cells with DNP-M γ G markedly reduced the response to DNP-Hy in these two representative experiments. Although the percentage of B cells that were susceptible to tolerance induction varied between experiments, in both instances >50% of the DNP-specific B cells were rendered unresponsive to antigen. Similar results were

TABLE I
*Susceptibility of Adult (CBA/N × DBA/2)F₁ Female and Male Splenic B
 Cells to In Vitro Tolerance Induction*

Experiment	Donor spleen cells	Tolerogen (10 ⁻⁶ M DNP- MγG)	Number of clones per 10 ⁶ cells transferred*	Percentage of control re- sponse
1	F ₁ female	-	1.83	95.6
	F ₁ male	-	1.25	
2	F ₁ female	+	0.41	32.8
		-	2.13	100.0
	F ₁ male	+	2.13	
		-	0.63	

* 4-6 × 10⁶ donor spleen cells were transferred to each recipient mouse. Recipient fragments were incubated in the presence or absence of 10⁻⁶ M DNP-MγG for 24 h, washed, and stimulated with DNP-Hy at 10⁻⁶ M DNP. DNP-specific clones were detected by radioimmunoassay of culture fluids with ¹²⁵I-labeled anti-mouse Fab (12).

obtained when fragment cultures that contained F₁ male spleen cells were preincubated with TNP-OVA and subsequently stimulated with TNP-Hy (data not shown). These results suggest that a subset of the splenic B cells in the adult immune defective F₁ male is similar to developing B cells in the neonate as assessed by the criterion of tolerance susceptibility.

Analyses of immunoglobulin isotypes from stimulated fragment cultures that contained day 3 neonatal splenic B cells or day 5 neonatal bone marrow B cells have previously demonstrated that preincubation with DNP-MγG reduced both the IgM- and IgG₁-producing DNP-specific clones (12, 13). However, preincubation of adult bone marrow B cells with DNP-MγG reduced only the IgM-producing precursor B cells (13, 14). Table II presents the results of a similar analysis of isotype distribution for fragment cultures derived from both F₁ female and F₁ male adult spleen cell donors. It is apparent that both IgM only and IgG₁ responses in the F₁ males were reduced by preincubation with DNP-MγG. In contrast, preincubation of adult F₁ female splenic B cells with DNP-MγG did not significantly affect the isotype distribution of the anti-DNP antibody responses in these mice. The reduction of both IgM only and IgG₁ responses in the F₁ male suggest that the splenic B cells in these mice are similar to B cells in the neonate rather than adult bone marrow B cells.

Discussion

A variety of studies have suggested that the immune defect(s) observed in CBA/N mice or their immunologically defective F₁ male progeny may result from an arrest in the maturation of the B-lymphocyte pool (7, 10, 11). If this is so, then the B cells in these adult mice should exhibit functional properties that are characteristic of immature B cells. This study uses in vitro tolerance susceptibility as a functional marker for immature B cells to assess the maturity of adult (CBA/N × DBA/2)F₁ female and male splenic B cells. The results demonstrate that >50% of the DNP-specific B cells in the immunologically defective F₁ male are immature (neonatal-like) by this criterion.

TABLE II
Effect of In Vitro Tolerance Induction on the Isotype of Monoclonal Anti-DNP Antibody Responses in (CBA/N × DBA/2)F₁ Females and Males

Donor spleen cells	Tolerogen (10 ⁻⁶ M DNP- M γ G)	Number of clones per 10 ⁶ cells trans- ferred*	Heavy-chain class	
			IgM	IgG ₁
			Number of clones per 10 ⁶ cells transferred	Number of clones per 10 ⁶ cells transferred‡
F ₁ females	-	1.98 ± 0.21	0.83 ± 0.17	0.58 ± 0.08
	+	1.94 ± 0.26	0.74 ± 0.37	0.56 ± 0.18
F ₁ males	-	1.44 ± 0.26	0.56 ± 0.06	0.43 ± 0.06
	+	0.52 ± 0.15	0.04 ± 0.03	0.16 ± 0.08

* 4-6 × 10⁶ donor spleen cells were transferred to each recipient mouse. Recipient fragment cultures were incubated in the presence or absence of 10⁻⁶ M DNP-M γ G for 24 h, washed, and stimulated with DNP-Hy at 10⁻⁶ M DNP. DNP-specific clones were detected by radioimmunoassay of culture fluids with ¹²⁵I-labeled anti-mouse Fab, IgM, c: IgG₁ (12).

‡ The frequency of DNP-specific clones that produced both IgM and IgG₁ anti-DNP antibody in the absence or presence of tolerogen is: F₁ female, 0.39 and 0.31; F₁ male, 0.25 and 0.25, respectively.

The capacity to render >50% of adult F₁ male splenic B cells unresponsive to antigen suggests that these tolerizable B cells may be functionally similar to immature populations of B cells of normal mice. The characteristics of the B cells in CBA/N mice that are susceptible to tolerance induction can be defined further by analysis of the heavy-chain class of antibody produced after preincubation with tolerogen and subsequent stimulation. In 3-d spleens of immunologically normal mice, both IgM only and IgG₁ responses are markedly reduced by tolerance induction (12). The precursors that produce only IgM are by far the most susceptible. In contrast, previous studies demonstrated that only the IgM-producing B cells were tolerizable in the adult bone marrow (14). The results of this study that demonstrate the susceptibility of both IgM- and IgG₁-producing precursor B cells to in vitro tolerance susceptibility in the adult F₁ male strongly suggest that the tolerizable B cells more closely resemble neonatal B cells than adult bone marrow B cells. Thus, CBA/N mice have two subpopulations of splenic B cells, one of which is functionally similar to early neonatal splenic B cells and a second that is functionally mature, when defined by the characteristics of their tolerance susceptibility. Furthermore, like neonates, CBA/N mice appear to lack the subpopulation of nontolerizable, i.e., mature, IgM only producing B cells that are found in normal mice.

Consistent with these findings are those of other studies that have demonstrated that the surface membrane markers of F₁ male B cells are characteristic of immature B cells. For example, F₁ male spleen cells have a high density of sIgM and do not express Lyb3, Lyb5, or Mls determinants (7-9). It should be noted that none of the F₁ male B cells express these membrane markers, whereas only a subset of the adult splenic B-cell population is tolerizable. Thus, in addition to the splenic population of F₁ males that exhibits the functional characteristics of immature B cells found in the spleens of neonatal mice, there is a second population that lacks the expression of various cell surface markers associated with mature adult B cells but that is mature by the criterion of their resistance to tolerance induction.

The presence or absence of various cell surface components on CBA/N (or F₁ male) B cells may also aid in the elucidation of the role of these markers in the susceptibility

of immature B cells to tolerance induction. Lyb3 and Lyb5 have been defined by their lack of expression on adult CBA/N B cells (8, 9). Thus, it would appear that although Lyb3 may have a role as a triggering molecule in some experimental systems (9), there is no apparent correlation between the expression of Lyb3/Lyb5 and the lack of *in vitro* tolerance susceptibility. Nevertheless, these data do not rule out the possibility that the absence of Lyb3 or Lyb5 may permit immature B cells to be tolerized.

Other studies have demonstrated that the surface μ : δ ratio in the F₁ male is unusual (11). More recent studies have indicated that the majority of F₁ male splenic B cells express increased amounts of μ , and that although the number of sIgM⁺ sIgD⁻ cells is increased, the density of δ on sIgM⁺ sIgD⁺ cells is normal (I. Scher. Unpublished data.). These observations, combined with the data presented in this paper, suggest that one of the important parameters in tolerance susceptibility may be the increased density of surface μ . Alternatively, the B cells that are tolerized in F₁ male splenic populations may be those that bear little or no sIgD.

Klinman and Press (15) have previously suggested that the neonatal B-cell repertoire represents a distinct subpopulation of B cells. The findings presented herein are consistent with this hypothesis but, more importantly, suggest that the entire B-cell population of the adult immune defective CBA/N mouse may be composed of a neonatal-like B-cell pool. When viewed in this light, all of the previous studies are supportive (1-11). This interpretation of the data is also consistent with the findings that F₁ males have a similar number of DNP-specific B cells as F₁ females when calculated on a per-B-cell basis (E. S. Metcalf and S. K. Pierce. Analysis of T-dependent B-cell responses in mice which express an X-linked B-cell defect. Evidence for a normal response in the splenic focus system. Manuscript in preparation.). Thus, it is possible that the B cells in the CBA/N mouse may represent a neonatal-like B-cell population present within an adult mouse and thus may facilitate the isolation, complete characterization, and functional analysis of neonatal B cells.

This report has established that the majority of DNP-specific splenic B cells in the adult F₁ male are susceptible to tolerance induction, whereas the adult F₁ female B cells are not susceptible. Furthermore, it would appear that the tolerizable B cells in the F₁ male resemble neonatal B cells more closely than adult bone marrow B cells. These findings are consistent with the hypothesis that the lymphoid population in the adult CBA/N mouse is characteristic of a neonatal B-cell population. Regardless, the susceptibility of the F₁ male B cells to tolerance induction defines two B-cell subsets, one of which is tolerizable. Thus, it may be possible to isolate these subpopulations and to formally correlate the expression of cell surface markers with the developmental status of any given lymphoid population and its susceptibility to tolerance induction.

Summary

Previous studies from this laboratory have indicated that the susceptibility to *in vitro* tolerance induction is restricted to B cells early in their development (12, 14). In this study, a modification of the *in vitro* splenic focus technique was used to determine whether 2,4-dinitrophenyl (DNP)-specific splenic B cells from adult (CBA/N × DBA/2)F₁ males are susceptible to *in vitro* tolerance induction. The results demonstrate that >50% of the DNP-specific B cells in the adult F₁ male are tolerizable and therefore immature by this criterion. Moreover, the findings define at least two

subpopulations in adult CBA/N mice, one of which is tolerizable. These findings are consistent with the hypothesis that the lymphoid population in the adult CBA/N mouse is characteristic of a neonatal B-cell population.

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