Brief Definitive Report

DEFECTIVE B CELL TOLERANCE IN ADULT (NZB × NZW)F_1 MICE*

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NZB and (NZB × NZW)F_1 (B/W) mice resist the induction of immunologic tolerance in vivo under certain conditions (1–3) but not others (4, 5). Adoptive transfer studies in such mice have indicated a resistance to tolerance induction at the T cell level (6, 7). Although NZB and B/W bone marrow-derived lymphocytes have been reported to display normal tolerance susceptibility (7–9), an age-dependent resistance to tolerance induction by bovine gamma globulin (BGG) in B/W bone marrow cells has been reported (8). In view of these divergent findings, as well as the more recent documentation of various B cell abnormalities in NZB and B/W mice (10–12), we have reexamined B cell tolerance in B/W mice in an entirely thymus-independent, in vitro system. With the tolerogen trinitrophenyl (TNP) human gamma globulin (HGG) (13), we report here that splenic B cells derived from B/W mice are less susceptible than B cells from three strains of normal mice to tolerance induction by a tolerogen with low, but not high, epitope density.

Materials and Methods

Animals. BDF_1 (C57BL/6 female × DBA/2 male F_1) (The Jackson Laboratory, Bar Harbor, Maine; Cumberland View Farms, Clinton, Tenn.; and Simonsen Laboratories, Gilroy, Calif.), CBA/J, and DBA/1J (The Jackson Laboratory) mice, 8–12 wk of age, were used in this study. 7- to 12-wk-old B/W mice were obtained from our colony at the University of Texas Southwestern Medical School (Dallas, Tex.). B/W mice bred from NZB and NZW mice, kindly provided by Dr. Alfred Steinberg, National Institutes of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md., were also tested.

Antigens. HGG Cohn fraction II, (Miles Laboratories, Inc., Elkhart, Ind.) was haptenated with 2,4,6-trinitrobenzene sulfonic acid (Sigma Chemical Co., St. Louis, Mo.) at substitution ratios of $TNP_{32}HGG$ (high epitope density tolerogen) and TNP_7HGG (low epitope density tolerogen) as determined spectrophotometrically. TNP-*Brucella abortus* (BA) was prepared as described previously (13). Heat-killed BA (U. S. Department of Agriculture, Ames, Iowa) was haptenated with fluorescein isothiocyanate (FITC) (Isomer I; Sigma Chemical Co.) as follows: 40 mg of FITC was added to 0.25 ml of washed, packed bacterial cells in carbonate/bicarbonate buffer, pH 9.2. The suspension was incubated at 26°C for 1 h before centrifugation (12,000 *g* for 10 min). The fluorescein (FL)-BA pellet was washed five times in Hanks' balanced salt solution (HBSS) (Microbiological Associates, Walkersville, Md.).

Plaque-forming Cells (PFC) Assay. PFC were determined by a slide modification (14) of the hemolysis in gel technique (15). TNP-BA-stimulated cultures were assayed against TNP-sheep erythrocytes (SRBC) (16); FL-BA-stimulated cultures were assayed against FL-SRBC (17).

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Induction and Assessment of Unresponsiveness. Pooled spleen cells derived from four or more syngeneic mice were treated with a monoclonal anti-Thy-1.2 reagent (New England Nuclear, Boston, Mass.) and guinea pig complement (Pel-Freeze Biologicals Inc., Rogers, Ark.) to kill T cells. The proliferative responses of treated cells to T cell mitogens (concanavalin A and phytohemagglutinin) were abolished entirely, whereas responsiveness to lipopolysaccharide remained intact. The cells were washed and cultured at 10^7 cells/ml in complete medium (13, 18) in the presence of log dilutions of either high or low epitope density tolerogens as previously described or underivatized HGG at 1 μg/ml (control) for 20–24 h on a rocker platform. The cells were then harvested, washed three times with HBSS, and resuspended in complete medium. 5×10^5 viable cells were placed in microtiter wells (Costar, Data Packaging, Cambridge, Mass.) to which 1×10^6 fresh, unprimed, syngeneic x-irradiated (1,500 R) spleen cells were added as filler cells. The cultures were immunized with either 0.001% TNP-BA or 0.001% FL-BA (vol/vol). After 3 d of incubation with antigen, direct anti-hapten PFC responses were assayed. The results of three to six replicate cultures are expressed as the arithmetic mean PFC response per 10^6 viable input B cells ± SE or as percent control PFC response.

Statistics. Four mouse strains were compared for tolerance susceptibility by using a three-factor analysis of variance with strain and tolerogen concentration as fixed factors and cell sample as a random factor nested within strain (19). When significance was attained, particular comparisons were made with Newman-Keuls multiple comparison procedure (19).

Results

As shown in Fig. 1 A, in pooled data from three to six experiments, 0.1–1.0 μg/ml of the high epitope density tolerogen induced 50% unresponsiveness to TNP in B cells from (autoimmune) B/W, and (normal) BDF_1, CBA/J, and DBA/1J mice. Statistical analysis revealed no significant difference between the strains at any concentration of the high epitope density tolerogen. Exposure to $TNP_{32}HGG$ did not affect the anti-FL response (Fig. 1 C), thus indicating that the tolerance was hapten specific. Furthermore, addition of the high epitope density tolerogen to B cells at the end of the 24-h tolerance induction period did not depress the control response by >20%. Thus, the tolerance observed required the generation of a time-dependent off-signal and was not a result of passive carry over of the tolerogen into the immunization phase of the cultures.

In contrast, when the same cells were exposed to the low epitope density tolerogen, the analysis of variance procedure yielded a significant difference among strains ($P < 0.0001$). With the multiple comparison techniques at $\alpha = 0.05$, B/W mice differed significantly from each of the three normal strains, and the normal strains did not differ significantly from each other in B cell tolerance susceptibility (Fig. 1 B). Exposure of cells to TNP_7HGG reduced the control anti-TNP response by 50% or more in each of the normal strains, but only 25% or less in B/W mice. A representative experiment is shown in Fig. 2. Of note, the resistance to the low epitope density tolerogen was evident when B cells from either male or female B/W mice were tested independently.

Discussion

The present studies demonstrate that B cells derived from spleens of adult B/W mice are resistant to tolerance induction when the epitope density of the tolerogen is low. Thus, when $TNP_{32}HGG$ was used, B cells from three normal mouse strains and B/W mice were not significantly different in tolerance susceptibility. In contrast, when TNP_7HGG was used, cells from B/W mice were significantly less sensitive than those from normal mice to the induction of B cell tolerance in vitro.

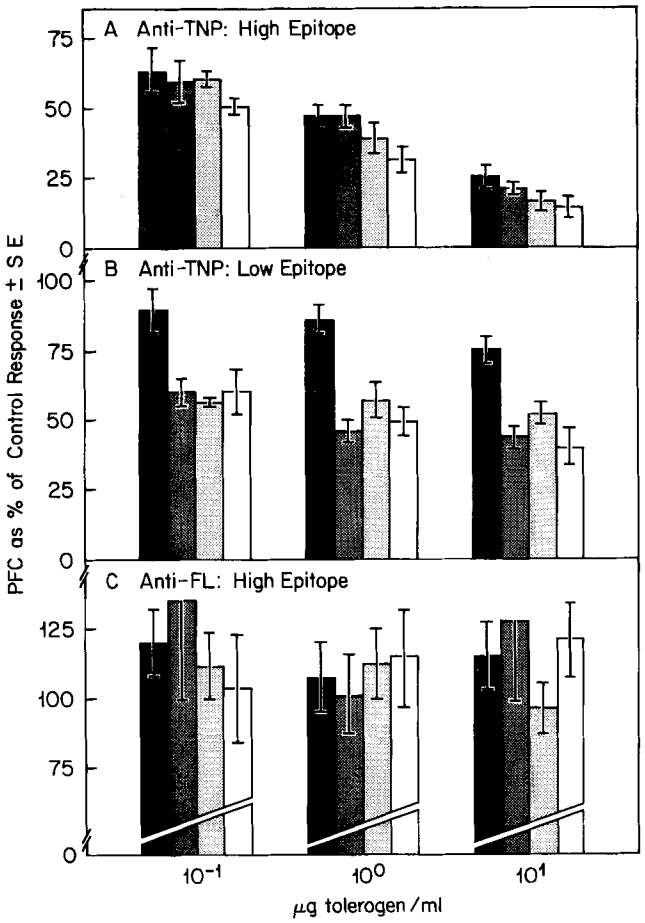

FIG. 1. Dose effect of tolerogens with either (A) high ($TNP_{32}HGG$) or (B) low (TNP_7HGG) epitope density on the anti-TNP PFC responses of B/W (■), BDF_1 (▩), CBA/J (▨), and DBA/1J (□) B cells. Arithmetic mean control anti-TNP PFC/10^6 ± 1 SE: B/W, 730 ± 283; BDF_1, 1,387 ± 640; CBA/J, 2,605 ± 380 and DBA/1J, 1,838 ± 164. (C) Anti-FL responses of cells exposed to $TNP_{32}HGG$. Arithmetic mean control anti-FL PFC/10^6 ± 1 SE: B/W, 454 ± 365; BDF_1, 457 ± 285; CBA/J, 335 ± 118; and DBA/1J, 225 ± 52.

Previous studies have shown a striking relationship between epitope density and tolerogenicity vs. antigenicity for hapten conjugates of proteins (20), polysaccharides (21), amino acid copolymers (21), immunoglobulin G (22, 23), and liposomes (24). Highly substituted conjugates are consistently tolerogenic, whereas those with lower substitution ratios are immunogenic or have no detectable effect. In agreement with these reports is the present study, which documents that TNP_7HGG is, to a varying degree, a less potent tolerogen than $TNP_{32}HGG$ for normal mouse B cells. Moreover, this is the first report to examine directly the relationship between epitope density and tolerogenicity in B cells from (autoimmune) B/W mice and to demonstrate a significant functional difference between these and B cells of normal mice.

Previous reports of B cell tolerance in autoimmune mice have relied on the adoptive transfer of bone marrow cells to irradiated recipients either alone (9) or together with

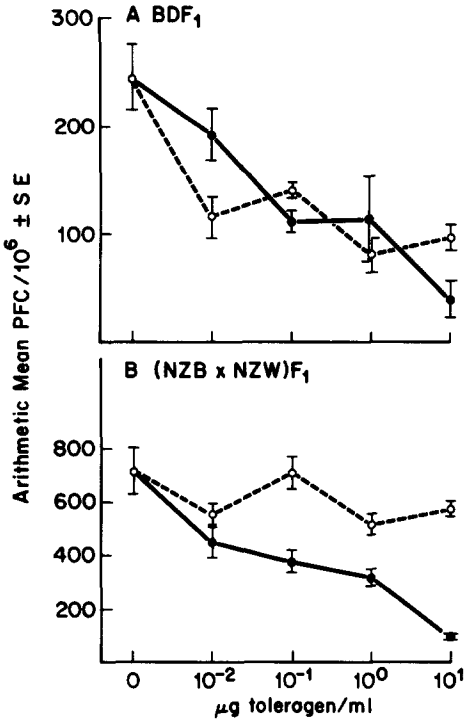

FIG. 2. A representative experiment showing the anti-TNP response of (A) BDF_1 or (B) B/W B cells after exposure to TNP-HGG with either high (●) or low (○) epitope density.

syngeneic thymocytes (7, 8). In no study was the bone marrow depleted of T cells before transfer. The susceptibilities of B/W or (NZB × BALB/c)F_1 bone marrow to the induction of high zone tolerance by SRBC with (7) or without (9) facilitation by cyclophosphamide proved to be entirely normal. Similarly, bone marrow appeared normally susceptible to tolerance induction by both pneumococcal polysaccharide (SIII) and levan (9). However, the tolerance susceptibility to BGG was normal only when cells from young (3-wk-old), but not adult (3- to 4-mo-old), mice were tested (8).

The present findings of normal B cell tolerance in B/W mice with multivalent, high, but not low, epitope density tolerogens suggest an explanation for the diverse outcomes in previous bone marrow transfer experiments. If, as in the case of various polysaccharide antigens, the epitopes are presented in a multivalent array, it would not be surprising that TNP_{32}HGG and SRBC are effective tolerogens for B cells of B/W mice. On the other hand, ultracentrifuged, monomeric BGG might be expected to behave as a low epitope density tolerogen. Thus, the B/W resistance to tolerance induction by TNP_7HGG would be consistent with previously observed resistance to BGG-induced unresponsiveness.

In conclusion, B cells from adult B/W mice are abnormally resistant to tolerance induction by TNP_7HGG, perhaps as a consequence of prior polyclonal activation (including TNP-specific clones) (10–12, 25). A similar loss of the susceptibility to

tolerance induction in B cells to self-antigens with low epitope density might lead to autoantibody formation and disease.

Summary

Hapten-specific tolerance was induced in vitro by trinitrophenyl-human gamma globulin ($TNP_{32}HGG$) to a comparable degree in B cells from adult autoimmune (NZB × NZW)F_1 (B/W) mice and normal BDF_1, CBA/J, and DBA/1J mice. When a lower epitope density tolerogen (TNP_7HGG) was used, B/W mice were significantly less sensitive than normal mice to the induction of B cell tolerance. This finding of defective B cell tolerance in adult B/W mice is consistent with previous reports that document other B cell abnormalities that may relate to the expression of autoimmune disease.

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