# ONTOGENY OF B-LYMPHOCYTE FUNCTION

## III. In Vivo and In Vitro Studies on the Ease of Tolerance Induction in B Lymphocytes from Fetal, Neonatal, and Adult Mice*

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It has generally been felt that tolerance can be induced more readily in neonatal than in adult animals. While this is clearly true in allograft tolerance induction (1), it has been less obvious with other antigens. Thus, Siskind et al. (2) and Howard and Hale (3) found no difference between neonatal and adult mice in regard to tolerance induction with polysaccharide antigens and Dresser (4) found that the dose of aggregate-free bovine gamma globulin (BGG)[1] required to induce tolerance in mice was the same in neonatal and adult animals. It has been unclear as to whether immature lymphocytes are or are not more sensitive to tolerance induction than are mature lymphoid cells. Recently, in vitro studies (5–7) have shown that tolerance could be induced in vitro in B lymphocytes from immature donors at antigen concentrations far lower than that required for tolerance induction in mature B lymphocytes.

In the studies reported here a cell transfer system was employed so that the susceptibility of B cells to tolerance induction could be assayed in the absence of T cells and in a constant, adult, in vivo environment. It was found that with two hapten-carrier conjugates neonatal cells were indeed more susceptible to tolerance induction, both in vivo and in vitro, than were adult B cells. However, with BGG as antigen no difference was detected between B cells from 17-day fetal mice, neonatal mice, and adult mice with regard to the ease of tolerance induction either in vivo or in vitro. The results thus suggest that the relative ease of tolerance induction in immature B cells may be limited to moderately polyvalent antigens such as hapten-carrier conjugates.

## Materials and Methods

*Antigens and Haptens.* BGG (Pentex Fr II) was obtained from Miles Laboratories Inc. (Elkhart, Ind.). The random copolymer of D-glutamic acid and D-lysine (D-GL; Miles-Yeda Ltd.,

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[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; D-GL, copolymer of D-glutamic acid and D-lysine; DNFB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; EACA, ε-amino-n-caproic acid; HBSS, Hanks' balanced salt solution; PFC, plaque-forming cell; SRBC, sheep erythrocytes; $TD_{50\%}$, dose of antigen for 50% depression of immune response.

Elkhart, Ind.) had a molar ratio of 60:40 and a mol wt of 34,300. Dinitrophenylated D-GL was prepared as described by Katz et al. (8) and had six hapten groups per molecule. The 2,4-dinitrophenyl (DNP) derivative of BGG was prepared by the reaction of 1-fluoro-2,4-dinitrobenzene (DNFB; Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) with BGG under alkaline conditions. The procedures for preparations and characterization were described previously (9). $DNP_{22}$-BGG was used for tolerance induction and the $DNP_{44}$-BGG was used for immunization. DNP-ε-amino-n-caproic acid (DNP-EACA) was prepared by the reaction of DNFB with EACA (Sigma Chemical Co., St. Louis, Mo.) as described previously (9).

*Animals and Immunization.* $LAF_1$ mice (The Jackson Laboratories, Bar Harbor, Maine) were used. Age was designated by taking the day of birth as day 0 and the day of vaginal plug detection as day 0 of fetal life. Neonatal liver was obtained within 12 h of birth.

Mice were immunized by the intraperitoneal injection of 100 μg BGG emulsified in complete Freund's adjuvant (CFA; containing 1.5 mg/ml *Mycobacteria butyricum*) so as to be in a final vol of 0.2 ml. The animals were boosted with a saline solution of BGG (500 μg) intraperitoneally 3 wk later and were sacrificed for plaque-forming cell (PFC) assay 13 days after boosting. Mice were immunized with $DNP_{44}$-BGG by a single intraperitoneal injection of 500 μg in CFA. Splenic anti-DNP PFC were assayed 13 days later.

*Cell Transfers.* Studies were carried out as previously described in detail (10, 11), by using lethally irradiated (800 R, from a gamma cell 40; Atomic Energy of Canada, Ltd.) thymectomized mice reconstituted with syngeneic lymphocytes. The source of fetal or neonatal B lymphocytes was the liver and the source of adult B cells was the spleen. Both were treated with anti-brain θ and complement. B cells from several donors were pooled. Recipients received the equivalent of the cells from one adult spleen (approximately $10^8$ nucleated cells) or from one fetal or neonatal liver (approximately $3 \times 10^7$ nucleated cells) 2–4 h after irradiation. The tolerance inducing injection of antigen was given 1 day after cell transfer. 3–13 days later all animals received $1 \times 10^8$ syngeneic adult thymus cells, pooled from several adult donors. The anti-brain θ antiserum was prepared by immunizing rabbits with CBA/J mouse brain in CFA as described and characterized previously (11).

*Assay of Number and Avidity of PFC.* Anti-BGG PFC in the spleen were determined by the method of Jerne et al. (12) as modified for slide assay by Dresser and Greaves (13). The slides were incubated for 1 h at 37°C. Freshly frozen guinea pig serum, absorbed with 50% sheep erythrocytes (SRBC) was added, at a 1/30 dilution, as a source of complement and the slides were incubated for an additional $2^1/_2$ h. Rabbit anti-mouse gamma globulin was used at 1:200 dilution to develop indirect plaques. The target cells to detect anti-BGG PFC were prepared as described by Golub et al. (14) by coupling 24 mg BGG to 0.5 ml washed, packed SRBC in phosphate-buffered saline, pH 6.0, by using 250 mg 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide HCl (Sigma Chemical Co.). Anti-DNP PFC were detected by use of picryl coated SRBC as described previously (10, 11, 15).

The avidity distribution of the PFC was assayed by inhibition of plaque formation by using various concentrations of ligand essentially according to the method of Andersson (16) as validated previously (17, 18). Plaque formation around high affinity antibody secreting cells is inhibited by a low concentration of hapten while plaque formation about low affinity antibody secreting cells is only inhibited by high concentrations of hapten. Concentrations of BGG ranging from $1 \times 10^{-11}$ to $1 \times 10^{-7}$ M and concentrations of DNP-EACA ranging from $1 \times 10^{-9}$ to $1 \times 10^{-5}$ M, both in half-log increments, were used.

*In Vivo Induction of Tolerance.* Tolerance to BGG was induced by a single intravenous injection of between 1 and 250 μg ultracentrifuged (133,575 *g* at middle for 150 min in a Beckman 50 Ti rotor, Beckman Instruments, Inc., Fullerton, Calif.) antigen dissolved in saline (30 mg/ml) essentially as described by Dresser (4). BGG concentration was determined from its absorbancy at 280 nm: $E^{1\%}_{1cm} = 12.1$ (19). 13 days later, all animals received $1 \times 10^8$ syngeneic adult thymus cells and thereafter were challenged with BGG in CFA. DNP-specific B-cell tolerance was induced in reconstituted irradiated mice by the intravenous injection of 5 μg of either $DNP_6$-D-GL (20) or ultracentrifuged $DNP_{22}$-BGG. 3–6 days later all animals were given $1 \times 10^8$ syngeneic adult thymus cells and 1 day later were challenged with $DNP_{44}$-BGG in CFA.

*In Vitro Induction of Tolerance.* Tolerance was induced in vitro as described by previous workers (6). Spleen cells from adult mice (6–8 wk old) or liver cells from neonatal mice were treated with anti-brain θ antiserum and complement. The cells were washed twice with sterile media consisting of Hanks' balanced salt solution (HBSS), $5 \times 10^{-5}$ M 2-mercaptoethanol, 100 U penicillin/ml and 100 μg streptomycin/ml, and 5% fetal calf serum. The cells were cultured, for 24

h at 37°C, under a 5% $CO_2$/balance air atmosphere, in 60-mm plastic Petri dishes containing 4 ml of sterile medium at a cell density of $1 \times 10^7$ nucleated cells/ml. Cells were cultured with or without 0.1 μg/ml ultracentrifuged BGG, $DNP_6$-D-GL, or $DNP_{22}$-BGG. After incubation for 24 h the cells were scraped off the Petri dishes; the tolerogen was removed by washing three times with cold HBSS; and the cells were injected intravenously into lethally irradiated syngeneic mice. Each recipient was given $2–3 \times 10^7$ cultured cells plus $1 \times 10^8$ normal thymus cells. 1 day after cell transfer all animals were challenged with BGG or $DNP_{44}$-BGG in CFA.

## Results

*Comparison of B Lymphocytes from Fetal, Neonatal, and Adult Mice with Regard to Ease of Tolerance Induction In Vivo with Deaggregated BGG.* The ease of tolerance induction of B lymphocytes from adult, neonatal, and fetal mice was assayed by determining the dose-response curve for tolerance induction with deaggregated BGG. Lethally irradiated, thymectomized mice were reconstituted with B cells from neonatal or fetal liver, or adult spleen or bone marrow. 1 day later they were injected with tolerogen. All animals received $1 \times 10^8$ thymus cells 13 days thereafter and were challenged with BGG in CFA 1 day later. The dose-response curves for B-cell tolerance induction in mice reconstituted with B lymphocytes from adult spleen, adult bone marrow, 8-day-old spleen, neonatal liver, and 17-day fetal liver were strikingly similar (Fig. 1). In each case the degree of unresponsiveness increased with increasing doses of deaggregated BGG: approximately 50% depression being obtained with 3 μg of tolerogen except in mice reconstituted with B lymphocytes from 14-day fetal donors which were relatively resistant to tolerance induction. In each case, except day 14 fetal liver, doses of tolerogen between 8 and 20 μg caused approximately a 60% depression in the immune response. Higher doses of tolerogen were only employed in recipients of 14-day fetal liver, neonatal liver, and adult spleen. Mice reconstituted with neonatal liver and adult spleen behaved indistinguishably: approximately 85% depression in the anti-BGG response being obtained after 100 μg of tolerogen. In contrast, a dose of 100 μg deaggregated BGG brought about only a 39% depression in the immune response of mice reconstituted with day 14 fetal liver.

From the dose-response curves one can estimate the dose of antigen ($TD_{50\%}$) required to bring about a 50% depression in splenic anti-BGG PFC. The value for the $TD_{50\%}$ offers a convenient parameter by which to compare the relative ease of tolerance induction in different B-cell populations (Fig. 2). It is clear that the ease of tolerance induction in B lymphocytes, with deaggregated BGG, is essentially identical in all groups except those animals reconstituted with 14-day fetal liver which were highly resistant to tolerance induction.

*Effect of Partial Tolerance on the Avidity of the Anti-BGG PFC.* The effect of partial tolerance (induced with the $TD_{50\%}$ dose of deaggregated BGG: 3 μg) on the distribution of avidities of PFC was studied in mice reconstituted with either anti-brain θ antiserum-treated adult spleen or neonate liver as the source of B lymphocytes (Fig. 3, Table I). A highly heterogenous response was obtained in control mice reconstituted with adult spleen cells. In contrast, as we have previously reported (10, 15), the control mice reconstituted with neonatal liver as the source of B lymphocytes produced a response which was relatively restricted in heterogeneity. In both cases, partial tolerance resulted in a statistically significant decrease in heterogeneity and in avidity. These findings, taken

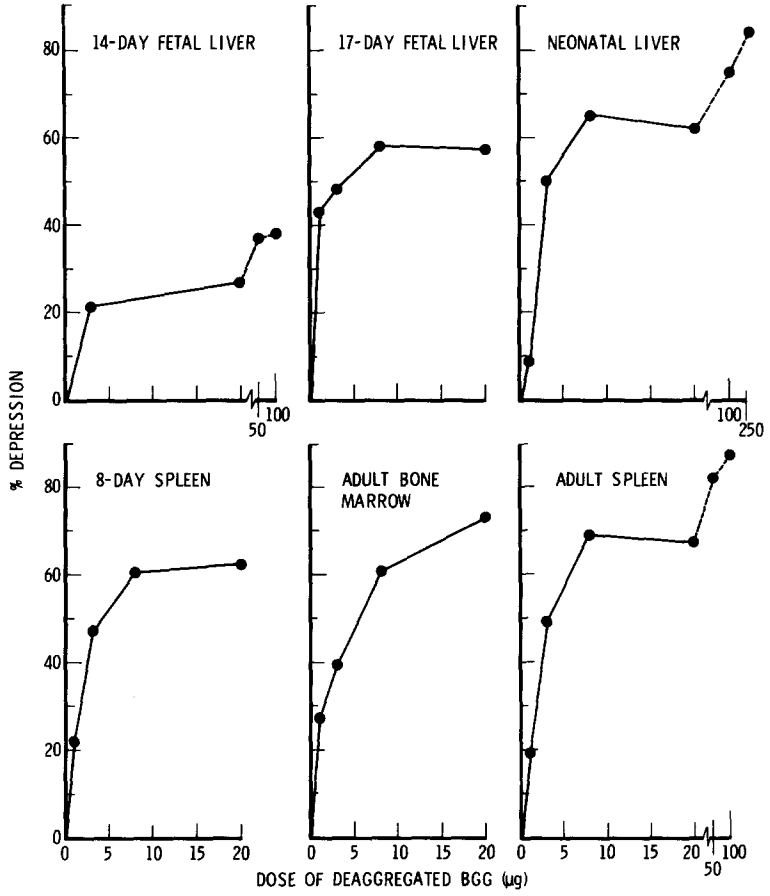


FIG. 1. Dose-response curves for tolerance induction with deaggregated BGG. Irradiated thymectomized mice were reconstituted with anti-brain $\theta$ antiserum-treated spleen, bone marrow, or fetal or neonatal liver as the source of B lymphocytes. 1 day after cell transfer groups of animals received doses of deaggregated BGG, intravenously, ranging from 1 to 250 $\mu$g. Control animals, which did not receive tolerogen, were included. 13 days later all animals received $1 \times 10^8$ syngeneic thymus cells, intravenously, and were immunized 24 h thereafter with 100 $\mu$g BGG in CFA, intraperitoneally. 3 wk later all animals were boosted with 500 $\mu$g BGG, intraperitoneally, and their splenic anti-BGG indirect PFC were assayed 13 days after boosting. The abscissa represents the dose of deaggregated BGG. The ordinate represents the percent depression of the number of splenic anti-BGG indirect PFC in tolerant animals as compared with the appropriate controls. Each point represents the percent depression of the arithmetic mean of the number of PFC, relative to controls, in groups of three to five mice. The results illustrated are in each case one representative example out of 2–13 independent dose-response curves obtained on mice reconstituted with each of the indicated sources of B lymphocytes.

together with the fact that tolerance was induced in the absence of T lymphocytes, suggest a B-cell clonal deletion type mechanism for this tolerant state.

*In Vivo Induction of B-Lymphocyte Tolerance with Polyvalent Antigens.* Recent in vitro studies with polyvalent hapten-carrier conjugates as tolerogens have indicated that B lymphocytes from neonatal donors are more easily rendered tolerant than are B cells from adult animals (6, 7). It was

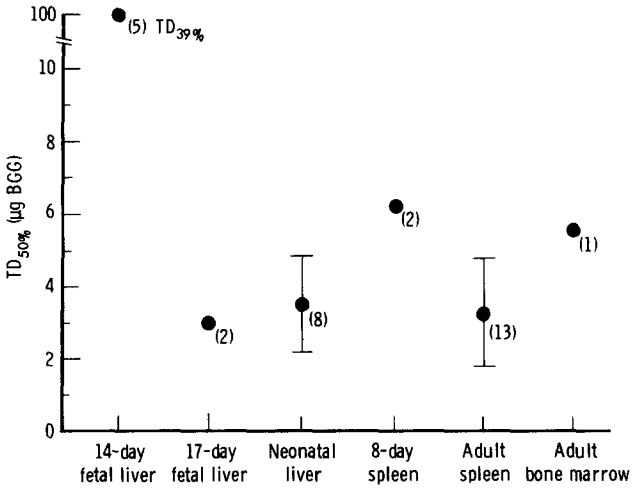


FIG. 2. Ontogeny of the ease of tolerance induction in mouse B lymphocytes with deaggregated BGG. The dose of deaggregated BGG required for a 50% depression of the indirect PFC response ($TD_{50\%}$), relative to appropriate controls, were obtained from dose-response curves such as those illustrated in Fig. 1. Along the abscissa is indicated the source of B lymphocytes used to reconstitute the lethally irradiated thymectomized mice and the ordinate represents the $TD_{50\%}$. The average value for $TD_{50\%}$ calculated from 2 to 13 independent dose-response curves is presented. The number in parentheses associated with each point indicates the number of dose-response curves obtained.

therefore deemed important to study the ease of tolerance induction in B lymphocytes, in vivo, by using such antigens. Tolerance induction with $DNP_6$-D-GL and ultracentrifuged $DNP_{22}$-BGG was studied in irradiated thymectomized mice reconstituted with B cells from neonatal or adult donors. It should be noted that tolerance induction took place in the absence of T lymphocytes. The data, presented in Table II, are consistent with the conclusion that B cells from neonatal mice are more readily rendered tolerant to these antigens than are B cells from adult donors.

*In Vitro Induction of B-Lymphocyte Tolerance.* Anti-brain θ antiserum-treated adult spleen or neonatal liver cells were maintained in culture for 24 h in the presence or absence of 0.1 μg/ml tolerogen ($DNP_6$-D-GL, deaggregated BGG, or $DNP_{22}$-BGG). The cells were then washed, mixed with normal thymus cells, and injected intravenously into lethally irradiated syngeneic mice. Each recipient received $3 \times 10^7$ cultured cells plus $1 \times 10^8$ normal adult thymus cells and was immunized with $DNP_{44}$-BGG or BGG in CFA 1 day later. There was no difference between B cells from neonatal and adult mice with regard to the ease of tolerance induction with ultracentrifuged BGG in vitro (Table III). In contrast, B cells from neonatal mice were markedly more susceptible than B cells from adult donors to in vitro tolerance induction with $DNP_{22}$-BGG or $DNP_6$-D-GL (Table III).

## Discussion

The susceptibility to tolerance induction of B lymphocytes from fetal, neonatal, and adult mice was studied in vitro and in vivo in a cell transfer system.

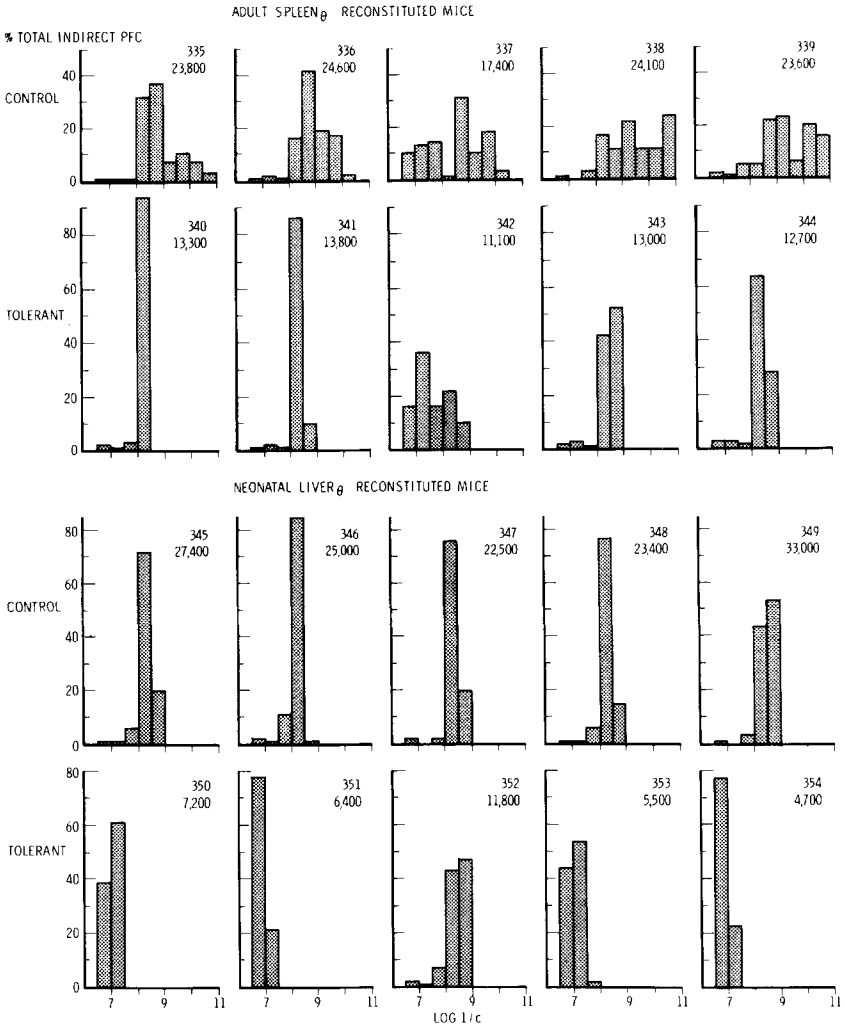


FIG. 3. Effect of partial tolerance on antibody avidity. Each histogram illustrates the distribution of anti-BGG indirect PFC with respect to avidity in the spleen of an individual mouse. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. The animal identification number (top) and total indirect PFC per spleen are given in the upper right corner of each histogram. Avidity increases to the right. Tolerant mice had received a tolerance inducing injection of 3 $\mu$g deaggregated BGG intravenously. Controls were injected with saline. 2 wk after the injection of tolerogen all animals were immunized with 100 $\mu$g BGG in CFA intraperitoneally and were boosted with 500 $\mu$g BGG, intraperitoneally, 3 wk later. The anti-BGG indirect PFC in the spleen were assayed 13 days after boosting. The top two rows illustrate data on tolerance induction in irradiated thymectomized mice reconstituted with anti-brain $\theta$ antiserum treated adult spleen cells. The bottom two rows illustrate data on tolerance induction in irradiated thymectomized mice reconstituted with anti-brain $\theta$ antiserum treated neonatal liver cells. The tolerance inducing injection of antigen was given 1 day after reconstitution with B cells and all animals received $1 \times 10^8$ adult syngeneic thymus cells 1 day before challenge with BGG in CFA.

TABLE I

*Effect of Partial Tolerance to BGG on Antibody Avidity and Heterogeneity**

| Experimental animals reconstituted with | Treatment (no. of mice) | Depression of PFC in tolerant mice | Avidity‡ $K_{50\%} \times 10^{-6}$ | Heterogeneity§ index | |
|---|---|---|---|---|---|
| | | % | | | |
| Adult spleen$_\theta$ | Control (10) | | 6.1 | 2.57 ± 0.25 | |
| | | 41 | | | $P < 0.002$ |
| | Tolerant (10) | | 0.9 | 1.57 ± 0.62 | |
| Neonatal liver$_\theta$ | Control (14) | | 0.8 | 1.47 ± 0.46 | |
| | | 55 | | | $P < 0.02$ |
| | Tolerant (9) | | 0.1 | 1.01 ± 0.30 | |

* Lethally irradiated thymectomized mice were reconstituted as indicated in the first column. Animals received anti-brain $\theta$-treated adult spleen cells or anti-brain $\theta$-treated neonatal liver cells. 1 day after cell transfer half of the mice received a tolerance inducing injection of 3 $\mu$g deaggregated BGG intravenously while the remaining mice were used as controls. 13 days later all animals received $1 \times 10^8$ syngeneic thymus cells intravenously and 1 day thereafter were immunized with 100 $\mu$g BGG in CFA intraperitoneally. 21 days later all animals were boosted with 500 $\mu$g BGG intraperitoneally and their splenic anti-BGG indirect PFC were assayed 13 days after boosting. The avidity distributions of some of these animals are illustrated in Fig. 3. The depression in number of PFC was found to be significant ($P < 0.002$) by the Mann-Whitney U test. A $t$ test was used to evaluate significance of differences in the heterogeneity indices.

‡ Geometric mean of the reciprocal of the concentration of hapten required for 50% inhibition of the number of indirect PFC.

§ The Shannon heterogeneity index (29) was used to describe the degree of heterogeneity of avidity of the anti-BGG indirect PFC population of individual animals. The average value ± standard deviation, of this index for each of the experimental groups is presented. The larger the index the greater the heterogeneity.

TABLE II

*In Vivo Induction of Tolerance in Neonatal or Adult Mouse B Lymphocyte Populations with $DNP_6$-D-GL and Deaggregated DNP-BGG**

| Exp. no. | Experimental animals | | Tolerogen | | | |
|---|---|---|---|---|---|---|
| | | | $DNP_6$-D-GL | | $DNP_{22}$-BGG | |
| | Reconstituted with | Treatment | Anti-DNP PFC | Depression | Anti-DNP PFC | Depression |
| | | | | % | | % |
| 1 | Adult spleen$_\theta$ | Control | 2,460 (2) | 29 | 2,000 (3) | 0 |
| | | Tolerogen | 1,740 (2) | | 2,850 (2) | |
| | Neonatal liver$_\theta$ | Control | 7,920 (2) | 89 | 1,245 (2) | 78 |
| | | Tolerogen | 908 (4) | | 270 (3) | |
| 2 | Adult spleen$_\theta$ | Control | 6,380 (5) | 9 | 6,380 (5) | 18 |
| | | Tolerogen | 5,790 (3) | | 5,220 (4) | |
| | Neonatal liver$_\theta$ | Control | 5,740 (6) | 75 | 5,740 (6) | 78 |
| | | Tolerogen | 1,460 (5) | | 1,270 (6) | |

* Lethally irradiated thymectomized mice were reconstituted as indicated in the second column. Animals received $3–5 \times 10^7$ anti-brain $\theta$ antiserum-treated neonatal liver cells or $7–10 \times 10^7$ anti-brain $\theta$ antiserum-treated adult spleen cells. 1 day after cell transfer half of the animals were injected intravenously with 5 $\mu$g of tolerogen ($DNP_6$-D-GL or deaggregated $DNP_{22}$-BGG). Controls were not injected with tolerogen. 3 days after $DNP_6$-D-GL or 6 days after $DNP_{22}$-BGG injection all animals received $1 \times 10^8$ syngeneic thymus cells, intravenously, and 1 day thereafter were immunized with 500 $\mu$g $DNP_{44}$-BGG in CFA. Splenic anti-DNP PFC were assayed 13 days after immunization. The results are presented as the average number of indirect PFC per spleen. The number of animals studied is indicated in parentheses. Two representative experiments with each tolerogen are presented.

TABLE III

*In Vitro Induction of Tolerance in Neonatal or Adult Mouse B-Lymphocyte Populations with Deaggregated BGG, $DNP_6$-D-GL, and $DNP_{22}$-BGG**

| Exp. no. | Cultured cells | In vitro treatment | Tolerogen | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | BGG | | $DNP_6$-D-GL | | $DNP_{22}$-BGG | |
| | | | Anti-BGG PFC (No. of mice) | Depression | Anti-DNP PFC (No. of mice) | Depression | Anti-DNP PFC (No. of mice) | Depression |
| | | | | % | | % | | % |
| 1 | Adult spleen$_\theta$ | Control | 62,530 (2) | 75 | 31,550 (2) | 48 | 5,330 (4) | 39 |
| | | Tolerogen | 15,830 (4) | | 16,240 (2) | | 3,660 (4) | |
| | Neonatal liver$_\theta$ | Control | 59,600 (2) | 74 | 2,660 (3) | 77 | 2,440 (4) | 74 |
| | | Tolerogen | 15,820 (5) | | 617 (3) | | 640 (3) | |
| 2 | Adult spleen$_\theta$ | Control | 94,350 (2) | 36 | 5,600 (5) | 0 | 5,600 (5) | 29 |
| | | Tolerogen | 60,150 (3) | | 6,450 (2) | | 3,950 (3) | |
| | Neonatal liver$_\theta$ | Control | 60,740 (3) | 20 | 3,180 (5) | 85 | 3,180 (5) | 81 |
| | | Tolerogen | 48,520 (3) | | 480 (2) | | 620 (2) | |

* Anti-brain $\theta$ antiserum-treated adult spleen cells or anti-brain $\theta$ antiserum-treated neonatal liver cells were maintained in tissue culture at a cell density of $1 \times 10^7$ cells/ml for 24 h with or without 0.1 μg/ml tolerogen. The cells were then harvested and were washed three times. $3 \times 10^7$ nucleated cells from each group were mixed with $1 \times 10^8$ syngeneic thymus cells and were injected intravenously into lethally irradiated syngeneic mice. Recipients were immunized 1 day later with either $DNP_{44}$-BGG or BGG. Splenic anti-DNP or anti-BGG PFC were assayed 13 days after immunization or boosting, respectively. The results are expressed as the geometric mean of the number of indirect PFC per spleen. The number of animals studied is indicated in parenthesis. Two independent experiments are presented with each tolerogen. In exp. 2, $DNP_6$-D-GL and $DNP_{22}$-BGG tolerance induction was done simultaneously by using a single group of controls.

It was found that with ultracentrifuged BGG, B cells from 17-day fetal, neonatal, 8-day old and adult mice were equivalent with respect to ease of tolerance induction in vivo. In addition, B cells from adult spleen and bone marrow were equally susceptible to tolerance induction, in vivo, with deaggregated BGG. Similarly, in vitro, B cells from neonatal and adult donors were equally susceptible to tolerance induction with deaggregated BGG. In marked contrast, B cells from neonatal donors were more readily tolerized with $DNP_6$-D-GL or ultracentrifuged $DNP_{22}$-BGG than were B cells from adult mice both in vivo and in vitro. It should be noted that tolerance was induced in the absence of T cells.

The partial tolerant state induced with deaggregated BGG in lethally irradiated, thymectomized mice reconstituted with neonatal or adult B cells was shown to be characterized by a marked depression in the avidity of the residual PFC. Furthermore, the tolerance was induced in T-cell depleted mice. Thus, it appears reasonable to regard this tolerant state as being mediated by a B-cell clonal deletion type mechanism. In previous studies (11) we established, by extensive cell transfer experiments, that the tolerant state induced in reconstituted irradiated adult mice with DNP-BGG is definitely due to a B-cell clonal deletion type mechanism. DNP-D-GL has been studied in detail and is known to be a B-cell tolerogen (20). Thus, it would appear likely that the models of tolerance studied here all represent B-cell clonal deletion type mechanisms and are therefore appropriate models to study the direct susceptibility of different B-lymphocyte populations to tolerance induction.

B cells from neonatal mice have been reported by previous workers to be far more susceptible to tolerance induction in vitro with hapten-carrier conjugates than are B cells from adult animals (6, 7). Immature B cells from the bone

marrow were also found to be more readily tolerized than mature peripheral B cells (5). In addition, immature B cells are more susceptible than mature B cells to suppression by anti-idiotype antibodies (21). Furthermore, immature B cells have been shown to require a higher concentration of anti-immunoglobulin antibody to bring about capping of surface immunoglobulin, and to fail to resynthesize surface immunoglobulin after capping as do mature B lymphocytes (22, 23). These observations have led workers to conclude that a high degree of sensitivity to tolerance induction is a basic property of immature B lymphocytes, and to assume that this property is critical for the normal establishment of self tolerance. There have been numerous observations which suggest that tolerance can be more readily induced in neonatal than in adult animals. On the other hand, studies with T-independent polysaccharide antigens have failed to demonstrate a similar enhanced susceptibility to tolerance induction in vivo during the neonatal period (2, 3). Furthermore, Dresser (4) found no difference in the dose of deaggregated BGG required to tolerize neonatal and adult mice. To some extent the studies reported here help to clarify these apparent discrepancies. It was found that with polyvalent hapten-carrier conjugates there is indeed a greater ease of tolerance induction in B cells from immature animals. This increased ease of tolerance induction was demonstrated both in vivo and in vitro. In contrast, with deaggregated BGG no difference in ease of tolerance induction was demonstrable either in vivo or in vitro when comparing B cells from immature and adult donors. Thus, a high susceptibility to tolerance induction cannot be regarded as a general property of immature B cells with respect to all antigens.

Immature B cells, after capping and shedding of surface immunoglobulin, fail to resynthesize surface immunoglobulin on further culture while mature B cells rapidly resynthesize surface immunoglobulin (22, 23). The failure to resynthesize surface immunoglobulin antigen receptors could result in a clonal deletion type tolerance after their capping and shedding as a consequence of cross-linking with a polyvalent antigen. It is with polyvalent antigens (hapten-protein conjugates) that we and previous workers (5–7) have detected a difference between immature and mature B cells in regard to the ease of tolerance induction. With an antigen, such as BGG, which does not contain multiple identical determinants, B-cell tolerance induction might involve other mechanisms than cross-linking of receptors, and with these mechanisms there is no difference between mature and immature B cells. Polysaccharide antigens clearly consist of multiple repeating determinants but immature B cells do not appear to differ from adult B cells with regard to susceptibility to tolerance induction (2, 3). It is possible that with these highly polyvalent antigens tolerance induction is through the formation of a stable cross-linked matrix (24) which takes place with equal facility in mature and immature B-cell populations. It should be noted that both BGG and DNP-BGG are T-dependent antigens (11 and unpublished observations). Therefore, T dependence of the antigen does not appear to be the critical factor in determining whether immature B cells will be more susceptible to tolerance induction with that antigen.

It is probable that several different processes can lead to an unresponsive B-cell population. Immature B cells appear to be more suceptible than mature B cells to tolerance induction by some of these mechanisms, but not by others. This

brings up the question of self tolerance. The clonal abortion theory (25) which emphasizes a tolerance susceptible phase during the development of B cells is very attractive as a model for self tolerance. However, the polyvalent hapten-carrier conjugates, with which the relative ease of tolerance induction in immature B cells can be demonstrated, probably represent poor models of the usual self antigen. It seems possible that BGG represents a better model for a self antigen. Since in tolerance induction with BGG no difference between mature and immature B cells is observed, one must seriously question the extent to which a difference in the ease of tolerance induction in immature and mature B cells plays an important role in controlling self tolerance induction. However, the possibility must also be considered that BGG actually represents an unusual example reflecting some special handling of immunoglobulins.

Finally a note should be made of the interesting finding that mice reconstituted with B cells from 14-day fetal donors were particularly resistant to tolerance induction. The precise mechanism is unclear. It is possible that this relative resistance to tolerance induction reflects the fact that B-cell surface antigen receptors are not yet fully expressed in the 14-day fetus (26). In addition, evidence has been reported which suggests that IgG precursor cells are more sensitive to tolerance induction than are IgM precursors (27, 28). It has been previously shown that cells from 14- or 16-day fetal mice produce mainly IgM PFC. Between day 16 and 17 of fetal life the capacity of B cells to produce indirect PFC develops (10, 15). It is also possible that rapid proliferation of this immature B-cell population is at least in part responsible for the failure to induce tolerance. If the cell population is rapidly proliferating, competent cells may quickly arise after clearance of the tolerogen and an unresponsive state will not be observed. In any event it is clear that even relatively early fetal B-cell populations are not particularly susceptible to tolerance induction with deaggregated BGG.

## Summary

The ease of tolerance induction in B lymphocytes from fetal, neonatal, and adult mice was studied in vivo, in a cell transfer system, and in vitro. Three different tolerogens were used: ultracentrifuged BGG, $DNP_6$-D-GL, and ultracentrifuged $DNP_{22}$-BGG. Irradiated thymectomized mice were reconstituted with B cells from fetal or neonatal liver or adult spleen or bone marrow. The mice were injected with tolerogen 1 day later. They were given normal thymus cells and challenged with either BGG or $DNP_{44}$-BGG between 4 and 14 days after tolerance induction. With BGG no difference in ease of B-cell tolerance induction was observed in mice reconstituted with B cells from 17-day fetal liver, neonatal liver, 8-day-old spleen, adult spleen, or adult bone marrow. B cells from 14-day fetal donors are relatively resistant to tolerance induction. In contrast, with $DNP_6$-D-GL and $DNP_{22}$-BGG B cells from neonatal donors were clearly more susceptible to tolerance induction than were B cells from adult donors. Comparable results were obtained in studies on tolerance induction in vitro. Neonatal B cells were more susceptible than adult B cells to tolerance induction upon culture with $DNP_6$-D-GL or $DNP_{22}$-BGG. However, neonatal and adult B cells were identical with respect to ease of tolerance induction in vitro with deaggregated BGG.

The results suggest that there are multiple mechanisms for B-cell tolerance induction. Immature B cells appear to be more susceptible to tolerance induction by some mechanisms but not by others. It is suggested that immature B cells are more susceptible to tolerance induction with moderately polyvalent antigens such as hapten-carrier conjugates. With antigens like BGG which do not have repeated epitopes no difference between mature and fetal B cells in regard to ease of tolerance induction is observed. These observations raise questions about the importance of relative ease of tolerance induction in immature B cells as a mechanism controlling the normal induction of self tolerance.

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