THE INDUCTION OF HAPTEN-SPECIFIC IMMUNOLOGICAL TOLERANCE AND IMMUNITY IN B LYMPHOCYTES

VI. Differential Tolerance Susceptibility in Adult Spleen as a Function of B-Cell Maturation Level*

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It has been generally accepted that neonatal animals are more susceptible to the induction of immunological tolerance than adults. This concept was originally based upon studies of intact animals performed before the realization that a number of interacting cells are involved in immune responses, any of which may serve as the target for tolerance induction (1–3). More recently, this enhanced neonatal suscepti-bility to tolerogenesis in humoral immunity has been studied at the cellular level. Although initially thought to be a result of increased suppressor thymic-derived $(T)^1$ cell activity in neonatal spleens (4), this heightened susceptibility to tolerance induction was recently shown to reside in neonatal B lymphocytes (5–8).

Studies of the ontogeny of the bone marrow (bursal equivalent)-derived (B) cell response to thymic independent (TI) antigens have led to the classification of B cells into two functional categories (9). Neonatal B cells respond to TI-1 antigens (such as trinitrophenylated lipopolysaccharide, TNP-LPS) and to the mitogen, LPS, but are refractory to stimulation by TI-2 antigens (like TNP-Ficoll) and the mitogen, purified protein derivative (PPD) (9, 10). B lymphocytes from adult mice, however, respond to all of these agents. Supporting the division of B lymphocytes into mature, adult type (TI-2) and immature, neonatal-type (TI-1) B cells is the responsiveness and lymphocyte constitution of the CBA/N mouse (9, 10). Bearing an x-linked defect in responses to TI antigens (11), CBA/N mice exhibit a decreased number of surface immunoglobulin (sIg)-bearing lymphocytes (12) and sIg and μ densities (13) suggestive of a deficit of mature B lymphocytes. Mice bearing this defect react to stimulation by TI-1 antigens and the mitogen, LPS (9, 10). However, they are unreactive to TI-2 antigens and are markedly hyporesponsive to mitogenic stimulation by PPD (9, 10). Thus, responses elicited by TI-1 or TI-2 antigens, or nonspecific polyclonal activators are characteristic of the maturation level of the B cells involved, and may then be

¹ *Abbreviations used in this paper:* B cell, antigen-sensitive bone marrow (bursal equivalent)-derived lymphocyte; BSS, balanced salt solution; BSS-FCS, balanced salt solution with 10% FCS; FCS, fetal calf serum; HRC, horse erythrocytes; LPS, lipopolysaccharide; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; PFC, plaque forming cell; PPD, purified protein derivative; sIg, surface immunoglobulin; SRC, sheep erythrocytes; T cell, antigen-reactive thymic-derived lymphocyte; TD, thymic dependent; TI, thymic independent; TNBS, trinitrobenzenesulfonic acid; TNP, trinitrophenyl.

used to functionally identify the state of maturity of the reactive B-lymphocyte subpopulations.

In this communication, the maturity of B cells involved in the induction of tolerance by trinitrobenzenesulfonic acid (TNBS) (14–16) was investigated. The maturation level of the B cells being studied was defined by the antigen or polyclonal activator which elicited a response. Thus, the magnitude of the hapten-specific response induced by TI-1 or TI-2 antigens, or the number of TNP-specific plaque-forming cells (PFC) resulting from stimulation by polyclonal activators was used as a measure of the tolerogenic effect of TNBS injection upon either the mature adult-type (TI-2) or immature neonatal-type (TI-1) B cells of the adult spleen. Tolerance to TNP was induced more rapidly after TNBS injection in immature than in mature B cells. Moreover, a lower dose of tolerogen was required to induce a state of unresponsiveness in neonatal-type than in adult-type B cells. These results support the concept of differential tolerance susceptibility in two subpopulations of B lymphocytes present in adult spleen, the immature neonatal-type B cells exhibiting heightened susceptibility in comparison with the mature adult-type B lymphocytes.

Materials and Methods

Mice.   Male CBA/CaJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, or were bred at the Scripps Clinic and Research Foundation, La Jolla, Calif. All mice were used at 8–12 wk of age and were generally the same age within an experiment.

Antigens and Polyclonal Activators.   The preparation, storage, and use of TNP-LPS (nine TNP residues per 100,000 daltons of LPS) were as described previously (17). 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP)-LPS (11 NIP residues per 100,000 daltons of LPS) was prepared by mixing 25 mg of LPS and 20 mg of NIP-azide in 0.1 M bicarbonate buffer for 12 h at 4°C with constant stirring. After extensive dialysis against phosphate-buffered saline, the conjugate was stored at −20°C. LPS, prepared from *Escherichia coli* 0111:B4 by the trichloroacetic acid (Boivin) extraction procedure, was purchased from Difco Laboratories, Detroit, Mich. TNP was conjugated to Ficoll (TNP-Ficoll, 68 mol TNP/mol of Ficoll) as described previously (18) using Ficoll 400 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). The immunizing dose of TNP-LPS, NIP-LPS, and TNP-Ficoll was 10 ng/1 ml culture of 10×10^6 viable spleen cells. PPD, extracted from *Mycobacterium tuberculosis* RT33, was obtained from Statens Seruminstitut, Copenhagen, Denmark. Sheep erythrocytes (SRC) and horse erythrocytes (HRC) were obtained in Alsever's solution from the Colorado Serum Co., Denver, Colo., and from a single sheep and horse maintained by the Scripps Clinic and Research Foundation. HRC were heavily conjugated with TNP (TNP-HRC) essentially as described by Kettman and Dutton (19), whereas the method of Rittenberg and Pratt was used for preparation of lightly conjugated TNP-HRC (20).

Tolerogen.   Tolerance to TNP was induced in vivo by the i.p. injection of TNBS (Eastman Kodak Co., Rochester, N. Y.) prepared by dissolving 100 mg of TNBS and 28.65 mg of Na-HCO₃ in 10 ml of 0.15 M NaCl (14, 21). The tolerogen was administered by injection of 0.5 ml/mouse of this TNBS solution or dilutions thereof in saline.

Tissue Culture.   Single cell suspensions were prepared as previously described (22, 23) in balanced salt solution (BSS) buffered with Hepes (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), isoosmotic with mouse serum (308 mosmol, equivalent to 0.168 M NaCl) (22, 24), and containing 10% fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N. Y.) (BSS-FCS). The cells were resuspended and cultured in serum-free medium consisting of: RPMI-1640 medium made from powder (Grand Island Biological Co.) to be isoosmotic with mouse serum, 0.034% NaHCO₃, 0.2 mM glutamine, and 0.95 mM sodium pyruvate (all Microbiological Associates, Walkersville, Md.), 100 U/ml penicillin (Eli Lilly & Co., Indianapolis, Ind.), 100 µg/ml streptomycin (Eli Lilly & Co.), and 10 mM Hepes.

A modification of the Mishell-Dutton culture system (25) was used as described previously (16). Plastic tissue culture trays (3008, Falcon Labware, Div. of Becton, Dickinson, & Co.,

Oxnard, Calif.) were used to culture 10×10^6 viable spleen cells in 1 ml of medium. Cells were prepared so that 0.9 ml of cell suspension was added per culture, which was then supplemented with 0.1 ml of antigen or polyclonal activator in medium. All experiments included cultures receiving medium only to serve as background controls.

In some cases, spleen cells were incubated without the addition of antigen for 24 h, followed by washing three times and the addition of antigen upon reculturing (16).

Assay of Antibody-Forming Cells. Direct anti-SRC, anti-TNP, and anti-NIP PFC were assayed by a modification of the Cunningham plaque technique as described previously (17, 23, 26, 27). High affinity TNP-specific PFC were assayed using lightly coupled TNP-HRC and subtracting the PFC produced against unconjugated HRC. The low affinity TNP-specific PFC response was determined using heavily conjugated TNP-HRC and subtracting the anti-HRC and high affinity anti-TNP responses.

Results

Kinetics of TNBS Tolerance. It was demonstrated previously that spleen cells from mice given 5 mg of TNBS 4 d earlier are specifically tolerant to the TNP hapten in response to immunogenic challenge with TI and thymic-dependent (TD) forms of the hapten in vivo and in vitro (14–16, 28). Although it has been suggested that hapten-specific B-cell unresponsiveness in systems similar to this is maintained by blocking of sIg molecules by tolerogen (29, 30), receptor blockade is clearly not responsible for the maintenance of tolerance in this system. Functional deletion of TNP-specific B cells appears to be the mode of TNBS-induced tolerance. Examination at shorter time intervals after the tolerizing injection was undertaken to obtain information on the induction phase of tolerance. Accordingly, spleen cells from mice injected with TNBS at one of a series of intervals before sacrifice were placed in vitro with TNP-LPS and NIP-LPS. As shown in Fig. 1, unresponsiveness to TNP was rapidly induced with the TNP-specific responsiveness being reduced by 89% 1 h after TNBS injection. A plateau level of virtually complete tolerance was obtained by 12 h after tolerogen administration which persisted through day 4 as shown previously (16). Responses to the control NIP hapten were not reduced by TNBS administration.

Cocultures of Tolerant and Normal Cells. To investigate the carryover of tolerogen by TNBS-treated spleen cells which might inhibit the response of, or induce tolerance in, other splenocytes, a cell mixing experiment was conducted with spleen cells from the experiments shown above. Spleen cells from mice injected with the TNBS from 1 h to 4 d before sacrifice were cultured with equal numbers of untreated control spleen cells. Based upon the results obtained in the above experiment (Fig. 1), any effect of one preparation upon the other cell population could be assessed. There was no significant reduction in the anti-NIP response of control splenocytes by tolerant spleen cells (Fig. 2). The 1-h TNBS-treated group reduced the anti-TNP response of the control cells such that only 3% of the expected PFC resulted. At 12 h, the reduction was 36%, and there appeared to be no significant decrease past this point. These results suggest that a large measure of the reduction in response observed with the 1-h group and part of that with the 12-h group may be a result of suppression mediated in vitro. The suppression could result from the elution of sIg-bound blockading moieties and binding to and inhibition of the response of other TNP-specific lymphocytes.

Antigen-Free Incubation of Unresponsive Cells. The concept of receptor blockade as the cause of hapten-specific unresponsiveness was supported in experiments of Gronowicz and Coutinho (29) and Aldo-Benson and Borel (30) in which antigen-free cultivation
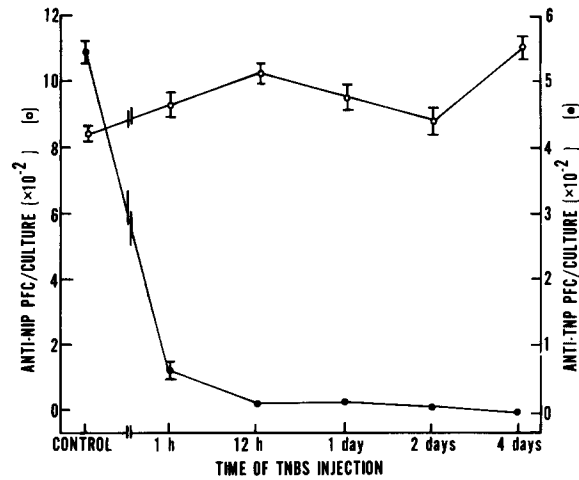
FIG. 1. Kinetics of TNBS tolerance assessed in vitro. Spleen cells from mice either injected i.p. with 5 mg of TNBS in 0.5 ml of saline at one of a series of intervals before sacrifice or left untreated were placed in culture with 10 ng of TNP-LPS and 10 ng of NIP-LPS. Cultures were harvested for assay on day 2. Values represent anti-NIP and anti-TNP PFC/culture ± SE of three cultures per group assayed individually. Data from four experiments are pooled. Background responses are subtracted. Abscissa, interval between TNBS injection and in vitro culture; ordinate, anti-NIP and anti-TNP PFC/culture.
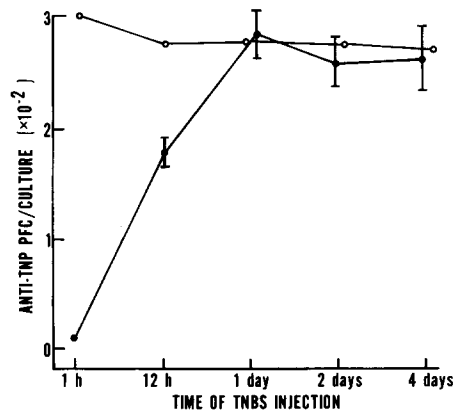

FIG. 2. Coculture of TNBS-treated and control spleen cells. Spleen cells from mice injected i.p. with 5 mg of TNBS in 0.5 ml of saline at one of a number of intervals before sacrifice were cultured together with spleen cells from untreated mice (5 × 10⁶ viable cells of each type/1 ml culture). Cultures received 10 ng of TNP-LPS and 10 ng of NIP-LPS and were harvested for assay on day 2. Values represent anti-NIP and anti-TNP PFC/culture ± SE of three cultures per group assayed individually. Data are pooled from four experiments. Background responses are subtracted. The expected response is the number of PFC that would be obtained if no interaction of either a suppressing or enhancing nature took place between the two types of spleen cells. The observed response ± SE and the expected response in terms of anti-NIP PFC/culture were as follows: TNBS day 4: 1026 ± 82 (977); TNBS day 2: 920 ± 39 (868); TNBS day 1: 883 ± 52 (901); TNBS 12 h: 787 ± 33 (937); and TNBS 1 h: 953 ± 51 (891). Abscissa, interval between TNBS injection and in vitro culture; ordinate, expected and observed anti-TNP PFC/culture. O, calculated response; ●, observed response.
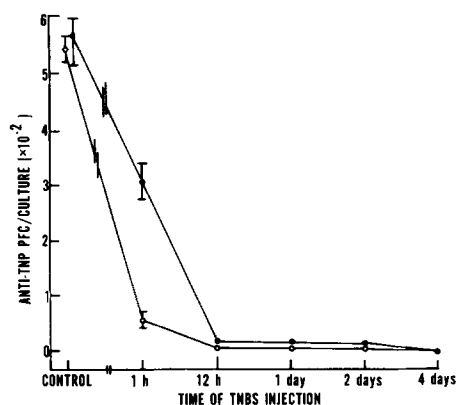
Fig. 3. Effect of antigen-free culture on TNBS tolerance. Spleen cells from mice either injected i.p. with 5 mg of TNBS in 0.5 ml of saline at one of a series of intervals before sacrifice or left untreated were placed in culture without antigen. After 24 h, the cultures were harvested, pooled within groups, and washed three times by dilution with BSS-FCS followed by centrifugation through FCS. After washing, the cells were diluted in medium and cultured with 10 ng of TNP-LPS and 10 ng of NIP-LPS per culture. All cultures were harvested for assay 2 d after the addition of antigen. Values represent anti-NIP and anti-TNP PFC per culture ± SE of three cultures per group assayed individually. Data are pooled from four experiments. Background responses are subtracted. The anti-NIP PFC/culture responses were: TNBS day 4: 910 ± 85; TNBS day 2: 696 ± 27; TNBS day 1: 1012 ± 41; TNBS 12 h: 900 ± 79; TNBS 1 h: 817 ± 34; and no TNBS: 928 ± 53. O, normal; ●, preincubation for 24 h.

was successful in reversing an apparent tolerant state. Although shown previously to be ineffective in breaking tolerance 4 d after TNBS injection, it was possible that the early stages of unresponsiveness could be reversed by such a maneuver.

To further investigate this point, the effect of 24-h antigen-free incubation of spleen cells from TNBS-injected mice, followed by extensive washing and challenge with antigen, was tested. The results of this experiment using spleen cells from the experiments described above are shown in Fig. 3. Confirming previous results (16), tolerance in the cultures of the day 4 group was not reversed by 24-h antigen-free incubation. Spleen cells from the mice sacrificed 1 h after tolerogen injection gave 55% of the control level TNP-specific response after the 24-h cultivation period. By 12 h after TNBS administration, however, there was no longer any significant recovery from TNBS-induced tolerance effected by antigen-free culture. Thus, in the period between 1 and 12 h after TNBS injection, during which splenocytes were losing the capacity to inhibit the response of other cells, the tolerance reached a stage where it could not be reversed by antigen-free incubation. Furthermore, tolerance induced by TNBS and assessed in vitro is initially characterized by a partially reversible blockade, and suggests that tolerogen carryover is responsible.

TNBS Tolerance and the TNP-Ficoll Response. Based upon an analysis of the maturation state of cells responding to TI antigens, it has been suggested that the B cells reactive with TNP-LPS (a TI-1 antigen) are less mature than B lymphocytes responding to a number of other TI antigens (9). To extend the above observations, the antigen-specific response to a TI antigen stimulating a more mature subset of B cells was investigated.

The TI-2 antigen TNP-Ficoll was used in an experiment identical to those shown
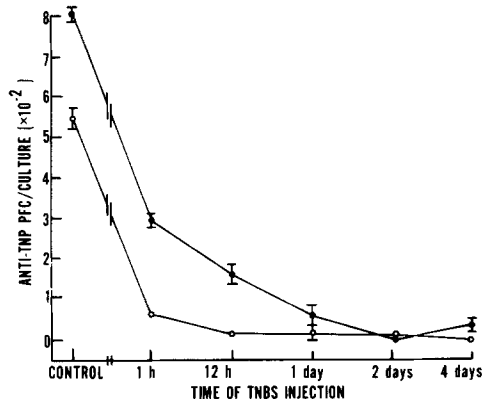
Fig. 4.   Kinetics of TNBS tolerance: challenge with TNP-Ficoll in vitro. Information is identical to Fig. 1 except that TNP-Ficoll was utilized rather than TNP-LPS. The anti-NIP PFC per culture responses were: TNBS day 4: 777 ± 26; TNBS day 2: 810 ± 33; TNBS day 1: 880 ± 53; TNBS 12 h: 986 ± 27; TNBS 1 h: 829 ± 36; and no TNBS: 760 ± 29. Data from two experiments are pooled. ○, TNP-LPS; ●, TNP-Ficoll.

in Figs. 1–3. Thus, spleens from mice injected with TNBS at one of a number of intervals earlier were placed in culture with TNP-Ficoll. As shown in Fig. 4, complete tolerance was evident when cells were cultured 1, 2, and 4 d after the TNBS injection. When examined 1 or 12 h after tolerogen treatment, tolerance was incomplete with 28 and 20% of the control response obtained, respectively. This compares to an 11 and 2% response at the 1- and 12-h points with TNP-LPS challenge. Thus, it appears that complete unresponsiveness to TNP was induced earlier in B cells responding to TNP-LPS than to TNP-Ficoll. This further suggests that TNBS induces tolerance more rapidly in those immature cells which are responsive to a TI-1 antigen than in the more mature cells reactive with a TI-2 antigen.

Coculture and the TNP-Ficoll Response.   To further investigate the response to TNP-Ficoll of TNBS-treated spleen cells, tolerant splenocytes were cultured in equal numbers with spleen cells from untreated mice. There was little or no reduction of the control anti-TNP response by the cells from mice treated with TNBS 1, 2, and 4 d earlier (Fig. 5). The only group causing significant reduction in the response from untreated spleen cells received TNBS 1 h before culture where a 60% reduction was evident. This result may be compared to the 97 and 36% reduction in the response to TNP-LPS caused by the 1 and 12 h groups, respectively. Thus, the response to TNP-Ficoll by control cells was inhibitable for a shorter time and to a lesser extent by cells from tolerant mice than the response to TNP-LPS.

Antigen-Free Culture and the TNP-Ficoll Response.   The effect of 24 h of antigen-free in vitro cultivation upon the PFC response to TNP-Ficoll was next investigated. As shown in Fig. 6, this incubation did not reverse the state of unresponsiveness induced by TNBS injection. Essentially the same level of response was obtained after a 24-h cultivation of TNBS-treated spleen cells as exhibited by splenocytes challenged with antigen at the initiation of culture (Fig. 4). Tolerance was maintained in the 2- and 4-d groups, whereas an anti-TNP PFC response of 38, 20, and 18% of the control level was induced by TNP-Ficoll in spleen cells from mice given TNBS 1, 12, and 24 h before culture, respectively. These results suggest that TNP-Ficoll-reactive (TI-2) B
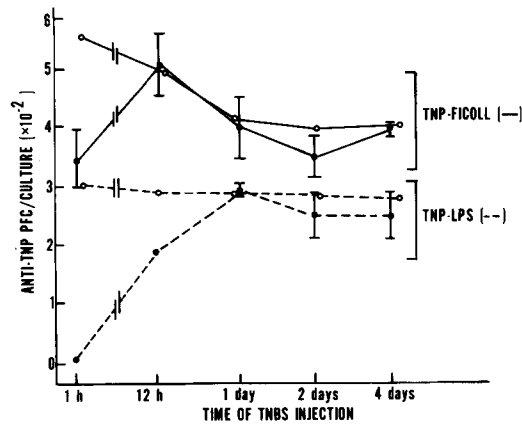
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Fig. 5. Coculture of TNBS-treated and control spleen cells: response to TNP-Ficoll in vitro. Information is identical to Fig. 2 except that TNP-Ficoll was used in place of TNP-LPS. The observed response ± SE and the expected response in terms of anti-NIP PFC/culture were as follows: TNBS day 4: 884 ± 45 (769); TNBS day 2: 796 ± 33 (785); TNBS day 1: 882 ± 38 (773); TNBS 12 h: 753 ± 38 (873); and TNBS 1 h: 914 ± 45 (794). Data are pooled from two experiments. O, calculated response; ●, observed response.

lymphocytes are less susceptible to the blockade that occurs early after tolerogen injection and require a longer period of time for the induction of tolerance than TI-1 B cells responsive to TNP-LPS.

Polyclonal Activation and TNBS Tolerance Kinetics. The lack of TNP-specific response by splenocytes from mice treated with TNBS 4 d before culture to the polyclonal activator LPS was used in a previous study to establish functional deletion as the mechanism of TNBS tolerance (16). To further investigate the induction of tolerance with TNBS, the response to polyclonal B-cell activation of spleen cells from mice injected with TNBS 1 h to 4 d previously was determined. In addition to LPS which activates cells in a less mature B-lymphocyte subset, the mitogen PPD was used as a polyclonal activator of more mature B cells (10, 31). HRC were conjugated lightly or heavily with TNP and used for the determination of low and high affinity TNP-specific PFC as described in Materials and Methods. In all cases, the PFC response to SRC induced by the polyclonal activator and used as a control for the hapten-specific effect of TNBS was relatively unchanged in the TNBS-treated groups (Fig. 7). The high affinity anti-TNP PFC induced by LPS, which are the same type assayed in specific anti-TNP responses, were not reduced by TNBS treatment 1 h before tissue culture. By 12 h, the high affinity PFC were reduced 55% and reached a low plateau level of 4–12% of the control level response 1–4 d after TNBS injection. The low affinity response was not greatly reduced (15 to 20% decrease) until 4 d after TNBS administration when it was 44% of the control level.

With PPD, the high affinity TNP response was at a high plateau level for 1 h to 1 d after TNBS injection. On days 2 and 4, the response was reduced to 12–17% of the control level. The low affinity response was stable at a high level from 1 h to 2 d after TNBS injection, with the response of cells taken 4 d after tolerogen administration dropping to 40% of the control level. A number of conclusions may be drawn from these data: (a) there is a greater effect of the tolerogen upon the production of high affinity than low affinity PFC, suggesting that the precursor B cells bearing high
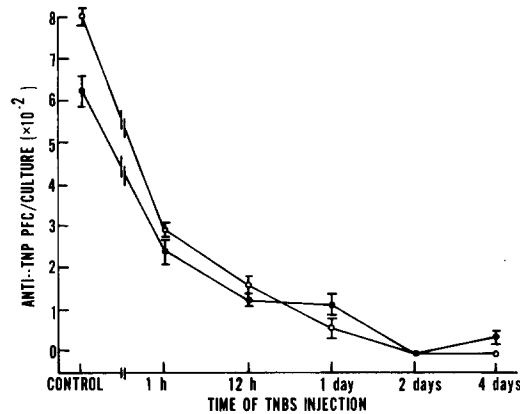
Fig. 6.   Effect of antigen-free culture on TNBS tolerance: TNP-Ficoll response. Information is identical to Fig. 3 except that TNP-Ficoll was used in place of TNP-LPS. The anti-NIP PFC per culture response was: TNBS day 4: 897 ± 53; TNBS day 2: 937 ± 39; TNBS day 1: 915 ± 41; TNBS 12 h: 958 ± 56; TNBS 1 h: 862 ± 39; and no TNBS: 884 ± 47. Data are pooled from two experiments. O, normal; ●, pre-incubation for 24 h.

affinity TNP-specific receptors are more susceptible to tolerogenesis than those bearing receptors of low affinity. (b) There is a kinetic difference in the effect of tolerogen upon the PFC of the two affinity classes with low affinity precursor cells being tolerized more rapidly than high affinity B cells. (c) The magnitude of the polyclonal B-cell response of control cells to PPD is greater than that to LPS. (d) The high affinity PFC produced in response to LPS were reduced earlier after TNBS injection than the response induced by PPD (12 h vs. 2 d), suggesting that the less mature cells responding to LPS are susceptible earlier to a greater extent to the tolerance-inducing activity of TNBS than the more mature B cells responding to PPD. (e) Conversely, no significant difference in the susceptibility to tolerance of the low affinity PFC response to these polyclonal B-cell activating substances was detected.

TNBS Dose Response Curve and Polyclonal Activation.   Another approach to the investigation of the cell populations involved in hapten-specific tolerogenesis is the determination of the tolerance dose-response relationship. The possibility of differential tolerance on a subpopulation basis was tested using polyclonal B-cell activation with LPS and PPD as shown above. Spleen cells were cultured 2 d after the donor mice received one of a series of TNBS doses because complete tolerance in the high affinity LPS and PPD responses was first attained at this time. As noted in Fig. 8, TNBS injection caused essentially no reduction in anti-SRC responses induced by LPS and PPD. The high affinity PFC produced in response to LPS were reduced 26 and 76% by the two lowest TNBS doses (0.01 and 0.1 mg, respectively) with a low plateau level of 4–10% in the control response remaining at the three highest doses. The high affinity TNP-specific response induced by PPD was much more resistant to tolerogenesis by TNBS, with 63–81% of the response remaining at 0.1–2.5 mg of TNBS injected and 84% tolerance finally being achieved at the 5-mg dose. When calculated in terms of the percentage of control high affinity response, the 50% tolerance level was achieved at a 100-fold lower dose of TNBS for LPS than for PPD. Thus, in addition to the kinetics of TNBS-induced tolerance being dissimilar for LPS- and PPD-responsive cell populations, the dose-response relationship also differed. LPS-
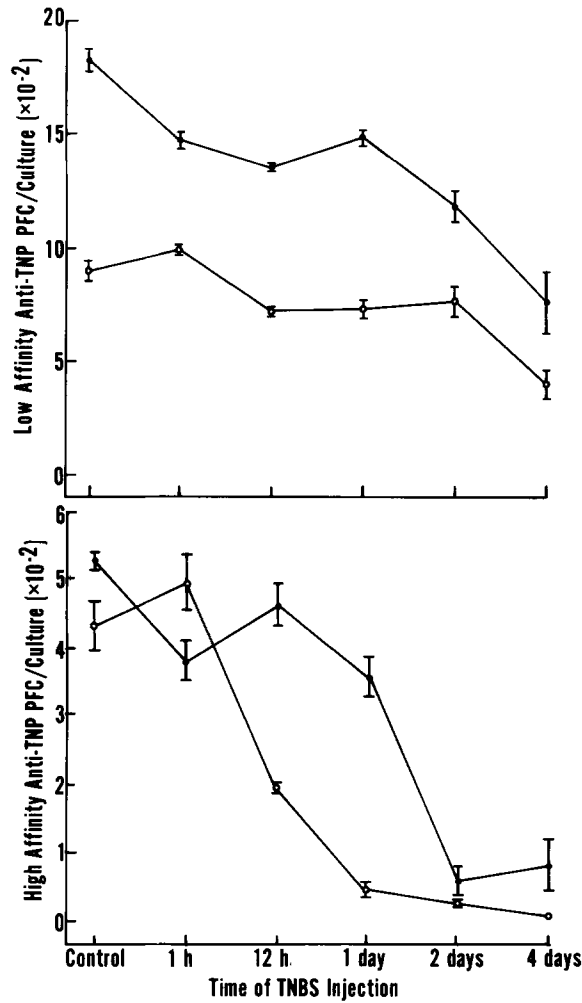
Fig. 7.   Polyclonal activation and kinetics of TNBS tolerance. Spleen cells taken from mice injected i.p. with 5 mg of TNBS in 0.5 ml of saline at various times previously were placed in tissue culture with optimal polyclonal activating concentrations of LPS (50 μg/ml) or PPD (250 μg/ml). Uninjected mice served as controls. Assays were performed on day 3 of culture. Values represent high and low affinity anti-TNP PFC/culture ± SE in groups of three cultures per experiment assayed individually. Data are pooled from six separate experiments. The anti-SRC PFC/culture responses were: LPS, TNBS day 4: 170 ± 19; TNBS day 2: 180 ± 21; TNBS day 1: 197 ± 12; TNBS 12 h: 158 ± 2; TNBS 1 h: 210 ± 6; no TNBS: 180 ± 9; and PPD, TNBS day 4: 311 ± 6; TNBS day 2: 303 ± 25; TNBS day 1: 292 ± 8; TNBS 12 h: 303 ± 26; TNBS 1 h: 349 ± 14; and no TNBS: 302 ± 27. Abscissa, interval between TNBS injection and in vitro culture; ordinate, high and low affinity anti-TNP PFC/culture. O, LPS; ●, PPD.

responsive (immature) B cells were tolerized at a significantly lower dose of tolerogen than the (more mature) PPD-reactive B cells.

Affinity Differences in Polyclonal Activation.   It is possible that differences in the affinity profiles of the TNP-specific PFC produced in response to LPS and PPD could account for the apparently greater susceptibility of LPS-reactive TNP-specific B cells to tolerance induction. This was tested using TNP-lysine inhibition of PFC produced by
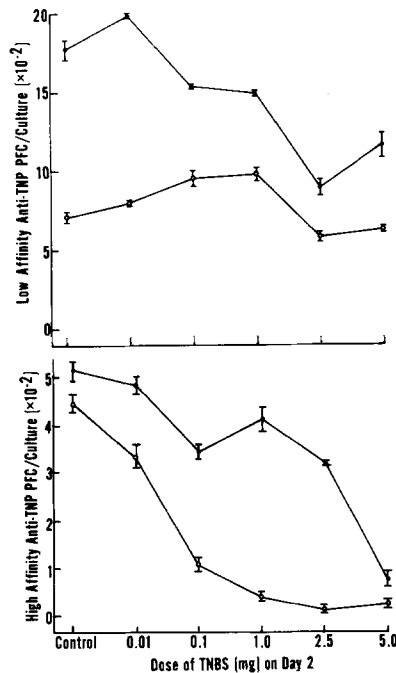
FIG. 8. Polyclonal activation and the TNBS dose response relationship. Spleen cells from mice given a dose of TNBS ranging from 0.01 to 5 mg in 5 ml of saline were placed in culture 2 d after the injection with the optimal polyclonal activating concentrations of LPS (50 μg/ml) or PPD (250 μg/ml). Cultures were harvested for assay on day 3. Values represent high and low affinity anti-TNP PFC/culture ± SE in groups of three cultures per experiment assayed individually. Data are pooled from three separate experiments. The anti-SRC PFC/culture responses were: LPS, no TNBS: 110 ± 10; 0.01 mg: 121 ± 8; 0.1 mg: 110 ± 4; 1.0 mg: 83 ± 2; 2.5 mg: 80 ± 6; and 5 mg: 110 ± 7; and PPD, no TNBS: 323 ± 16; 0.01 mg: 405 ± 25; 0.1 mg: 282 ± 11; 1.0 mg: 243 ± 15; 2.5 mg: 267 ± 13; and 5 mg: 319 ± 17. Abscissa, dose of TNBS given to spleen donors; ordinate, high and low affinity anti-TNP PFC per culture. O, LPS; ●, PPD.

LPS and PPD stimulated spleen cells with heavily conjugated TNP-HRC. A deficiency in higher affinity PFC produced by stimulation with PPD would bias the data toward the result obtained above. This was not the case (data not shown). Thus, a relative paucity of high affinity TNP-specific PFC is not responsible for the difference in tolerance induction observed between LPS- and PPD-reactive B cells.

Discussion

TNBS induces TNP-specific unresponsiveness when tested under a variety of conditions (14–16, 28). When assessed in vitro, unresponsiveness was complete during the 1st wk after TNBS injection, with the TNP-LPS response rising to 26% of the control level by 4 wk (16). Closer scrutiny of the early portion of the time-course, as reported here, revealed that complete unresponsiveness was induced within 12 h after tolerogen treatment, with a small but significant response being obtained with spleen cells treated 1 h before culture (Fig. 1). However, the partial recovery of the TNP-LPS response of the 1-h group resulting from antigen-free incubation (Fig. 3) suggests that an initial blockade of TNP-LPS reactive B cells was caused by the injection of TNBS. This conclusion is supported by the results of the coculturing experiments

(Fig. 2). The anti-TNP response of normal cells was totally inhibited by incubation with spleen cells from mice given TNBS 1 h earlier, although no reduction was caused by cells from mice receiving TNBS >12 h earlier. Partial elution of the cell-bound blockading moiety apparently occurred during 24 h of culture allowing a response from spleen cells in the 1-h treated group to take place. Unresponsiveness in B cells tested early after tolerogen treatment is thus maintained by an easily reversible or readily bypassed receptor blockade. The unresponsive state becomes well established as the tolerance becomes irreversible and leads to functional deletion of TNP-specific B lymphocytes. It is unclear whether tolerant B cells perish, or persist in an immunologically null state. In direct contrast to the tolerance models of Gronowicz and Coutinho (29) and Aldo-Benson and Borel (30), however, receptor blockade is not responsible for the maintenance of TNBS tolerance. Whereas blockade is involved in the early events of tolerogenesis, functional deletion is responsible for B-cell tolerance induction by TNBS.

The maturation level of the B cells being tolerized by TNBS injection was investigated using three separate approaches. Initially, the study involved the use of TNP-Ficoll, classified as a TI-2 antigen by Mosier et al. (9) on the basis of the late emergence of responsiveness during ontogeny and the lack of reactivity in CBA/N mice. The TI-1 antigen, TNP-LPS, induces responses at birth and in CBA/N mice (9). The tempo of tolerance induction was demonstrably slower in the mature TI-2 B cells responding to TNP-Ficoll than in immature TNP-LPS-reactive B lymphocytes. Unlike the result with TNP-LPS, 24 h of antigen-free culture did not increase the response to TNP-Ficoll. Furthermore, the PFC response to TNP-Ficoll of control cells was much less inhibitable by coculture with cells from TNBS-treated mice than was the TNP-LPS response, suggesting that blockade contributed little to the observed unresponsiveness to TNP-Ficoll. Thus, judging by the effect upon responses to TI antigens, immature TI-1 B cells were more susceptible to tolerogenesis by TNBS than more mature TI-2 B lymphocytes. In the second approach, the polyclonal response of tolerant spleen cells was studied using two B-cell activators that stimulate cell populations differing in maturation level (10, 31). It was concluded in a recent study of the ontogeny of B-cell mitogenesis that LPS activates immature B cells whereas the mitogen PPD stimulates predominantly mature B lymphocytes (10). The response to PPD arose later than that to LPS in ontogeny and in the reconstitution of lethally irradiated recipients by fetal liver stem cells (10, 31). Additionally, the unusually low PPD/LPS response ratio of CBA/N mice supports this conclusion (10). Tolerance was induced in the less mature TNP-specific B cells responding to LPS by the production of high affinity antibody earlier than in more mature, PPD-reactive B lymphocytes. The third approach was the investigation of the dose-response relationship of tolerogenesis and reactivity to LPS and PPD. The immature B cells stimulated to high affinity TNP-specific antibody formation by LPS were rendered tolerant at a 100-fold lower dose of TNBS than the more mature PPD reactive B cells. Thus, by three criteria, the immature B cells in the spleen prove to be more susceptible to tolerance induction with TNBS than the more mature B lymphocytes.

Differences in reactivity to a hapten-specific tolerogen were detected using polyclonal stimuli. It has been suggested by Coutinho and Moller (32, 33) that the only rigorous test of central B-cell tolerance is reactivity to nonantigen-specific polyclonal B-cell activators which stimulate lymphocytes via non-Ig surface receptors. Neither

LPS (16) nor PPD could reverse the tolerance induced by TNBS injection 4 d before tissue culture. The distinction between mature PPD-responsive and immature LPS-reactive populations appeared when kinetics and dose-response relationships of tolerogenesis were studied. A much greater effect of the tolerogen was exerted upon the production of high affinity than low affinity TNP-specific PFC. This suggests that the precursors of PFC which bear low affinity TNP-specific receptors are more resistant to the tolerizing effects of TNBS injection than are the B cells with high affinity receptors, as would be predicted by cell selection theories for the regulation of antibody affinity (34). In the studies which have supported this hypothesis (35–37), an antigen-specific stimulus was used to test the state of responsiveness, thereby allowing a second encounter with the tolerated determinant(s) which would exert selective pressure on the responding cell populations (34). Nonspecific stimulation with LPS and PPD was utilized in the experiments reported here, allowing a more direct assessment of the immunoreactive potential of B cells from tolerogen-treated mice without any preferential selection of affinity subsets by antigen.

It is clear from in vitro (5–8) and in vivo (15) experiments that neonatal B cells, in contrast to adult B lymphocytes, are very susceptible to tolerance induction. Although the differences in resistance to tolerogenesis may be a result of the maturation of B cells from $\mu + \delta-$ to $\mu + \delta+$ lymphocytes (38–41), a conclusion which is supported by the effect of modulation of δ-receptors (42–45), a number of other surface markers appear in parallel with the δ-surface receptor (i.e., CR, Ia, LyB$_5$) (41, 46–49). The rate at which neonatal B cells acquire Ia is very similar to the rate that B cells from young mice lose susceptibility to tolerance induction (5, 41). Furthermore, development of susceptibility to inhibition by anti-μ antiserum correlates to both of these changes (50–52). The late acquisition of immunocompetence to TNP-Ficoll (9) is temporally related to the appearance of these surface markers, and signals the emergence of a second B-cell subpopulation (the mature, adult-type B lymphocyte).

This conclusion is supported by results with the CBA/N mouse. In addition to a number of other B-cell defects, mice of this strain exhibit an unusually low δ/μ ratio (13) and cannot respond to TNP-Ficoll (9). Accordingly, these mice respond to TI-1 antigens but lack B cells that are reactive with TI-2 antigens (9). CBA/N mice are also susceptible to hapten-specific blockade of TD responses by polysaccharide conjugates (53, 54), lack the adult-type mature B-lymphocyte subpopulation, and possess B cells which exhibit high levels of sIgM but apparently normal amounts of sIgD. CBA/N B cells are similar to neonatal B lymphocytes in function, surface antigens, and receptor display with the exception of sIgD, which is present in the former but absent in the latter. Because B-cell neogenesis is continually underway in the adult, the immature B cells, apparently identical to those found in neonatal spleen, exist in the differentiation pathway as precursors of the mature adult B cells which are absent from the CBA/N mouse. The conversion from immature neonatal-type to mature adult-type B cells would then involve acquisition of the differing reactivity patterns and surface markers characteristic of these cells. Not only do B lymphocytes from different ontogenic levels differ in the profile of responsiveness to antigen-specific and nonspecific stimuli as well as the level of susceptibility to tolerogenesis, but the data reported here show that both immature neonatal-type and mature adult-type B lymphocytes with these characteristics are present in adult mouse spleen.

Summary

The maturation level of the B-lymphocyte subpopulations involved in trinitro-phenyl (TNP)-specific immunological tolerance in adult mice induced by the injection of trinitrobenzenesulfonic acid (TNBS) was investigated using in vitro antigen-specific and nonspecific polyclonal stimulation. The maturity of the B-cell subsets being studied was defined by the antigen or polyclonal activator which evoked a response. Thus, the thymic independent (TI-1) antigen TNP-lipopolysaccharide (TNP-LPS) and the polyclonal stimulant LPS were used to activate immature, neonatal-type B lymphocytes, whereas mature, adult-type B cells were responsive to the TI-2 antigen, TNP-Ficoll, and the nonspecific activator, purified protein derivative (PPD).

Whereas unresponsiveness in TNP-LPS-reactive (immature) B cells 4 d after TNBS treatment was previously shown to be the result of functional deletion, partially reversible receptor blockade was detected in this study early after tolerogen treatment. By the 24-h point, tolerance was irreversible, as assessed by 24-h of antigen-free incubation and cocultivation of tolerant cells with control splenocytes. Tolerance was induced more rapidly in immature, TI-1 B cells than in mature TI-2 B lymphocytes. B lymphocytes reactive to TNP-Ficoll were also less susceptible to receptor blockade. Using LPS as a nonspecific probe for immature B cells, 60% tolerance in high affinity TNP-specific cells was induced within 12 h of TNBS treatment, and complete unresponsiveness by 24 h. In contrast, no significant decrease in response to the mature B-cell activator, PPD, occurred until day 2. Furthermore, the 50% tolerance level was achieved in TNP-specific LPS-reactive B cells by 100 times less tolerogen than required for PPD-responsive cells.

Thus, TNBS-induced unresponsiveness in cells reactive to TNP-LPS is initially a result of reversible receptor blockade which leads within 4 d to functional deletion. Immature, TI-1 B lymphocytes, which give polyclonal responses to LPS and antigen-specific responses to TNP-LPS, are rendered tolerant to TNBS more rapidly and at lower tolerogen doses than mature, TI-2 mouse B cells which react polyclonally to PPD and specifically to TNP-Ficoll. Moreover, these data show that both the immature and the mature B lymphocytes with these characteristic tolerance suscep-tibilities and specific and nonspecific immune response patterns are present in the adult mouse spleen.

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