IN VITRO TOLERANCE INDUCTION OF NEONATAL MURINE B CELLS*

By ELEANOR S. METCALF and NORMAN R. KLINMAN

(From the Department of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174)

Immunologic tolerance was originally defined as specific hyporesponsiveness to an antigen which results from prior contact with that antigen (1, 2). The concept of natural tolerance to self-antigens was perceived by Burnet (3) as the deletion of clones reactive to autologous antigens after contact of these antigens with the reactive cells' specific antigen receptors during the development of the cell's immunologic competence. If such a theory were to have physiological relevance, then several predictions should be realized: (a) developing cells should be significantly more susceptible to tolerance induction than their mature counterparts; (b) tolerance should be inducible over a wide range of antigen concentrations, since the concentrations of different self-antigens present presumably vary; (c) since the spectrum of self-antigens is diverse, the development of unresponsiveness should be plausible with a wide variety of antigenic structures; (d) since bone marrow-derived precursors to antibody-forming cells (B cells) presumably mature independently of other cell types, induction of B-cell tolerance should occur in the absence of antigen-specific thymus-derived lymphocytes (T cells); (e) the discrimination between self and nonself would be absolute, and therefore, tolerance should exhibit exquisite determinant specificity.

Several models have been developed to examine B-cell tolerance; however, these approaches have generally been restricted to the analysis of adult responses, since responses by neonatal cells and the necessary controlled comparisons of neonatal and adult cells have been technically difficult to achieve (4). Furthermore, the requirement for T cells in the elicitation of maximal B-cell stimulation has engendered considerable uncertainty over the cellular level of any unresponsiveness induced (4-6). The demonstrated role of suppressor cells or soluble factors in the development of unresponsiveness has added to the difficulties in determining whether tolerance occurs at the B- or T-cell level (7-10). Finally, antigen-induced redistribution of cells from their normal migratory pathways or temporary antigen "blockade" of cell receptors may be misconstrued as a state of tolerance (11).

T-cell tolerance, suppressor T cells, and receptor blockade may represent valid biological mechanisms and may reflect important regulatory controls on B-cell responsiveness. However, the existence of such mechanisms does little to elucidate the possible role of a "clonal abortion" mechanism in B-cell responses (11). Furthermore, systems in which putative B-cell tolerance has been achieved usually involve the exposure of B cells to high concentrations of antigens, at least for induction (4), and have been limited to such antigens as serum proteins, particularly immunoglobulins (4-6), haptenated homologous serum proteins, erythrocytes or undegradable synthetic copolymers (12-18), and a few "T-independent" antigens (19, 20). Lastly, in these models, B-cell tolerance is often reversible with factors derived from T cells or mitogens and has not been highly specific (6, 16, 21, 22).

Recent comparative studies of neonatal and adult B cells have demonstrated differences in susceptibility to anti-immunoglobulin or anti-idiotype antibody-induced suppression (23–26), implying that neonatal (i.e., immature or developing) cells may be highly susceptible to tolerance induction. In addition, Nossal and Pike have demonstrated that developing B cells in the adult bone marrow may indeed be susceptible to tolerance induction (27). Therefore, a model of B-cell tolerance which compares neonatal (immature) and adult (mature) B-cell responses should permit a physiologically relevant approach to the study of self-recognition mechanisms.

Previous studies from this laboratory have demonstrated that the splenic focus technique permits detectable stimulation of individual neonatal or adult clonal precursor cells (28–32). Since tolerogens can be added to fragment cultures and removed before stimulation, this system provides a unique opportunity to compare the interaction of tolerogen with individual neonatal and adult cells. The results demonstrate that in this model of B-cell tolerance: (a) splenic B cells are susceptible to tolerance induction only during the first few days after birth; (b) tolerance of developing B cells can be established by hapten at concentrations ranging from 10^{-5} M to 10^{-9} M on a wide variety of carriers, including those generally assumed to be immunogenic; (c) the tolerance induced is highly specific, discriminating even between the dinitrophenylated (DNP)[1] and trinitrophenylated (TNP) determinants; and (d) this tolerance induction is T-cell independent, time dependent, and cannot be reversed by removal of the tolerogen. These findings suggest intrinsic differences between neonatal and adult B cells and may provide a model for the study of tolerance to self-antigens.

Materials and Methods

Hapten-Carrier Conjugates. The preparation of *Limulus polyphemus* hemocyanin (Hy), dinitrophenylated hemocyanin (DNP-Hy, 10 mol of DNP per 100,000 g of Hy), and trinitrophenylated hemocyanin (TNP-Hy, 10 mol of TNP per 100,000 g of Hy) have been previously described (29, 31). Human gamma globulin (HγG, Cohn fraction II, Nutritional Biochemicals Corporation, Cleveland, Ohio) was purified by the method of Chiller et al. (33). Mouse gamma globulin (MγG) was prepared from ascites fluid of mice carrying the plasmacytoma 31 C (κ, γ_1) by the procedure described by Rowe and Fahey (34). Chicken albumin (OVA, Fraction V, Pentex, Inc., Kankakee, Ill.) was used without further purification. The following DNP conjugates were prepared and assessed for DNP content as previously described (29, 35): DNP-HγG (10 mol of DNP per 150,000 g of HγG), DNP-MγG (15–20 mol of DNP per 150,000 g of MγG), and DNP-OVA (4 mol of DNP per 44,000 g of OVA). The random copolymer of D-glutamic acid and D-lysine (D-GL, Miles-Yeda, Ltd., Rehovot, Israel) had a ratio of G:L of 3:2.05 and was conjugated to trinitrobenzene sulfonic acid (Nutritional Biochemicals Corporation) by a modification of the procedure of Rittenberg and Amkraut (36). 50 mg of 2,4,6-trinitrobenzene sulfonate was added to 150 mg D-GL dissolved in 10 ml of 0.28 M cacodylic acid, pH 7.2, stirred overnight at room temperature, and dialyzed extensively against distilled water at 4°C for 48 h: (TNP-D-GL, 20 mol of TNP per 40,600 g of D-GL). 5-dimethylaminonaphthalene-1-sulfonyl-N-acetyl-β-alanylglycylglycine Boc hydrazide-hemocyanin (DANS-AGG-Hy), a dansyl-tripeptide spacer-Hy conjugate, a gift from Mr. Nolan Sigal, was prepared by the method of Inman et al. (37), and contained 10 mol of hapten per mol of Hy. DNP$_1$-S-papain was the generous gift of·Dr. S. J. Singer (38). Aggregates were removed by passage through a Sephadex G-50 column.

---

[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; D-GL, D-glutamic acid and D-lysine; DANS-AGG-HY, 5-dimethylaminonaphthalene-1-sulfonyl-N-acetyl-β-alanylglycylglycine Boc Hydrazide-hemocyanin; DMEM, Dulbecco's modified Eagle's medium; DNP-Hy, dinitrophenylated-hemocyanin; HγG, human gamma globulin; Hy, hemocyanin; MγG, mouse gamma globulin; OVA, chicken albumin; TNP-Hy, trinitrophenylated-hemocyanin.

Animals.   6-8-wk-old BALB/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine or Carworth Division, Becton Dickinson & Co., Rutherford, N. J. BALB/cAnN were acquired through the Institute for Cancer Research, Philadelphia, Pa. Mice from all three sources were used interchangeably in these studies. Neonatal mice were obtained from breeding pairs of BALB/cAnN mice in our own mouse colony as well as from pregnant BALB/cAnN females obtained from the Institute for Cancer Research, Philadelphia, Pa.

Immunizations.   6-8-wk-old mice received an intraperitoneal injection of 0.1 mg of Hy in complete Freunds' adjuvant (CFA) 6-12 wk before use as irradiated, carrier-primed recipients for adoptive adult or neonatal cell transfer.

Preparation of Cell Suspensions.   Suspensions of neonatal or adult BALB/c spleen cells were prepared by either extensively flushing the spleens with RPMI 1640 plus 25 mM HEPES and 10% fetal calf serum (FCS) or by gently pressing small sections of spleen between two glass slides into media. Large cell clumps were removed by filtration through a nylon screen, and viability of nucleated cells was determined by the trypan blue exclusion method.

Cell Transfers and In Vitro Tolerance Induction.   A modification of the in vitro splenic focus technique (29-32) was developed for the study of tolerance induction of neonatal and adult spleen cells. 4×10^6 spleen cells from unimmunized neonatal or adult mice were injected intravenously into Hy carrier-primed recipients which had been irradiated at 1,300 R, 4-6 h earlier. 16-18 h after transfer, the recipient spleens were removed and sliced into 1-mm fragments by a McIlwain Tissue Chopper (Brinkmann Instruments, Inc., Westbury, N. Y.). Fragments were transferred individually into wells of microtiter plates (Linbro Chemical Co., New Haven, Conn.) and cultured for various time intervals in either Dulbecco's modified Eagle's medium (DMEM) or with various hapten-carrier conjugates diluted in DMEM. The fragments were subsequently washed thoroughly with DMEM and then stimulated with either DNP-Hy, TNP-Hy, or DANS-AGG-Hy at hapten concentrations of 1×10^{-6} or 5×10^{-7} M. Culture fluids were changed every 3 days.

Radioimmunoassay.   20 μl of culture fluids collected 10 or 13 days after stimulation were quantitatively assayed for antihapten antibody. The radioimmunoassays were performed by detecting immunoglobulin bound to 2,4-dinitrophenyl-lysine-bromoacetylcellulose immunoadsorbent (DNP-LYS-BAC), 2,4,6-trinitrophenyl-lysine-BAC (TNP-LYS-BAC), or dansyl-lysine-BAC (DANS-LYS-BAC) prepared by general procedures described previously (29-31). Bound immunoglobulins were quantified with ^{125}I-labeled purified rabbit antimouse Fab or goat antimouse IgM (μ) or IgG$_1$ (γ_1) antibody (39, 40).

Depletion of T Lymphocytes.   Donor spleen cell suspensions were treated with AKR anti-θ C3H serum (a gift from Dr. C. A. Janeway, Jr.) and guinea pig complement as previously described (41). These conditions have previously been shown to be capable of completely abrogating "helper" activity of donor T-lymphocyte populations and lack cytotoxicity for B cells (41).

Results

Parameters of Tolerance Induction in Neonatal Splenic B Cells.   The efficiency of tolerance induction by preincubation of fragment cultures with 10^{-6} M DNP-MγG is summarized in Table I. It is clear that adult splenic B cells were not tolerized, whereas both IgM and IgG$_1$ responses in day 3 splenic B cells were markedly reduced. Furthermore, the neonatal B cells capable of yielding clones producing only IgM antibodies appeared most susceptible to tolerance induction.

The specificity of tolerance induction in neonatal B cells was examined, and the results indicate that preincubation with MγG alone did not influence the number of hapten-specific clonal precursor cells specific for DNP (94% of control response). Most significant, however, is the fact that the tolerance induced in neonatal B cells by DNP-MγG caused no measurable diminution in the response of DANS-reactive cells or even of cells reactive to the closely related hapten TNP as demonstrated in Table II.

Age Dependence of Tolerance Susceptibility.   Fig. 1 summarizes the normalized data obtained from the analysis of culture fluids from fragment cultures

TABLE I

The Effect of Incubation with DNP-MγG on the Frequency and Isotype of Monoclonal Anti-DNP Antibody Responses

Age of donor spleen cells	Tolerogen (10⁻⁶ M DNP-MγG)	Number clones per 10⁶ spleen cells transferred*	Heavy chain class					
			IgM		IgG₁		IgM + IgG₁	
			Number clones per 10⁶ cells transferred	Total clones	Number clones per 10⁶ cells transferred	Total clones	Number clones per 10⁶ cells transferred	Total clones
				%		%		%
Adult (8–10 wk)	–	1.92 ± 0.21	0.28	16.2	1.29	74.5	0.16	9.3
	+	1.77 ± 0.19	0.39	27.1	0.95	65.7	0.10	7.0
Neonatal (3 days)	–	1.34 ± 0.13	0.47	40.5	0.65	55.7	0.03	2.9
	+	0.36 ± 0.03	0.02	6.8	0.32	93.1	0.0	0.0

* 4 × 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-MγG for 24 h, washed, and stimulated with DNP-Hy at 10⁻⁶ M DNP. Clones were detected by radioimmunoassay of culture fluids with ¹²⁵I-labeled antimouse Fab, IgM, or IgG₁.

TABLE II

Specificity of Tolerance Induced in Neonatal and Adult Spleen Fragment Cultures

Age of donor spleen cells	Total number of donor cells analyzed × 10⁻⁶ *	Tolerogen (10⁻⁶ M)	Stimulating antigen	Number of clones per 10⁶ cells transferred	Percent of control response
Adult (8–10 wk)	124	–	DNP-Hy	1.92 ± 0.21	
	128	DNP-MγG	DNP-Hy	1.77 ± 0.19	92.1
	40	–	TNP-Hy	1.63 ± 0.31	
	24	DNP-MγG	TNP-Hy	1.82 ± 0.48	111.6
	16	–	DANS-AGG-Hy	0.56 ± 0.06	
	16	DNP-MγG	DANS-AGG-Hy	0.56 ± 0.06	100.0
Neonatal (3 days)	86.4	–	DNP-Hy	1.34 ± 0.13	
	86.4	DNP-MγG	DNP-Hy	0.36 ± 0.03	26.8
	30.4	–	TNP-Hy	1.08 ± 0.11	
	30.4	DNP-MγG	TNP-Hy	1.08 ± 0.13	100.0
	16.0	–	DANS-AGG-Hy	0.52 ± 0.25	
	16.0	DNP-MγG	DANS-AGG-Hy	0.62 ± 0.37	119.2

* 4–8 × 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-MγG for 24 h, washed, and stimulated with either DNP-Hy at 10⁻⁶ M DNP, TNP-Hy at 10⁻⁶ M TNP, or DANS-AGG-Hy at 5 × 10⁻⁷ M DANS. Clones were detected by radioimmunoassay of culture fluids with ¹²⁵I-labeled antimouse Fab, IgM, or IgG₁.

containing donor cells derived from mice of varying ages. All of the cell populations responded in the absence of putative tolerogen treatment. Furthermore, the response of adult spleen cells was unaffected by in vitro culture with DNP-MγG. In contrast, the response of fetal liver cells at 18 days of gestation was entirely abrogated after tolerogen treatment, and greater than 90% of the DNP-specific clones from day 1 spleen cells were susceptible to tolerance induction. However, the frequency of cells not susceptible continues to increase thereafter and reaches adult levels of resistance to tolerance induction by day 7 after birth. These results suggest that the susceptibility to tolerance induction is restricted to B cells early in their development.

To establish that neonatal suppressor T cells (42) do not play a role in
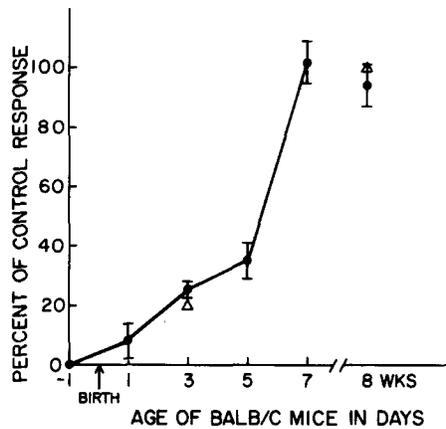
FIG. 1. The dependence of in vitro tolerance induction on the age of the B-cell donor. Fragment cultures containing spleen or liver cells of donors of various ages were exposed to DNP-MγG for 24 h before stimulation with DNP-Hy (●—●). Each point represents data obtained from a minimum of 8×10^6 donor cells ± standard error. (△) represents similar experiments with donor cells treated with anti-θ antiserum and C' before transfer. 100% of the control response represents the total response of DNP-specific cells cultured in the absence of tolerogen.

tolerance induction, day 3 neonatal and adult spleen cells were pretreated with anti-θ antiserum and C' before cell transfer. The results of this analysis are presented as the additional points at day 3 and 8 wk in Fig. 1 and indicate that the same degree of unresponsiveness was observed in treated and untreated day 3 neonatal cells in fragment cultures.

Time Dependence of Tolerance Induction. To establish the requirements for optimal unresponsiveness in this system, day 3 neonatal or adult spleen fragments were exposed to DMEM or 10^{-6} M DNP-MγG for various time intervals, washed, and stimulated with 10^{-6} M DNP-Hy. The normalized results (Fig. 2) show that whereas 24-h exposure to DNP-MγG had no effect on adult splenic B cells, a significant reduction in neonatal B-cell responsiveness was seen after only 6 h of culture in the presence of DNP-MγG, and maximal reduction was achieved after 12–24 h of culture. However, it is apparent that a critical time period is required for the neonatal cells to be rendered unresponsive. In addition, tolerance induction apparently involves more complex interactions than simple binding of tolerogen, since exposure to tolerogen for 2 h did not render most neonatal cells unresponsive.

Antigen Concentration and Carrier Independence of Tolerance Induction. Previous studies have provided evidence that B-cell tolerance induction is dependent on high antigen concentrations (4, 5, 43) and limited to a few carriers (12–20). From the results presented in Table III it is evident that DNP-MγG failed to induce tolerance in adult splenic B cells at any concentration up to 10^{-5} M haptenic determinant. This is in marked contrast to the response of day 3 splenic B cells in which significant reduction in the number of responsive cells was evident at concentrations as low as 10^{-9} M DNP-MγG.

If the unresponsiveness observed in fragment cultures of neonatal splenic B cells after preincubation with DNP-MγG represents a generalized characteristic
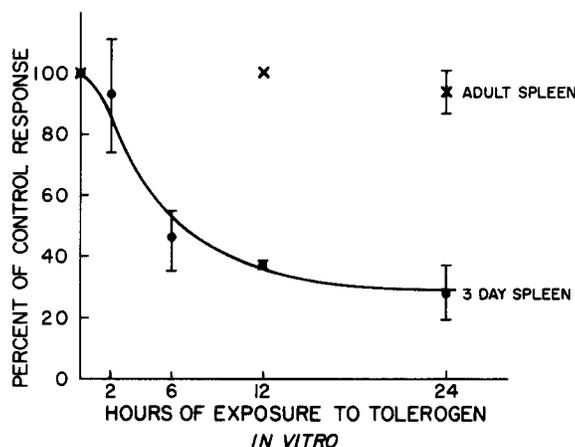
FIG. 2. The induction of in vitro tolerance as a function of time. Fragment cultures containing day 3 neonatal (● — ●) or adult (× — ×) spleen cells were exposed to 10^{-6} M DNP-MγG for various time intervals before stimulation with DNP-Hy. Each point represents data obtained from a minimum of 8×10^6 donor cells ± standard error. 100% of the control response represents the total response of DNP-specific cells cultured in the absence of tolerogen.

of neonatal (immature) cell populations and not a property of a particular tolerogen, then presumably the majority of these cells should also be rendered unresponsive after culture with other hapten-protein conjugates. Table III also presents results demonstrating that while adult splenic B cells remain unaffected by any of the hapten-carrier conjugates, day 3 neonatal splenic B cells were tolerized by a wide variety of hapten-carrier conjugates. In addition, the results suggest an apparent requirement for multivalent presentation of the relevant hapten for the induction of tolerance, since neither DNP-lysine nor DNP_1-S-papain (which is mainly in the monomer form) are effective tolerogens. It should be noted that in all of these studies the quantity of antibody produced by responding fragments after tolerogen treatment was the same as untreated fragments. Thus, treatment of mature spleen cells with conjugates highly tolerogenic in other systems (14, 16, 18) appears irrelevant to either the number of responding cells or total antibody production under conditions described here. This is presumably the result of either efficient removal of tolerogen or extremely efficient stimulation in an environment primed to the relevant carrier.

Discussion

These studies were undertaken to determine whether B lymphocytes, during their development, mature through a stage in which they are extremely susceptible to tolerance induction. To examine this proposition, the response of splenic B cells from neonatal mice was analyzed. Neonatal spleens are presumably enriched in immature, developing cells and have been demonstrated to be highly susceptible to suppression by anti-immunoglobulin (23–25) or anti-idiotypic antibodies (26). The results in the present study indicate that B cells, early in their development, are readily tolerized in vitro in a highly specific manner, by low concentrations of hapten on a wide array of carriers, while the

TABLE III

The Induction of Tolerance in Fragment Cultures of Neonatal and Adult Spleen Cells by Various Hapten and Hapten-Protein Conjugates

Age of donor spleen cells	Total number of cells analyzed × 10^{-6} *	Tolerogen‡	Concentration	Stimulating antigen	Percent of control response
			moles hapten		
Adult (8–10 wk)	24	—	—	DNP-Hy	
	24	DNP-MγG	10^{-5}	DNP-Hy	90.0 ± 30.0
	16	DNP-HγG	10^{-5}	DNP-Hy	106.8 ± 14.0
	8	DNP-OVA	10^{-5}	DNP-Hy	100.0 ± 20.0
	16	TNP-D-GL	100 μg/ml	TNP-Hy	106.8 ± 37.2
	8	DNP-lysine	10^{-5}	DNP-Hy	100.0 ± 20.0
Neonatal (3 days)	24	—	—	DNP-Hy	
	24	DNP-MγG	10^{-6}	DNP-Hy	29.8 ± 10.0
	22.4	DNP-MγG	10^{-8}	DNP-Hy	24.0 ± 8.3
	14.4	DNP-MγG	10^{-9}	DNP-Hy	16.0 ± 4.0
	15.2	DNP-MγG	10^{-10}	DNP-Hy	72.2 ± 2.2
	16	DNP-HγG	10^{-6}	DNP-Hy	19.6 ± 10.0
	8	DNP-OVA	10^{-6}	DNP-Hy	20.0 ± 10.0
	8	TNP-D-GL	100 μg/ml	TNP-Hy	20.0 ± 10.0
	16	DNP-lysine	10^{-6}	DNP-Hy	125.0 ± 25.0
	8	DNP$_1$-S-papain	10^{-6}	DNP-Hy	70.0 ± 10.0

* 4×10^6 donor spleen cells were transferred to each recipient mouse.

‡ Fragments were cultured for 24 h in the presence or absence of hapten or hapten-carrier conjugates, washed, and stimulated with either DNP-Hy at 10^{-6} M DNP or TNP. Clones were detected by radioimmunoassay of culture fluids with ^{125}I-labeled antimouse Fab, IgM, or IgG$_1$.

vast majority of adult splenic B cells remain unaffected by antigen at these concentrations and concentrations which are four orders of magnitude greater.

These studies were facilitated by a modification of the in vitro splenic focus technique in which fragment cultures are derived from the spleens of irradiated, carrier-primed, syngeneic adult recipients of neonatal or adult B cells. These recipient fragment cultures permit the antigenic stimulation of almost all resident transferred hapten-specific precursor cells by providing all ancillary mechanisms of stimulation (32). Furthermore, hapten-specific B-cell tolerance can be studied without the complications of T-cell tolerance by using one hapten carrier for tolerance induction followed by challenge with the same hapten on a second carrier to which the recipient has been primed. In addition, the stimulation of each B cell in culture can be assessed in the absence of ambiguities introduced by cell population effects or the influence of cell localization patterns. Finally, donor cells are transferred in limited dilutions which minimizes the interaction between donor cells in the recipient fragments. Thus, tolerance induction or immune induction of neonatal and adult B cells can be compared at the level of individual B cells. In addition, this technique is currently the only available method that permits measurable stimulation of neonatal B cells under defined conditions and allows the characterization of the isotype and clonotype (individual antigenic specificity) of the antibody product of each stimulated B cell (29–32).

The results demonstrate that, at birth, more than 90% of DNP-specific splenic B cells are readily tolerized. Thereafter, the proportion of cells not susceptible to tolerance induction (adult-like B cells) constantly increases and attains adult levels by the 7th day after birth. Since the parameters of stimulation of BALB/c neonatal splenic B cells have been previously characterized (29) and the clonotypes of the majority of neonatal BALB/c cells responsive to DNP and TNP identified (30–32), it is now possible to compare precisely the parameters of tolerance induction and stimulation of these B cells. The findings show that the range of antigen concentrations capable of inducing tolerance (10^{-5} M–10^{-9} M haptenic determinant) closely reflects the concentrations that are optimal for the stimulation of these cells. The striking similarities between the two mechanisms is even more apparent when the exquisite specificity of tolerance and immune induction of these cells is compared. Previous studies from this laboratory (31, 32) have demonstrated that even though the antibodies, and presumably the cell receptors, of the three predominant neonatal anti-TNP clonotypes can bind DNP, nevertheless, DNP-Hy is unable to stimulate cells of these clonotypes. The interpretation of these findings was consistent with earlier postulates (28, 32) that the binding of antigen to cell receptors is necessary but not sufficient for stimulation. Furthermore, the specificity of antigenic stimulation of B cells appeared to be dependent on the intrinsic affinity of the receptor-antigen interaction (28, 32). Since the induction of tolerance in neonatal cells is equally specific and thus also consistent with these interpretations, these results strongly suggest that neonatal tolerance may also be viewed as a highly specific and receptor affinity-dependent event. Therefore, the ability of a cell to respond to either an immunogenic or tolerogenic signal would depend solely on the maturational stage of the B cell and the presence or absence of antigen-specific T cells. If tolerance induction is a significant physiological mechanism designed to eliminate B-cell specificities reactive to self-determinants, then it appears reasonable to expect that the induction of such a mechanism would require only low antigen concentrations, and that the specificity would be exquisite. In contrast to these findings, most previous demonstrations of induced hyporeactivity of adult B cells required relatively high antigen concentrations (4, 5, 43), and tolerance appeared to be less specific than stimulation. For example, the adult response to TNP-Hy is reduced by DNP-induced tolerance (16), and HyG tolerance, which eliminates most HyG binding B cells (2–4%), may eliminate many more B cells than comprise the HyG-specific precursor pool (22).

The ability to tolerize neonatal B cells by a wide range of effective carrier moieties is also consistent with the predictions for a physiologically relevant tolerance system. If the establishment of tolerance towards a vast array of self-constituents is to be effective, then a tolerance mechanism which would be limited to the narrow range of antigenic moieties thus far found to be effective in inducing adult B-cell unresponsiveness, such as immunoglobulins or amino acid copolymers, would seem too restricted. It would appear from the studies presented here that tolerance of neonatal B cells can be induced with hapten on almost any carrier, provided that the antigen is multivalent for the haptenic determinant. Therefore, tolerance induction, like immune induction, appar-

ently requires cross-linkage of receptors, as well as a receptor affinity for antigen that exceeds a minimal threshold, but no special requisites for the nature of the carrier moiety.

Tolerogenic vs. Immunogenic Signals. It is of considerable interest that hapten-carrier conjugates stimulated, rather than tolerized, developing B cells if the fragment cultures were derived from recipients primed to that carrier. Thus, antigen-specific T cells apparently play a pivotal role in the stimulation of neonatal B cells. This finding is reminiscent of the two-signal model of B-cell stimulation espoused by Bretcher and Cohn (44) which postulates that the interaction of antigen with cell receptors is tolerogenic unless a second signal is delivered optimally by an antigen-specific T cell. However, the results of the present study are contrary to the predictions of this theory in two major aspects. Firstly, tolerance induction appears to pertain only to developing B-cell populations. This is reassuring since if this theory were applicable to the adult B cell, the frequency of the two-party interaction of the antigen and B cell would far exceed the frequency of the three-party interaction involving the antigen, B cell, and specific T cell required for stimulation. Thus antigen contact with B cells, particularly before antigen priming of T cells, would presumably result in tolerance of all specific B cells. However, a "one signal" tolerance mechanism, allowing the elimination of self-reactivities in developing B cells, could be a highly efficient physiological process.

The evidence presented in this study also confirms previous reports that demonstrated that soluble antigens may bind to mature B cells and even inhibit stimulation, but, if the antigen is removed before stimulation, the B cells remain unaffected (45, 46). Therefore, it appears that the "one signal" mode (antigen binding to B cells) is irrelevant to mature B cells but tolerogenic to developing B cells (Table IV). However, for both cell types, interactions with antigen in conjunction with carrier-specific T cells is stimulatory. These results suggest that developing B cells may be prevented from being tolerized in the presence of carrier-specific T cells, and thus the recruitment of new (developing) B cells during an ongoing immune response would be permitted. It should be noted that a role for specific T cells in the induction of mature B-cell tolerance is currently a highly controversial area (4, 7, 12, 21). However, since tolerance induction of mature B cells is not observed in the splenic fragment culture system, the controversy is irrelevant to our findings. It would appear, however,

TABLE IV
Two and Three Party Interactions of Neonatal and Adult B Cells

Signal	Neonatal (immature) B cell	Adult (mature) B cell
Univalent antigens	Irrelevant	Irrelevant
Multivalent antigens*	Tolerogenic	Irrelevant
Multivalent antigens plus carrier-primed environment	Stimulatory	Stimulatory

* Antigen with multiple, identical determinants.

that tolerance induction of neonatal B cells does not require the presence of antigen-specific T cells.

The findings of this study differ from the classical two-signal model in a second important aspect: the demonstration that multivalent presentation of haptenic determinants maximizes tolerance induction in developing B cells. Even at 10^{-6} M DNP, neither DNP-lysine nor monovalent DNP_1-S-papain is an efficient tolerogen. Thus, the "first signal" apparently requires the interlinkage of receptors (multivalent binding) rather than the simple occupation of receptor sites. This result is contrary to the previous studies of adult spleen and bone marrow B cells which report that oligovalent antigens or protein monomers are effective tolerogens (2, 6, 12). Finally, since the neonate presumably does not have specific T cells or antibody with which to polymerize antigens, neonatal tolerance may pertain to antigens, such as those on cell surfaces, in which each determinant is presented multivalently, and not to some monomeric protein antigens.

Tolerance Induction and B-Cell Subpopulations. The neonatal spleen cells analyzed in this study are presumably a generative population, doubling in number approximately every 24 h, and should thus contain a higher percentage of developing cells than the adult spleen. The disparity in the tolerizability of cells from 1–3-day neonates vs. 7-day neonates or adult mice confirms this presumption and strongly suggests that the vast majority of splenic B cells are mature, as determined by their resistance to tolerance induction, by the 7th day after birth. This would imply that by 1-wk postparturition, the generative cell pool may reside primarily in the bone marrow. This is consistent with the finding that a sizeable percentage of the bone marrow cells are tolerizable well into the 2nd wk after birth and maintain at least 20% of the cell population susceptible to tolerance even in adulthood. (E. S. Metcalf and N. R. Klinman, unpublished observations, 27).

Previous studies have established that the specific antibody produced after stimulation of fetal and perinatal B cells is primarily of the IgM isotype (31, 32, 47). Although in 3-day spleens both the IgM and IgG_1 responses are markedly reduced by tolerance induction, the precursors that produce only IgM are by far the most susceptible cells. This result provides additional evidence that the least mature precursor cells are the most easily tolerized. Unfortunately, it is not yet possible to correlate tolerance susceptibility with other "differentiation markers." Recent reports have demonstrated that 10–20% of adult spleen cells are Ia negative within the limits of the experimental design, and this population represents the majority of B cells whose clonal progeny produce only IgM antibodies (48, 49). However, since IgM-producing clones are apparently not affected by tolerance induction of adult spleen cells, it is unlikely that the absence of Ia determinants on B cells correlates with the capacity to be tolerized. A similar argument would presumably apply to the 20% of adult splenic B cells which are surface IgM positive but lack the "IgD" analogue (50). Furthermore, the resistance to tolerance susceptibility does not correlate at all with the published kinetics of the neonatal acquisition of "IgD" which is present on only a small minority of splenic B cells even at day 7 after birth (51).

The findings reported here extend previous reports that neonatal (developing)

B cells have several properties which distinguish them from adult primary B cells and indicate that they may constitute a definable subpopulation (32). It is conceivable that the ability to be tolerized in vitro may eventually serve as the ultimate marker for developing B cells and B-cell maturation. This criterion has already established that the majority of B cells in the adult murine spleen, even those that are apparently Ia or "IgD" negative, are mature, whereas some neonatal B cells capable of IgG$_1$ production may be immature. Perhaps the most important question in this regard is whether the bone marrow serves as an important generative source of cells throughout life. The findings of Nossal and Pike (27) and preliminary results from our laboratory indicate that bone marrow does contain cells, primarily precursors to clones producing only IgM antibodies, which are susceptible to tolerance induction. A detailed analysis of such cells and their antibody clonotypes may greatly increase our understanding of the ongoing generation of specific B cells.

Tolerance Induction and the Acquisition of the B-Cell Specificity Repertoire. Recent evidence, derived principally from the analysis of neonatal splenic B cells, has argued that the acquisition of the B-cell specificity repertoire is a highly ordered, genetically determined process (31, 32, 52-54). "Germ line" theories of diversity generation, even those postulating the predetermined somatic variation of "germ line" information (32, 52, 53), rely heavily on evolutionary selective forces to provide a highly selected repertoire. Within this context, the existence of a tolerance mechanism, which eliminates potentially harmful specificities during development, would seem anachronistic. However, given the apparent genetic polymorphic expression of various antigenic determinants, particularly cell-associated antigenic determinants, all theories of diversity generation would require a mechanism whereby the F$_1$ generation recognized parental determinants as self. In this regard, a highly specific, widely applicable, and extremely sensitive tolerance mechanism which pertains to developing B cells would seem ideal.

Thus it has been established in this report that only during their development can B cells be tolerized in vitro in a highly specific manner, by low concentrations of a wide array of multivalent antigens. This in vitro tolerance system should provide a model with which the molecular mechanism(s) of tolerance induction to self-antigens may be more thoroughly investigated.

Summary

The susceptibility of neonatal and adult B lymphocytes to tolerance induction was analyzed by a modification of the in vitro splenic focus technique. This technique permits stimulation of individual hapten-specific clonal precursor cells from both neonatal and adult donors. Neonatal or adult BALB/c spleen cells were adoptively transferred into irradiated, syngeneic, adult recipients which had been carrier-primed to hemocyanin (Hy), thus maximizing stimulation to the hapten 2,4-dinitrophenyl coupled to Hy (DNP-Hy). Cultures were initially treated with DNP on several heterologous (non-Hy) carriers and subsequently stimulated with DNP-Hy. Whereas the responsiveness of adult B cells was not diminished by pretreatment with any DNP conjugate, the majority of the neonatal B-cell response was abolished by in vitro culture with all of the

DNP-protein conjugates. During the 1st wk of life, the ability to tolerize neo-natal splenic B cells progressively decreased. Thus, tolerance in this system is: (a) restricted to B cells early in development; (b) established by both tolerogens and immunogens; (c) achieved at low (10^{-9} M determinant) antigen concentrations; and (d) highly specific, discriminating between DNP- and TNP-specific B cells. We conclude that: (a) B lymphocytes, during their development, mature through a stage in which they are extremely susceptible to tolerogenesis; (b) the specific interaction of B-cell antigen receptors with multivalent antigens, while irrelevant to mature B cells, is tolerogenic to neonatal (immature) B cells unless antigen is concomitantly recognized by primed T cells; and (c) differences in the susceptibility of immature and mature B lymphocytes to tolerance induction suggest intrinsic differences between neonatal and adult B cells and may provide a physiologically relevant model for the study of tolerance to self-antigens.

References

1. Medawar, P. B. 1956. A discussion on immunological tolerance. *Proc. Roy. Soc. London Ser. B.* 146:1.
2. Dresser, D. W., and N. A. Mitchison. 1968. The mechanism of immune paralysis. *Adv. Immunol.* 8:129.
3. Burnet, F. M. 1959. The clonal selection theory of acquired immunity. Cambridge University Press, New York.
4. Weigle, W. O. 1973. Immunological unresponsiveness. *Adv. Immunol.* 16:61.
5. Taylor, R. B. 1969. Cellular cooperation in the antibody response of mice to two serum albumins: specific function of thymus cells. *Transplant. Rev.* 1:114.
6. Chiller, J. M., J. A. Louis, B. J. Skidmore, and W. O. Weigle. 1974. *In* Immunological Tolerance. Mechanisms and potential therapeutic applications. D. H. Katz, and B. Benacerraf, editors. Academic Press, Inc., New York. 373.
7. Gershon, R. K., and K. Kondo. 1971. Infectious immunological tolerance. *Immunology* 18:723.
8. Tada, T., and T. Takemori. 1974. Selective roles of thymus-derived lymphocytes in the antibody response. I. Differential suppressive effect of carrier-primed T cells on hapten-specific IgM and IgG antibody responses. *J. Exp. Med.* 140:239.
9. Basten, A., J. F. A. P. Miller, J. Sprent, and C. Cheers. 1974. Cell-to-cell interaction in the immune response. X. T-cell-dependent suppression in tolerant mice. *J. Exp. Med.* 140:199.
10. Benjamin, D. C. 1975. Evidence for specific suppression in the maintenance of immunological tolerance. *J. Exp. Med.* 141:635.
11. Nossal, G. J. V., and B. L. Pike. 1974. Immunological tolerance. *In* Mechanisms and Potential Therapeutic Applications. D. H. Katz and B. Benacerraf, editors. Academic Press, Inc., New York. 351.
12. Schrader, J. W. 1975. The in vitro induction of immunological tolerance in the B lymphocyte by oligovalent thymus-dependent antigens. *J. Exp. Med.* 141:962.
13. Havas, H. F. 1969. The effect of the carrier protein on the immune response and on the induction of tolerance in mice to the 2,4-dinitrophenyl determinant. *Immunology.* 17:819.

14. Borel, Y., and L. Kilham. 1974. Carrier determined tolerance in various strains of mice: the role of isogenic IgG in the induction of hapten-specific tolerance. *Proc. Soc. Exp. Biol. Med.* 145:470.

15. Walters, C. S., J. W. Moorehead, and H. N. Claman. 1972. Immunity and tolerance to a hapten (NIP) coupled to an isologous carrier (mouse γ-globulin). *J. Exp. Med.* 136:546.

16. Schrater, A. F. 1976. Induction of hapten-specific unresponsiveness at the level of the clonal precursor B cell. *Fed. Proc.* 35:1582 (Abstr.).

17. Hamilton, J. A., and J. F. A. P. Miller. 1974. Hapten-specific tolerance in mice. I. Induction with hapten-coupled syngeneic erythrocytes. *Eur. J. Immunol.* 4:261.

18. Katz, D. H., T. Hamaoka, and B. Benacerraf. 1972. Immunological tolerance in bone marrow-derived lymphocytes. I. Evidence for the inactivation of hapten-specific precursors of antibody-forming cells. *J. Exp. Med.* 136:1404.

19. Howard, J. G. 1972. Cellular events in the induction and loss of tolerance to pneumococcal polysaccharides. *Transplant. Rev.* 8:50.

20. Feldmann, M. 1972. Induction of immunity and tolerance in vitro by hapten-protein conjugates in vitro. I. The relationship between the degree of hapten conjugation and the immunogenicity of dinitrophenylated polymerized flagellin. *J. Exp. Med.* 135:735.

21. Kindred, B. 1975. Specific unresponsiveness in nude mice given antigen before T cells. *Eur. J. Immunol.* 5:609.

22. Louis, J. A., J. M. Chiller, and W. O. Weigle. 1973. Fate of antigen binding cells in unresponsive and immune mice. *J. Exp. Med.* 137:461.

23. Raff, M. C., J. J. T. Owen, M. D. Cooper, A. R. Lawton III, M. Megson, and W. E. Gathings. 1975. Differences in the susceptibility of mature and immature mouse B lymphocytes to anti-immunoglobulin-induced immunoglobulin suppression in vitro. *J. Exp. Med.* 142:1052.

24. Kearney, J. F., M. D. Cooper, and A. R. Lawton. 1976. B lymphocyte differentiation induced by lipopolysaccharide. III. Suppression of B cell maturation by anti-mouse immunoglobulin antibodies. *J. Immunol.* In press.

25. Sidman, C. L., and E. R. Unanue. 1975. Receptor-mediated inactivation of early B lymphocytes. *Nature (Lond.)* 257:149.

26. Strayer, D. S., W. M. F. Lee, D. A. Rowley, and H. Köhler. 1975. Anti-receptor antibody. II. Induction of long-term unresponsiveness in neonatal mice. *J. Immunol.* 114:728.

27. Nossal, G. J. V., and B. L. Pike. 1975. Evidence for the clonal abortion theory of B-lymphocyte tolerance. *J. Exp. Med.* 141:904.

28. Klinman, N. R. 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. *J. Exp. Med.* 136:241.

29. Press, J. L., and N. R. Klinman. 1973. Enumeration and analysis of antibody-forming cell precursors in the neonatal mouse. *J. Immunol.* 111:829.

30. Press, J. L., and N. R. Klinman. 1974. Frequency of hapten-specific B cells in neonatal and adult murine spleens. *Eur. J. Immunol.* 4:155.

31. Klinman, N. R., and J. L. Press. 1975. The characterization of the B-cell repertoire specific for the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl determinants in neonatal BALB/c mice. *J. Exp. Med.* 141:1133.

32. Klinman, N. R., and J. L. Press. 1975. The B cell specificity repertoire: its relationship to definable subpopulations. *Transplant. Rev.* 24:41.

33. Chiller, J. M., G. S. Habicht, and W. O. Weigle. 1970. Cellular sites of immunologic unresponsiveness. *Proc. Natl. Acad. Sci. U. S. A.* 65:551.

34. Rowe, D., and J. L. Fahey. 1965. A new class of human immunoglobulins. II. Normal serum IgD. *J. Exp. Med.* 121:185.

35. Eisen, H. N. 1964. Some methods applicable to the study of experimental hypersensitivity. *Methods Med. Res.* 10:94.
36. Rittenberg, M. B., and A. A. Amkraut. 1966. Immunogenicity of trinitrophenyl hemocyanin. Production of primary and secondary anti-hapten precipitins. *J. Immunol.* 97:421.
37. Inman, J. K., B. Merchant, and S. E. Tacey. 1973. Synthesis of large haptenic compounds having a common functional group that permits covalent linkage to proteins, cell surfaces, and adsorbents. *Immunochemistry.* 10:153.
38. Trump, G. N., and S. J. Singer. 1970. Electrophoretically homogeneous anti-DNP antibodies with restricted isoelectric points elicited in mice by immunization with the antigen papain-S-DNPL. *Proc. Natl. Acad. Sci. U. S. A.* 66:411.
39. Klinman, N. R., and R. Taylor. 1969. General methods for the study of cells and serum during the immune response: the response to dinitrophenyl in mice. *Clin. Exp. Immunol.* 4:473.
40. Press, J. L., and N. R. Klinman. 1973. Monoclonal production of both IgM and IgG$_1$ antihapten antibody. *J. Exp. Med.* 138:300.
41. Janeway, C. A., Jr. 1975. Cellular cooperation during *in vivo* antihapten antibody responses. I. The effect of cell number on the response. *J. Immunol.* 114:1394.
42. Mosier, D. E., and B. M. Johnson. 1975. Ontogeny of mouse lymphocyte function. II. Development of the ability to produce antibody is modulated by T lymphocytes. *J. Exp. Med.* 141:216.
43. Kettman, J. 1974. In vitro induction of specific unresponsiveness against the 2,4,6-trinitrophenyl determinant. I. The induction of unresponsiveness. *J. Immunol.* 112:1139.
44. Bretscher, P. A., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science (Wash. D. C.).* 189:1042.
45. Klinman, N. R. 1971. The secondary immune response to a hapten in vitro: antigen concentration and the carrier effect. *J. Exp. Med.* 133:963.
46. Feldmann, M. 1972. Induction of immunity and tolerance in vitro by hapten-protein conjugates. III. Hapten inhibition studies of antigen binding to B cells in immunity and tolerance. *J. Exp. Med.* 136:532.
47. Klinman, N. R., and J. L. Press. 1975. Expression of specific clones during B cell development. *Fed. Proc.* 34:47.
48. Press, J. L., N. R. Klinman, C. Henry, L. Wofsy, T. L. Delovitch, and H. O. McDevitt. 1975. Ia antigen on B cells. Relationships to B cell precursor function and to surface immunoglobulin. *In* Proceedings of the International Symposium on Membrane Receptors of Lymphocytes. M. Seligmann, J. L. Preud'homme, and F. M. Kourilsky, editors. Elsevier, Amsterdam. 274.
49. Press, J. L. 1975. Splenic expression of Ia alloantigens during murine neonatal development. *Fed. Proc.* 34:945 (Abstr.)
50. Vitetta, E. S., and J. Uhr. 1975. Immunoglobulin-receptors revisited. *Science (Wash. D. C.). 189:*964.
51. Vitetta, E. S., U. Melcher, M. McWilliams, M. E. Lamm, J. M. Phillips-Quagliata, and J. Uhr. 1975. Cell surface immunoglobulin. XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. *J. Exp. Med.* 141:206.
52. Klinman, N. R., J. L. Press, N. H. Sigal, and P. J. Gearhart. 1976. The acquisition of the B cell specificity repertoire: the germ line theory of predetermined permutation of genetic information. *In* The Generation of Diversity: A New Look. A. J. Cunningham, editor. Academic Press, Inc., New York. In press.
53. Sigal, N. H., P. J. Gearhart, J. L. Press, and N. R. Klinman. 1976. Late acquisition of a germ line antibody specificity. *Nature (Lond.).* 259:51.
54. Sherwin, W. K., and D. T. Rowlands, Jr. 1975. Determinants of the hierarchy of humoral immune responsiveness during ontogeny. *J. Immunol.* 115:1549.