The Journal of Immunology
RESEARCH ARTICLE | APRIL 01 1980

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J Immunol (1980) 124 (4): 1983–1989.
https://doi.org/10.4049/jimmunol.124.4.1983

0022-1767/80/1244-1983$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 124, No. 4, April 1980
Printed in U.S.A.

IDIOTYPE-SPECIFIC TOLERANCE: PREFERENTIAL ALTERATION OF IDIOTYPE EXPRESSION AFTER NEONATAL EXPOSURE TO TOLEROGEN[1]

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The development of HOPC-8 idiotype-positive and HOPC-8 idiotype-negative B cell subpopulations involved in the immune response to a single PC antigen, has been monitored during normal differentiation and in situations where neonatal tolerance had been induced. It was found that H8 id$^+$ and H8 id$^-$ anti-PC antibody-secreting cells appeared at approximately the same time in ontogeny and these late maturing subsets constituted equal fractions of the immune response in CBA/CaJ × BALB/c F_1 (JBF_1) mice. However, when JBF_1 mice were injected at birth with graded doses of PC-FGG, responses obtained in 4-week-old mice were predominantly of the H8 id. This PC-specific, idiotype-restricted, unresponsive state was stable upon adoptive transfer. Specific depletion of Thy-1.2-positive cells and *in vitro* "mixing" experiments involving combined ratios of tolerant and nontolerant spleen cells suggest that the unresponsive state measured 4 weeks after tolerogen administration does not involve suppressor cells. It would appear that intrinsic differences between these idiotypically defined B cell subsets account for the observed differential susceptibility to tolerance induction among PC-responsive B cells.

Examination of tolerance characteristics in B cell subpopulations has provided useful information concerning the normal course of B lymphocyte differentiation (1–7) and has contributed to our understanding of the developmental heterogeneity of B cell subpopulations. For instance, it has been documented that immature B cells undergo clonal deletion/abortion due to their hypersusceptibility to tolerogens (1–3, 5, 6). Our own studies (7) demonstrate that the previously recognized neonatal susceptibility to tolerogenesis in responses to thymus-dependent (TD)[3] antigens (1, 3, 5) extends to some, but not all, thymus-independent (T1) antigen-induced responses. Specifically, our results indicate that the response to TNP-LPS is easily tolerizable in spleen cell cultures of neonatal mice (4 days of age), whereas the response in B cells from the same neonatal mice to TNP-*Brucella* is not readily abolished. These findings correlate with the studies on tolerance characteristics in CBA/N mice that have an X-linked defect of B lymphocyte differentiation (8–11). We have demonstrated that adult CBA/N mice display tolerance hypersusceptibility in B cells responding to TNP-LPS, but not in TNP-*Brucella* stimulated B cells (7). Therefore tolerance hypersusceptibility is characteristic of a fraction of neonatal and adult CBA/N antigen-reactive B lymphocytes. To study further the basis of differential tolerance susceptibility among B cells, we have induced neonatal tolerance to the hapten phosphorylcholine (PC) and studied its effects on idiotype expression in murine anti-PC PFC responses. Thus, in contrast to our previous work in which different antigens were used to evaluate the extent of tolerance susceptibility among B cells responding to a given antigen, we have selected for the present studies an idiotypically defined system in which the same antigen could be utilized to stimulate different B cell subpopulations.

Results presented here reveal that within the immune response to a single antigenic species, PC-specific B cell subsets can be shown to possess different tolerance susceptibilities in spite of remarkable similarities in ontogenetic development and avidity of secreted antibody. The differential or selective unresponsiveness of HOPC-8 idiotype-negative B cell clones could not be attributed to suppressor cell function and would appear to be determined by intrinsic differences at the B cell level, which could be important in the regulation of idiotype expression.

MATERIALS AND METHODS

Animals. (CBA/CaJ × BALB/c) F_1 (JBF_1) mice, from our breeding colony, were used at different ages (ranging from birth to 10 weeks of age) as indicated in the text. CBA/CaJ (Jackson Laboratories, Bar Harbor, Maine) and BALB/c mice (Cumberland View Farms, Clinton, Tenn.), 8 to 12 weeks of age, were used in selected experiments. (CBA/N × BALB/c) F_1 (NBF_1) male mice were used as recipients in adoptive transfer experiments as previously described (12). CBA/N mice were obtained from our colony, which originated from pairs kindly supplied by Dr. C. Hansen, NIH, Bethesda, Md.

Received for publication November 14, 1979.
Accepted for publication January 14, 1980.
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[1] This work was supported by National Institutes of Health Grant 1-R01-AI-14530-03, a Basil O'Connor Research Starter Grant from the National Foundation March of Dimes, and a Research Career Development Award 1-K04-AI-00268-03.
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[3] Abbreviations used in this paper: TD, thymus-dependent; TI, thymus independent; PC, phosphorylcholine; TNP, 2,4,6-trinitrophenyl; JBF_1, (CBA/CaJ × BALB/c) F_1; NBF_1, (CBA/N × BALB/c) F_1; PnCs, C-polysaccharide extract of a *Streptococcus pneumoniae* mutant; H8 id, idiotype specific determinants of the HOPC-8/TEPC-15 plasmacytoma; R, rads; FGG, fowl γ-globulin.

Antigens. The phosphorylcholine (PC)-containing bacterial vaccine *Streptococcus pneumoniae* strain R36a, and the soluble PC-containing C-polysaccharide extract of the Cs-encapsulated *Streptococcus pneumoniae* mutant strain (PnCs),[4] were used to evoke anti-PC PFC responses by injecting i.v. 10^8 R36a bacteria or by adding 20 μl of a $1:10^4$ dilution to spleen cell cultures while optimal responses to PnCs were obtained following i.v. immunization with 0.25 μg of soluble C-polysaccharide.

2,4,6-Trinitrophenyl-conjugated *N*-(2-aminoethyl)carbamylmethyl Ficoll (TNP_{50}AECM-Ficoll) and TNP_{61}-AECM-Dextran were prepared according to the method of Inman (13) and used at the dose of 10 μg i.v. per mouse (12). TNP-conjugated heat killed *Brucella abortus* (5) (TNP-*Brucella*) was a kind gift from Dr. J. Cambier, Duke University, Durham, N. C. It was utilized as an *in vitro* specificity control at a $1:10^5$ dilution (14). The antigens listed above have been shown to be capable of stimulating PFC responses in the absence of T cells (12, 14, 15).

Anti-idiotypic antiserum. The preparation and specificity of A/HeJ antiserum directed against the HOPC-8 idiotype determinant(s) has been reported previously (16). Briefly, A/HeJ mice received multiple injections of the purified HOPC-8 myeloma protein (17), and the resultant antiserum was adsorbed on a column containing normal mouse sera conjugated to Sepharose 4B (18). Normal mouse sera were previously adsorbed on a PC-Sepharose 4B column (19) to remove all anti-PC antibodies.

Irradiation and cell transfer. NBF_1 male adult mice received 300 rads (R) total body irradiation from a ^{137}Cs source at a rate of 200 R min^{-1}. The parameters of this adoptive transfer system have been detailed previously (12). Reconstitution experiments reported here utilized inocula of 2×10^7 cells per recipient.

Plaque-forming cell assay and plaque inhibition. PC-conjugated sheep red blood cells (PC-SRBC) and TNP-conjugated SRBC (TNP-SRBC) were used as indicator cells in the Cunningham-Szenb. g modification (20) of the hemolytic plaque technique (21). PC-SRBC were prepared by coupling the C-polysaccharide to SRBC with chromium chloride (22) as previously described (15); TNP-SRBC were prepared according to Rittenberg and Pratt (23).

Avidity of secreted antibody was measured by inhibiting plaque formation with free hapten. Phosphorylcholine chloride (Sigma, St. Louis, MO.) was incorporated into the plaquing mixture at various molar concentrations, and the number of PFC in triplicate chambers was averaged and expressed as percentage PFC inhibited.

Inhibitions of PFC with anti-H8 id serum were carried out in a similar manner with the antiserum described above. A 1:250 dilution of anti-H8 id serum was optimal for inhibition of H8 id^+ PFC.

Tissue culture. Miniature Mishell-Dutton lymphocyte cultures (24) were set up in 0.2 ml total volume in COSTAR 3596 plates. Lymphoid cells were cultured at a concentration of 10^6 cells per culture, unless noted otherwise, in RPMI 1640 (GIBCO, Grand Island, N. Y.) supplemented with fresh glutamine, 5×10^{-5} M 2-mercaptoethanol, penicillin and streptomycin (50 units/ml each), and 10% fetal calf serum. All cultures were plated in triplicate and maintained at 37°C in an atmosphere of 5% CO_2, 7% O_2, 88% N_2, and 95% humidity until the time of assay.

In vivo tolerance induction. Neonatal (less than 24-hr old) JBF_1 mice were rendered tolerant by i.p. injection of specified doses of PC-conjugated fowl γ-globulin (FGG, Miles Biochemicals, Elkhart, Ind.). Preparation of PC-conjugated proteins has been previously described (15, 19). PC-FGG used in this study contained an average of 15 haptenic groups per FGG molecule. Normal, or nontolerant, mice were littermates that received no injection at birth, or received 500 μg FGG, since both groups of control mice made identical anti-PC and anti-TNP PFC responses.

Statistics. Data were analyzed by application of the Student *t*-test whenever required.

[4] Prepared according to H. C. Anderson and M. McCarty. J. Exp. Med. 93, 25, 1951.

RESULTS

Characterization of JBF_1 anti-PC responses. To compare the developmental characteristics of HOPC-8 idiotype-positive (H8 id^+) PFC with HOPC-8 idiotype-negative (H8-id^-) anti-PC PFC, it seemed desirable to find a situation in which these idiotypically defined subsets of PC-responsive B cells existed within the same animal in order that: 1) expression of both subsets occurred without experimental manipulation of the host environment and 2) the subsets be represented as a sizeable fraction of the total anti-PC response.

As shown in Table I, the requirements listed above were satisfied in (CBA/CaJ × BALB/c) F_1 (JBF_1). Thus, in JBF_1 mice two distinct B cell subpopulations responsive to the same antigen could be identified since approximately 50% of the anti-PC PFC response was characterized by antibodies bearing the H8 id-specific determinant(s), whereas the other 50% of the anti-PC PFC response was found to be lacking the H8 id-specific determinant(s).

Of considerable importance to the interpretation of findings to be presented later was the observation that these two subsets (H8 id^+ and H8 id^-) of PC-responsive B cells apparently produced antibodies of similar avidity. PFC inhibition experiments, designed to measure the relative avidity of H8 id^+ and H8 id^- anti-PC antibodies (25), were performed by comparing predominantly H8 id^+ BALB/c, H8 id^- CBA/CaJ, and "mixed" H8 id^+ and H8 id^- JBF_1 anti-PC responses. Figure 1 shows that the avidities of these different anti-PC antibodies did not differ greatly since the concentration of free PC hapten needed to achieve 50% inhibition of PFC was not significantly different from one response to another.

An additional parameter of the anti-PC response we wished to characterize in JFB_1 mice was the timing of the acquisition of immunocompetence for H8 id^+ and H8 id^- anti-PC PFC. The ontogeny of anti-PC responses was examined by marking the

TABLE I

Inhibition of anti-PC PFC by anti-H8 id serum[a]

Mouse Strain	Anti-PC PFC/Spleen	Anti-PC PFC/Spleen not Inhibited by Anti-H8 Id Serum	% Inhibition
BALB/c	392,900 (5.59 ± 0.09)	3,140 (3.50 ± 0.07)	99
CBA/CaJ	121,620 (5.09 ± 0.08)	109,020 (5.04 ± 0.05)	10
JBF_1	194,480 (5.29 ± 0.17)	91,200 (4.96 ± 0.17)	53

[a] Anti-PC PFC were determined in individual mice 5 days after injection of *Streptococcus pneumoniae* strain R36a. Clonotypic analysis was performed by incorporating a 1:250 dilution of anti-H8 id serum into the plaquing mixture as described in *Materials and Methods*. Data represent the antilog of the geometric means and the S.E.M. for groups of 5 to 10 mice.

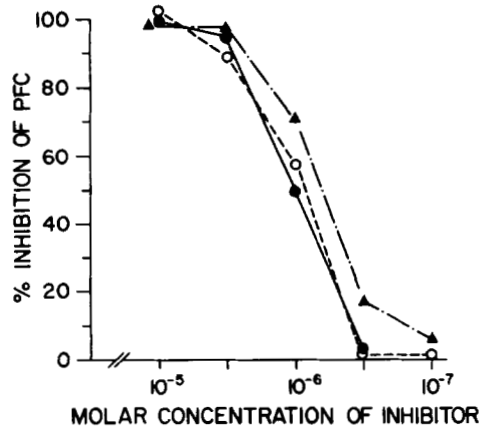

Figure 1. Hapten inhibition of PFC responses. Splenic anti-PC PFC responses in BALB/c (●——●); CBA/CaJ (O-----O); and CBA/CaJ × BALB/c (JBF_1) mice (▲–·–▲) immunized 5 days previously with *Streptococcus pneumoniae* strain R36a were inhibited with half-log dilutions of free PC hapten. Spleen cells from five individual mice were pooled in each determination. Data represent the mean of triplicate PFC counts.

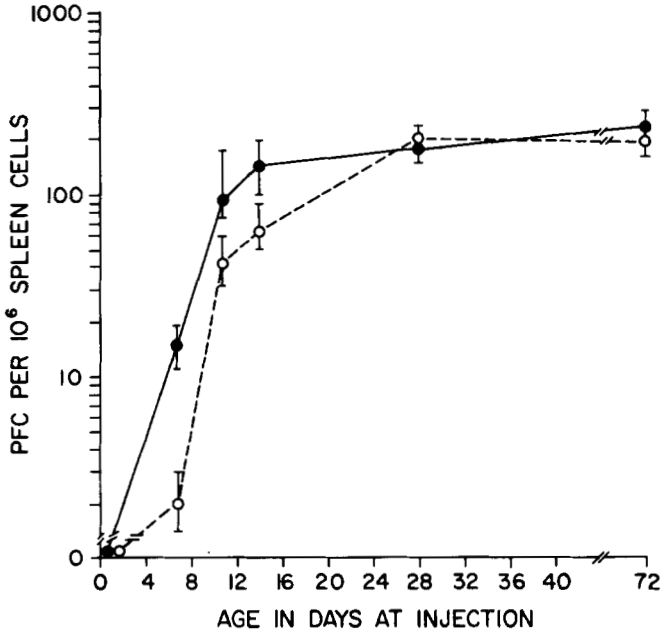

Figure 2. Onset of responsiveness to *Streptococcus pneumoniae* strain R36a in JBF_1 mice. JBF_1 mice (5 to 10 per group) were injected with an immunogenic dose (see *Materials and Methods*) of R36a at various ages and assayed 5 days later. Anti-PC PFC inhibited with anti-H8 id serum ($H8id^+$) (●——●), and anti-PC PFC not inhibited with anti-H8 id serum ($H8id^-$) (O-----O), were enumerated in individual mice as described in *Materials and Methods*. Data represent the antilog of the geometric means and the S.E.M.

onset of responsiveness of H8 id^+ and H8 id^- anti-PC PFC after *in vivo* immunization of neonatal JBF_1 mice of different ages with the thymus-independent antigen *Streptococcus pneumoniae* strain R36a.

It can be seen in Figure 2 that H8 id^+ and H8 id^- PFC became detectable at approximately the same time in development. In the first 2 weeks of life, the contribution of the H8 id^- PFC to the total anti-PC response seems to be relatively smaller than at later stages. However, using the Student *t*-test, we failed to find any significance to these differences between the magnitude of H8 id^+ and H8 id^- anti-PC responses ($0.4 > p > 0.2$), and therefore we are led to conclude that these two subsets acquire immunocompetence at essentially the same period in ontogeny as originally documented by Sigal *et al.* (26). In addition, our results show that JBF_1 splenic B cell responses to PC antigens were acquired relatively late in ontogeny, as were responses obtained in adoptive transfer experiments that analyzed the reconstitution of responsiveness in lethally irradiated syngeneic adult hosts grafted with neonatal (less than 24 hr old) JBF_1 liver cells (J. P. McKearn, unpublished results).

Therefore, responses to R36a in JBF_1 mice resembled the anti-PC immune response of other murine strains (26–29) since: 1) immunocompetence was acquired late in ontogeny, 2) H8 id^+ PFC appeared at the same time in ontogeny as anti-PC PFC that secreted antibodies lacking the H8 id determinant(s), and 3) H8 id^+ and H8 id^- anti-PC PFC secreted antibodies of similar avidity.

Tolerance profiles of PC-responsive B cells. The effects of exposure to tolerogen were assessed in neonatal JBF_1 mice. Specifically, newborn JBF_1 mice (less than 24 hr old) were injected with graded doses of PC_{15}-FGG and assayed for their ability to mount PFC responses at 4 weeks of age after *in vivo* immunization with R36a. When immune responsiveness was examined in these mice, substantial alterations in the clonal profile of PC-responsive B cells were observed.

Four-week-old JBF_1 mice that received no tolerogen produced 23,940 ± 4450 H8 id^+ PFC and 25,070 ± 3450 H8 id^- anti-PC PFC when immunized 5 days earlier with R36a (Fig. 3). However, JBF_1 mice of the same age exposed to a single injection of 500 μg PC_{15}-FGG at birth generated much lower anti-PC responses. The number of H8 id^+ PFC was reduced to 10,970 ± 1800 whereas H8 id^- anti-PC PFC were reduced to 1900 ± 440 PFC. This decrease in responsiveness amounted to a 54% reduction in the H8 id^+ response ($0.025 > p > 0.010$) and a 92% reduction in the H8 id^- anti-PC response ($p < 0.001$) when compared to the responses of the control group. Larger differences in the relative tolerance susceptibility of H8 id^+ and H8 id^- anti-PC PFC were noted in JBF_1 mice exposed to 5 μg PC_{15}-FGG at birth. R36a immunization of these mice resulted

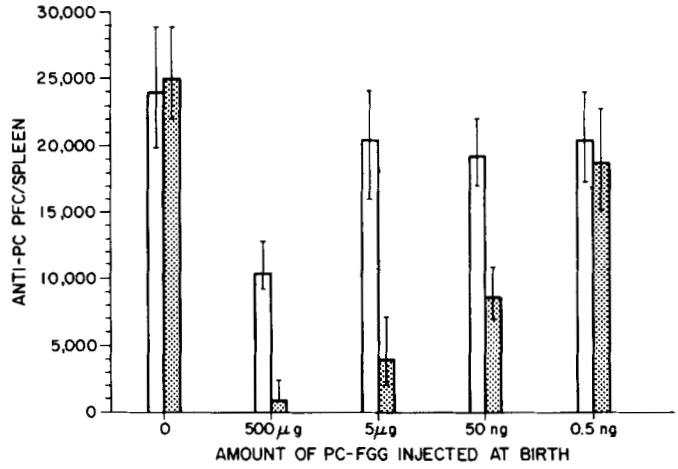

Figure 3. Tolerance in R36a-responsive B cells from JBF_1 mice. JBF_1 mice, injected as neonates with 0, 500 μg, 5 μg, 50 ng, or 0.5 ng PC_{15}-FGG, were immunized at 4 weeks of age with R36a and assayed for anti-PC PFC 5 days later. Each group contained 6 to 12 mice which were assayed individually to determine the number of anti-PC PFC inhibited with anti-H8 id serum (H8 id^+ PFC, *open bars*), and anti-PC PFC not inhibited with anti-H8 id serum (H8 id^- PFC, *shaded bars*) as described in *Materials and Methods*. Data represent the antilog of the geometric mean and the S.E.M

in 20,950 ± 5250 H8 id$^+$ and only 4210 ± 2400 H8 id$^-$ anti-PC PFC (a 12 and an 83% reduction, respectively). This pattern of differential tolerance susceptibility was also found in the group of JBF_1 exposed to 50 ng PC_{15}-FGG neonatally (*t*-test values for H8 id$^+$ $0.4 > p > 0.2$; H8 id$^-$ $p < 0.001$) and finally subsided in mice injected at birth with 0.5 ng PC_{15}-FGG. This last group made anti-PC responses that were not significantly different from uninjected control mice ($0.4 > p > 0.2$ for both H8 id$^+$ and H8 id$^-$). Tolerance hypersusceptibility within the H8 id$^-$ B cell population is not restricted to JBF_1 mice since BALB/c mice, neonatally suppressed with anti-H8 id serum, express H8 id$^-$ B cells that are hypersusceptible to PC-specific tolerance induction (unpublished results).

To establish the specificity of the tolerant state induced by neonatal exposure to PC_{15}-FGG, responses to an antigen bearing an unrelated hapten specificity were studied. As before, JBF_1 mice that received no tolerogen, 500 μg, or 5 μg at birth were immunized *in vivo* 4 weeks later. In these experiments, responses were measured after immunization with a TNP antigen, TNP-AECM-Ficoll, and the PC-containing polysaccharide extract of a *Streptococcus pneumoniae* mutant (PnCs) (12). It is apparent from data presented in Table II that the magnitude of anti-TNP PFC responses was unaffected in PC-unresponsive mice and that anti-PC responses in PnCs-immunized mice were similar to those obtained in R36a-immunized mice. Namely, the H8 id$^-$ fraction of the response to a PC antigen was highly sensitive to tolerance induction as compared to the H8 id$^+$ fraction. Therefore, the differential tolerance susceptibility among H8 id-positive and -negative PC-responsive B cells was not peculiar to R36a-induced PFC responses, and the unresponsive state induced by PC_{15}-FGG did not affect the immune response to an unrelated hapten specificity.

Assessment of the tolerant state in 4-week-old neonatally tolerized JBF_1 mice. Although the experimental results described above demonstrated unequal tolerance sensitivities among H8 id$^+$ and H8 id$^-$ anti-PC PFC elicited by thymus-independent antigens, it is not clear whether this disparity was a reflection of fundamental differences at the B cell level or the selective operation of tolerogen-induced suppressor cells. We performed two types of experiments to distinguish between these two possibilities.

The first method made use of the adoptive transfer of T cell-depleted spleen cells from tolerant and nontolerant JBF_1 mice into sublethally irradiated (CBA/N × BALB/c) F_1 (NBF_1) male recipients. As previously demonstrated (12), NBF_1 male mice represent a unique transplantation model due to a genetically linked defect that precludes B cell responsiveness to certain antigens (e.g., PC antigens and TNP-AECM-dextran). However, these mice possess normal PC-specific helper T cells (30), are fully capable of supporting B cell maturation when grafted with normal B lymphocytes (12, 31, 32), and provide adequate T cell help for both H8 id$^+$ and H8 id$^-$ PC-specific B cells (12). 2×10^7 T cell-depleted spleen cells from 4-week-old JBF_1, exposed to 500 μg PC_{15}-FGG as newborns or normal JBF_1 mice, were transplanted to 300 R irradiated NBF_1 male recipients that were immunized with R36a and TNP-AECM-dextran. The ability of the transplanted cells from normal and tolerant mice to respond to PC and TNP was evaluated 5 days later. Results shown in Table III illustrate that despite T cell removal, B cells from mice injected at birth with PC_{15}-FGG remained largely unresponsive to PC, whereas responsiveness to an unrelated hapten, TNP, was not different from responses in control mice.

The second method used to evaluate the role of suppressor cells in maintenance of PC_{15}-FGG-induced tolerance in JBF_1 neonatal mice involved *in vitro* mixing experiments in which whole spleen cell populations from tolerant and nontolerant mice were combined at different ratios to study the effect on immune responsiveness. The rationale behind these experiments relied on the observation that "infectious" tolerance, or

TABLE II

Specificity of PC-FGG induced tolerance[a]

Treatment	No. of Animals	Antigens	Anti-PC PFC/Spleen		Anti-TNP PFC/Spleen
			H8 id$^+$ PFC	H8 id$^-$ PFC	
None	5	PnCs + TNP-Ficoll	155,810 (5.19 ± 0.10)	139,320 (5.14 ± 0.10)	271,640 (5.43 ± 0.05)
500 μg PC-FGG	3	PnCs + TNP-Ficoll	43,680 (4.64 ± 0.21)	25,510 (4.41 ± 0.30)	363,080 (5.56 ± 0.20)
5 μg PC-FGG	5	PnCs + TNP-Ficoll	144,430 (5.16 ± 0.10)	26,200 (4.42 ± 0.15)	243,220 (5.39 ± 0.09)

[a] Anti-PC and anti-TNP PFC responses were evaluated in normal and tolerant 4-week-old JBF_1 mice 5 days after immunization with PnCs and TNP-AECM-Ficoll. These mice received 0-, 500-, or 5-μg PC_{15}-FGG at birth. Anti-PC and anti-TNP PFC assays were performed as described in *Materials and Methods*. Results are expressed as geometric means with the log of the mean and S.E. given in parentheses.

TABLE III

Inability of anti-Thy 1.2 plus C-treated spleen cells from tolerant mice to restore PC responsiveness upon transfer to adoptive hosts[a]

Treatment of Donor	Antigens	Anti-PC PFC/Spleen		Anti-TNP PFC/Spleen
		H8 id$^+$ PFC	H8 id$^-$ PFC	
None	R36a + TNP-Dextran	5,070 (3.71 ± 0.06)	5,290 (3.72 ± 0.07)	14,400 (4.16 ± 0.04)
500 μg PC-FGG	R36a + TNP-Dextran	1,500 (3.18 ± 0.11)	510 (2.71 ± 0.35)	15,190 (4.20 ± 0.14)

[a] Two × 10^7 anti-Thy 1.2 plus guinea pig C-treated spleen cells from 4-week-old, normal or neonatally tolerized, JBF_1 mice were injected i.v. into 300 R irradiated NBF_1 male mice as described previously (12). Groups of six recipient mice were immunized with R36a and TNP-AECM-Dextran, and 5 days later anti-PC and anti-TNP PFC were enumerated and expressed as described in Table II.

TABLE IV

Assessment of the ability of tolerant spleen cells to induce tolerance among normal spleen cells in vitro[a]

Spleen Cells/Culture	PFC/Culture			
	Expt. No. 1		Expt. No. 2	
	Anti-PC PFC	Anti-TNP PFC	Anti-PC PFC	Anti-TNP PFC
10^6 nontolerant	129	585	90	449
10^6 tolerant	22	590	3	492
5×10^5 nontolerant +	[76]	[588]	[47]	[471]
5×10^5 tolerant (1:1)	73	629	42	553
5×10^5 nontolerant +	[87]	[883]	[48]	[717]
10^6 tolerant (1:2)	97	1193	57	745
5×10^5 nontolerant +			[50]	[963]
1.5×10^6 tolerant (1:3)	ND[b]	ND	55	666

[a] Spleen cells from 4-week-old normal or neonatally tolerized (injected i.p. with 500 μg PC_{15}-FGG), JBF_1 mice were cultured separately or in combination at specified ratios and stimulated with R36a or TNP-*Brucella abortus* (14). Anti-PC and anti-TNP PFC responses were measured 4 days later. The values enclosed in boxes represent numbers of PFC predicted if tolerant cells could not induce tolerance among normal spleen cells (i.e., non-"infectious" tolerance). Values not enclosed by boxes represent the observed PFC response per culture. All cultures were performed in triplicate and the data represent the mean PFC determination on PC-SRC or TNP-SRC minus background PFC responses. Background responses were measured in cultures which received no antigen.

[b] ND, not determined.

the ability of tolerant spleen cells to induce unresponsiveness among a previously nontolerant population was indicative of suppressor cell involvement in the maintenance of a tolerant state (33–35). *In vitro* mixing experiments, presented in Table IV, demonstrated that: 1) tolerance induced in JBF_1 neonates was stable upon *in vitro* immunization and was specific for PC, and 2) mixing tolerant and nontolerant spleen cells at 1:1, 2:1, or 3:1 ratios resulted in PFC responses nearly identical to those that would be predicted if tolerance were not "infectious."

These experiments strongly favor the notion that neither suppressor cells nor tolerance among accessory cells were responsible for the differential tolerance induced by PC_{15}-FGG in thymus-independent antigen-responsive B cells from JBF_1 mice. We conclude that intrinsic differences at the B cell level were responsible for the observed pattern of differential tolerance susceptibility among H8 id^+ and H8 id^- PC-specific B cells.

DISCUSSION

Previous studies dealing with immunologic tolerance have explored a variety of parameters to define critical aspects of tolerance induction and their subsequent influence on immune responses. Results from *in vivo* and *in vitro* assays have emphasized the importance of the molecular properties of tolerogens (36, 37), the antigen used to trigger cells suspected to be tolerant (5, 7, 37, 38), the time-course of tolerance induction in particular cell types (1, 6), the structural arrangement of the immune system (39), and the donor origin of lymphoid cells (40, 41). Studies on B cell tolerance have indicated that the presence or absence of helper T cells (1), as well as the developmental stage (1–3, 5, 6), greatly influence the tolerance susceptibility of B cells. Our previous work (7, 14) has focused on the differential behavior of thymus-independent B cell subpopulations. In these studies, hapten-specific, carrier-determined antigen responsiveness and tolerance characteristics were analyzed. In contrast to our earlier studies, experiments reported herein have dealt with tolerance in the response to a single antigen capable of stimulating separate, idiotypically defined B cell subsets. This system enabled us to focus our studies on inherent properties of different B cell subpopulations.

Evaluation of the tolerant state within defined PC-responsive B cell subsets has revealed differences in the functional behavior of H8 id^+ and H8 id^- B cells. JBF_1 mice, injected at birth with graded doses of PC_{15}-FGG and subsequently challenged with thymus-independent PC antigens 4 weeks later, produced PFC responses predominantly of the H8 id whereas control mice produced sizeable H8 id^+ and H8 id^- anti-PC PFC responses. The marked reduction in responsiveness among neonatally tolerized JBF_1 mice was specific for PC-reactive B cells since normal responses to TNP antigens were generated. B cells remained tolerant upon transfer to adoptive hosts or to an *in vitro* environment. Since anti-Thy 1.2 plus C-treated spleen cells from tolerized mice remained unresponsive in adoptive hosts and *in vitro* mixing experiments employing different ratios of tolerant and nontolerant spleen cells failed to demonstrate T cell influence on responsiveness, it seems unlikely that suppressor cells were responsible for the maintenance of tolerance.

It should be noted that experiments detailed in this report were aimed at defining the status of PC-specific B cells in neonatally tolerized mice at a point where tolerance was likely to be stable, 4 weeks of age, so that we could scrutinize directly the functional deletion of PC-specific B cells. *In vitro* mixing experiments and the ineffectiveness of anti-Thy 1.2 plus C treatment in abrogating unresponsiveness support the conclusion that a central effect of tolerogen on PC-responsive B cells, rather than the induction of suppressor cells, maintained tolerance at this point in development. Similarly, cell transfer experiments in progress, which analyze responsiveness of spleen and bone marrow cells from adult BALB/c mice neonatally suppressed with anti-H8 id serum, clearly demonstrate that in this model, long-term unresponsiveness to PC is also the result of clonal deletion of antigen-reactive B cells. Thus, it is likely that the functional deletion of specific B cell clones establishes tolerance without the additional influence of suppressor cells. The expression of suppressor cell activity is probably related to recovery of the B cell compartment from tolerance. The recent findings of Waters *et al.* (42) demonstrate the appearance of suppressor cells that emerge at, or near, the time when recovery from clonal deletion B cell tolerance begins. It will be of interest to determine whether the thymus-independent PC-responsive B cells studied in this paper are eventually subject to regulatory mechanisms similar to those documented by others (34, 42).

Although it is generally accepted that cells capable of secreting high avidity antibodies are preferentially tolerized when compared with B cell clones secreting lower avidity antibodies (43–45), avidity differences do not seem to influence tolerance susceptibility within the idiotypically defined B cell subsets studied here and the B cell subpopulations triggered by different TI TNP antigens (7, 14). Tolerance experiments examining PC-specific B lymphocytes (this report) as well as neonatal and adult B lymphocytes responsive to TNP-LPS (7, 14) demonstrated that B cells that secrete antibodies with identical avidity profiles differed greatly in their susceptibility to tolerance induction. Although our determinations of avidity were measured in antibody-secreting cells and do not necessarily reflect the

avidity of receptors at the cell surface, our inability to correlate avidity with tolerance susceptibility suggests that in addition to antibody avidity, other factors must account for tolerance characteristics of different B cell subpopulations. The use of anti-idiotypic antibody to delineate B cell subsets in tolerance studies should enable one to isolate the populations involved and define the role of various cell surface structures in mediating tolerance signals. Thus, it should be possible to elucidate the molecular basis for alterations of clonotype expression by tolerogens. Relative densities of cell surface IgM:IgD (46), the differential role of IgD (47–49), the involvement of Fc receptors (50, 51), and the total density of antigen-binding Ig receptors (46) could be important elements in determining the fate of these B cell subsets after interactions with tolerogen.

An important question that might be raised concerns the pertinence of data presented here to the problem of idiotypic clonal dominance (27, 52–55). It has been established that in certain cases (e.g., BALB/c mice), greater than 95% of the anti-PC antibodies are of the H8 id, although these mice have the potential to express other idiotypes (12, 17, 55). One may therefore ask whether hypersusceptibility to tolerance induction in the H8 id^- clones contributes to the dominance of the H8 id in anti-PC responses in some strains of mice (26–29). Although our experiments have not addressed this question directly, it would seem unlikely that tolerance sensitivity differences at the B cell level can be held solely accountable for the establishment of clonal dominance for certain idiotypes since other idiotype-specific regulatory mechanisms have been shown to influence clonotype expression (56–58).

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