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J Clin Invest. 1986;78(3):784-789. <https://doi.org/10.1172/JCI112641>.

Research Article

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Defects in Antigen-specific Immune Tolerance in Continuous B Cell Lines from Autoimmune Mice

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Abstract

B cell hyperactivity and resistance to tolerance induction are well-recognized immunologic abnormalities associated with both human and murine models of systemic lupus erythematosus. Studies evaluating the role of B cells in these defects have been complicated by the difficulties of consistently isolating large numbers of B cells from T cells and other host-derived regulatory factors. We have recently developed continuous cell lines of B lymphocytes with a high degree of specificity for the antigen dinitrophenyl (DNP) from both New Zealand black times New Zealand white F₁ hybrid (BWF₁) and BALB/c mice, and we used them to study intrinsic B cell defects in autoimmunity. We found that the kinetics of the immune response to the antigen DNP-Ficoll of both the BWF₁ and BALB/c B cell lines are not different. In addition, the BWF₁ cell lines, like the BALB/c cell lines and normal B cells, require nonspecific T cell-derived factors as well as antigen to produce an immune response. Tolerance was tested in the BWF₁ B cells by preincubating them with DNP-murine IgG_{2a} (MGG), which can induce tolerance in BALB/c cell line lymphocytes. The BWF₁ B cell lines were resistant to tolerance induction by DNP-MGG and required 50-fold higher dose of DNP-MGG than BALB/c cell lines for suppression. They were also relatively resistant to tolerance with trinitrophenyl-*d*-glutamyl lysine. Thus, DNP-specific B cells from autoimmune mice have an inherent defect in tolerance induction.

Introduction

Our understanding of the immunologic defects associated with systemic lupus erythematosus has been greatly advanced by the use of several murine strains that develop an autoimmune disease very similar to systemic lupus erythematosus. Using these inbred strains of mice, several investigators have studied the phenomenon of immunologic tolerance in an attempt to pinpoint specific cellular defects associated with this autoimmune state (1-5). Most of these investigators have found that in general there is a relative resistance to tolerance induction in several strains of autoimmune mice. Several studies have attempted to determine whether this tolerance resistance is due to a defect in the B cell or in regulatory T cells. However, the results have been conflicting. For example, Goldings found a defect of tolerance in New Zealand

black (NZB)¹ B cells while Laskin et al. found a defect in T cells (2, 3). Studies of specific cellular defects have been hindered by the difficulty in obtaining model systems in which large numbers of antigen-specific B cells can be isolated from other host cells and T cell-derived factors. Even in vitro studies in which T cell-depleted splenic lymphocytes were used cannot consistently avoid small amounts of T cell contamination (4). In addition, these splenic B cells have matured in vivo under the influence of possibly abnormal host cells and T cell-derived factors, and this influence may last for several days after the cells are isolated in vitro (5). To overcome these difficulties we have developed continuous pure B cell lines from NZB times New Zealand white (NZW) F₁ hybrid mice (BWF₁) that have a significant proportion of cells that are specific for the hapten dinitrophenyl (DNP). These cells have been maintained in tissue culture for more than one year. These cell lines have no T cell or macrophage contamination and they are no different than previously described BALB/c cell lines (6) in terms of spontaneous [³H]thymidine and [³H]uridine uptake in the unstimulated state (7). These cells can be used as a model system for studying intrinsic B cell abnormalities in autoimmune mice.

The present studies make use of these DNP-specific B cell lines from both autoimmune BWF₁ and normal BALB/c mice to compare the antigen-specific immune response and tolerance induction between the BWF₁ and BALB/c cell lines. We found that the requirement of the BWF₁ cell lines for nonspecific T cell factors in order to produce an immune response after stimulation with specific antigen was the same as that noted for BALB/c mice. We also found that the kinetics of the anti-DNP response to DNP-Ficoll was the same for normal and autoimmune cell lines. To investigate antigen-specific tolerance in the cells from autoimmune mice we used the system of tolerance induction in which the hapten DNP is bound to mouse IgG_{2a} (DNP-MGG). DNP-MGG has been previously shown to induce tolerance in both normal splenic B lymphocytes and in cell line B lymphocytes from normal BALB/c mice (8, 9). We found that BWF₁ B cells from several pure B cell lines were resistant to tolerance induction by both DNP-MGG and trinitrophenyl-*d*-glutamyl lysine (TNP-dGL). However, the resistance was not absolute, since higher doses of DNP-MGG or higher substitution ratios of TNP-dGL could be effective in tolerance induction. Thus we have demonstrated that B cells from autoimmune BWF₁ mice have an inherent defect that makes them relatively resistant to tolerance induction.

Methods

Animals. 8-wk-old female BALB/c mice were used for the propagation of the plasmacytoma RPC5. 3-6-wk-old female Sprague-Dawley rats

1. **Abbreviations used in this paper:** BWF₁, NZB times NZW F₁ hybrid mice; dGL, *d*-glutamyl lysine; DME, Dulbecco's modified Eagle's medium; DNP, dinitrophenyl; MGG, murine IgG_{2a}; NZB, New Zealand black; NZW, New Zealand white; PMA, phorbol myristate acetate, PFC, plaque-forming cell; TNP, trinitrophenyl.

A preliminary report of this paper was published in abstract form (*Arthritis Rheum.* 285:26S).

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Received for publication 4 February 1986 and in revised form 22 April 1986.

J. Clin. Invest.

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0021-9738/86/09/0784/06 \$1.00

Volume 78, September 1986, 784-789

were used as a source of irradiated normal thymus filler cells. 8–14-wk-old female C57BL/6 mice were used for the passage and maintenance of the EL-4 lymphoma cell line. All of these animals were obtained from Harlan Industries, Indianapolis, IN. BWF₁ mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and used to develop the cell lines.

T cell-derived growth and differentiation factors. EL-4 lymphoma cells were passaged by intraperitoneal injection into C57BL/6 mice. The cells were harvested from the peritoneum. 2×10^6 /ml sterile EL-4 cells were then grown in culture for 24 h in serum-free RPMI medium supplemented with penicillin, streptomycin, and 10 ng/ml of phorbol myristate acetate (PMA), washed thoroughly to remove the PMA, and placed back in RPMI medium. The supernatant was collected after 48 h of further growth without PMA and stored in aliquots at -70°C . This EL-4 supernatant has been previously shown to be required for both continuous growth of the cell lines and for an immune response in DNP-Ficoll-stimulated cell line lymphocytes (6, 10).

DNP-specific B cell lines. B lymphocyte lines from normal BALB/c 8–12-wk-old female mice were isolated and propagated as previously described (6). The characteristics of these BALB/c cell lines have been described elsewhere. (11) Briefly, the cell lines are nonmalignant and not transformed and are propagated in the absence of antigen. They have resting B cells as well as some B cells in the activated state, and thus are similar to splenic B cells, as described by Thompson et al. (11, 12). Similar continuous B cell lines were isolated from autoimmune BWF₁ mice and propagated as described for BALB/c mice (7) (manuscript in preparation). Briefly, splenic lymphocytes from 8-wk-old female BWF₁ mice were enriched for DNP-specific B cells by the method of Haas and Layton (13). The enriched cells were cloned in soft agar by a modification of the method of Pillai and Scott (14). After 24 h incubation the cells were layered over with complete Dulbecco's modified Eagle's medium (DME) with 20% EL-4 supernatant. As the colonies became macroscopic they were picked from the agar with the aid of a dissecting microscope and placed in individual wells of a 96-well culture plate (Falcon Labware, Div. of Becton-Dickinson & Co., Oxnard, CA). When cells had grown to confluency they were transferred to 24-cm tissue culture flasks.

These cells are pure B cell lines with no macrophage or T cell contamination (6, 7) (manuscript in preparation). The cell lines are maintained in DME supplemented with 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 5×10^{-5} M 2-mercaptoethanol, 0.1 mM nonessential amino acids, 100 mM L-glutamine, and 10% fetal calf serum (HyClone Sterile Systems, Inc., Logan, UT). 20% EL-4 supernatant was required for the continued growth of these cell lines in culture. The medium was changed twice weekly and the cultures split when they became confluent. There is no antigen or lipopolysaccharide present in the medium.

Antigen stimulation of cell line lymphocytes. 10^5 cell line lymphocytes from either the BALB/c or the BWF₁ cell lines were placed in individual wells of a 96-well microculture plate. DNP-Ficoll (20 ng/ml) was added to each well of the antigen-stimulated groups and the volume of the well was brought up to 250 μl using complete DME. Except where indicated in results, 20% EL-4 supernatant and 10^5 freshly obtained normal rat thymus cells that had been irradiated with 2,000 rad 1–2 h before culture were added to each well. Background response was determined by culturing line cells with EL-4 supernatant and filler cells without antigen. After 4 d in culture the cells were washed thoroughly to remove the DNP-Ficoll and placed back in culture overnight with 250 μl complete DME. The following day the number of antibody-forming cells was assayed by the Cunningham slide modification of the Jerne plaque-forming cell (PFC) assay using trinitrophenyl (TNP)-coated sheep red blood cells as target cells (15). Irradiated thymus cells alone were also cultured with antigen and produced no significant PFC.

Cell line kinetics. 10^5 cell line lymphocytes were placed in individual wells of a 96-well microculture plate with 20 ng/ml DNP-Ficoll, 10^5 irradiated rat thymus filler cells, and 250 μl DME complete medium containing 20% EL-4 supernatant. At various times after the initiation of these cultures, sample wells were washed thoroughly to remove the DNP-Ficoll and a PFC assay was performed using Cunningham slides as previously described. The response of the BALB/c and BWF₁ cell line

lymphocytes to DNP-Ficoll at various times were compared with each other and to normal splenic lymphocytes from BALB/c mice.

Tolerance induction. 10^5 cell line lymphocytes were placed in individual wells of a 96-well microculture plate with varying doses of DNP-MGG or TNP-dGL. The total volume of each well was 250 μl . After 48 h of preincubation with DNP-MGG, the cell line lymphocytes were washed three times to remove all tolerogen and placed back in culture with 20 ng/ml of the antigen DNP-Ficoll plus 10^5 rat thymus filler cells irradiated with 2,000 rad. After 4 d in culture the antibody-forming cells were assayed as noted above. 20% EL-4 supernatant was also present during preincubation with tolerogen. Cultures preincubated in medium without tolerogen and then cultured with DNP-Ficoll were used as immune controls. Cultures incubated without either tolerogen or DNP-Ficoll were used as background controls.

In some experiments cells were grown for 30 d in the presence of DNP-MGG. Controls were treated in the same fashion but did not have DNP-MGG in the medium. At the end of this 30-d period the cells were washed free of tolerogen and placed back in culture. At varying numbers of days after removal of DNP-MGG, aliquots of cells from control or DNP-MGG groups were cultured with antigen in the presence of filler cells and EL-4 supernatant, and the PFC response assayed 5 d later.

Hapten-protein conjugates. 2,4-Dinitrobenzene sulfonic acid was twice recrystallized according to the method of Eisen and bound to murine IgG_{2a} as previously described (16). Murine IgG_{2a} was separated from the ascites of mice bearing the plasmacytoma RPC5 by starch block electrophoresis as previously described (17). DNP-Ficoll was obtained from Biosearch, San Rafael, CA. TNP₇ dGL was the generous gift of Dr. Eliot Goldings (University of Texas Health Science Center at Dallas, TX). TNP₂₇ dGL and TNP₁₁₆ dGL were made by conjugating TNP to the dGL polymer by the method of Goldings and Mosier (18).

Viability assay. 10^5 cell line lymphocytes were incubated for 24 h in DME with 20% EL-4 growth factors and varying concentrations of DNP-MGG. The cells were then tested for viability by trypan blue dye exclusion.

Statistics. Values expressed are the means \pm standard errors of 3–4 separate cultures. Each experiment was performed 2–3 times to document reproducibility of data. In most cases only data from one representative experiment is shown. Groups were statistically compared using a one-tailed Student's *t* test.

Results

Previous results in our laboratory have shown that the nonautoimmune BALB/c cell lines require filler cells and nonspecific T cell-derived factors to produce an immune response upon stimulation with DNP-Ficoll (11). This requirement for soluble T cell-derived factors for antibody production to occur after stimulation with T-independent antigen is similar to that found in normal B cells (18–20). The possibility was raised that such a requirement may be absent in the BWF₁ cell lines, since these mice have an increased number of B cells spontaneously secreting antibody and their B cells have been shown to be hyperactive in vivo (21). To test this we cultured BWF₁ cell line lymphocytes with 20 ng/ml of DNP-Ficoll both with and without EL-4 supernatant and irradiated normal rat thymus filler cells. The BWF₁ B cell lines show no increase over background in antibody-forming cells after stimulation with DNP-Ficoll in the absence of EL-4 supernatant and filler cells (Fig. 1). However, a significant immune response occurs when these cell line lymphocytes are stimulated with DNP-Ficoll in the presence of these factors ($P < 0.05$).

The kinetics of the immune response in these cell line lymphocytes was measured at various times after stimulation of the cells with DNP-Ficoll in the presence of EL-4 supernatant and filler cells. The peak response of the BWF₁ cells occurs at 4–5 d after antigen-specific activation, and there is a return to background levels of antibody-forming cells by 8 d after stimulation

CELL LINE BWF₁ 37

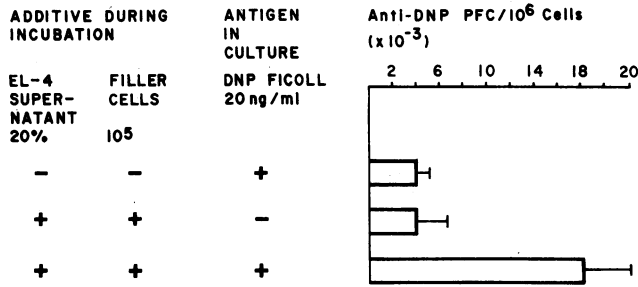


Figure 1. Requirements for an immune response in BWF₁ B lymphocyte lines. Cell line lymphocytes were cultured for 4 d with either 20 ng/ml DNP-Ficoll, with filler cells and EL-4 supernatant alone, or with all three. Only the cell line cultures that contained all three constituents demonstrated a significant PFC response ($P < 0.05$).

(Fig. 2, A and B). This response is similar to that found for the BALB/c cell line lymphocytes and is also similar to that found in freshly obtained splenic lymphocytes from BALB/c mice.

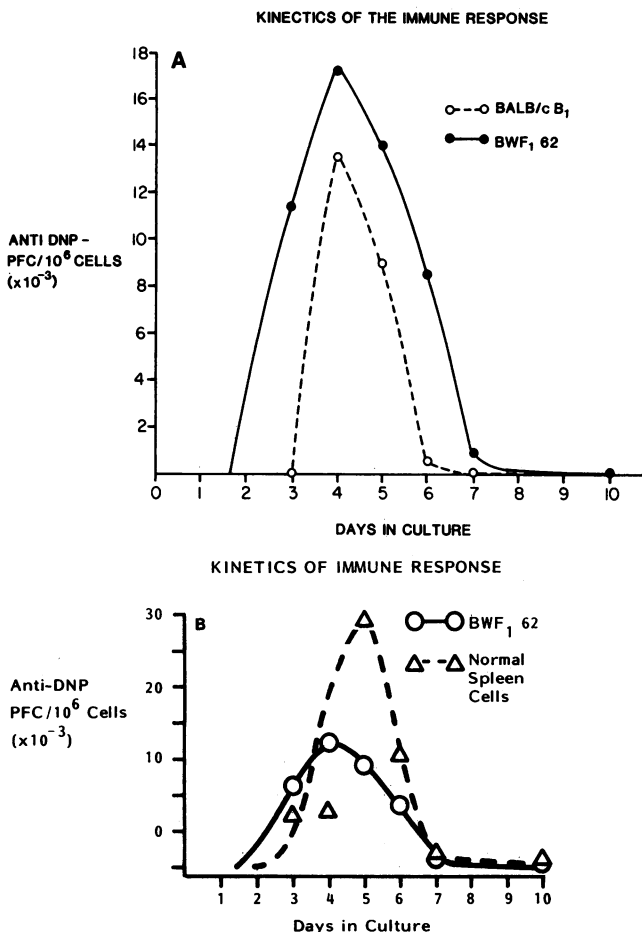


Figure 2. Kinetics of immune response in BWF₁ cell lines compared with BALB/c cell lines and normal spleen cells. (A) Both BALB/c and BWF₁ cell line lymphocytes were cultured with antigen, filler cells, and EL-4 supernatant. The PFC response was assayed on varying days after the start of culture. The PFC response to DNP-Ficoll antigen peaked on day 4 for both BALB/c and BWF₁ cell line lymphocytes. (B) The kinetics of BWF₁ cell line is compared with that of normal spleen cells. The PFC response of BWF₁ cell line cells again peaked on day 4, which is one day earlier than normal spleen cells from BALB/c mice peaked.

To study the phenomena of antigen-specific tolerance we used DNP-MGG as a tolerogen because, in previous studies, we have shown that preincubation of BALB/c cell lines for 48 h with DNP-MGG renders them tolerant to further stimulation with DNP-Ficoll (9). This tolerance in BALB/c cell line lymphocytes is antigen specific, occurs with or without nonspecific T cell factors present in the preincubation period, and occurs at a concentration of 10–200 $\mu\text{g/ml}$ of DNP-MGG (9). To test whether there was a tolerance defect inherent in the BWF₁ B cells, cells from both the BALB/c and the BWF₁ cell lines were preincubated for 48 h with 200 $\mu\text{g/ml}$ DNP-MGG, washed free of tolerogen and then cultured with DNP-Ficoll, filler cells and EL-4 supernatant for 4 d, washed, and assayed for PFC. Appropriate immune and background controls were set up as described for each cell line. There was a statistically significant suppression ($P < 0.05$) of the BALB/c cells preincubated with DNP-MGG when compared with the immune controls (Fig. 3). However, BWF₁ cell lines preincubated with DNP-MGG were not significantly suppressed compared with control BWF₁ cells. Similar experiments with three other BWF₁ cell lines and three BALB/c lines gave identical results (data not shown).

Goldings has shown that in B lymphocytes isolated from spleens of BWF₁ mice, resistance to tolerance induction is partially dependent upon the epitope density of the antigen carrier complex (3). To test whether a similar dependence on the epitope density of DNP-MGG was involved in the resistance to tolerance seen here, the experiments were repeated using DNP₂₆ MGG and DNP₁₂ MGG. Tolerance in the BWF₁ cells was not induced at either epitope density (Fig. 4). Since it is known that DNP-MGG at epitope densities above 30 will not induce tolerance in nonautoimmune lymphocytes, we did not test DNP-MGG with a very high substitution ratio (22).

The BWF₁ cell line lymphocytes were preincubated with DNP-MGG at various concentrations ranging from 200 to 1,000 $\mu\text{g/ml}$. As before, the BWF₁ cells preincubated with 200 $\mu\text{g/ml}$ DNP-MGG showed no statistically significant difference in their response to stimulation with DNP-Ficoll. However, there was a significant difference in the PFC response of those cells preincubated with DNP-MGG at concentrations of 500 and 1,000 $\mu\text{g/ml}$ (Fig. 5). A cytotoxicity assay using trypan blue exclusion showed that incubation of the cells with 500 $\mu\text{g/ml}$ of DNP-MGG produced no significant cell death, but a mild degree of cytotoxicity was found in some cell lines when tested with 1,000

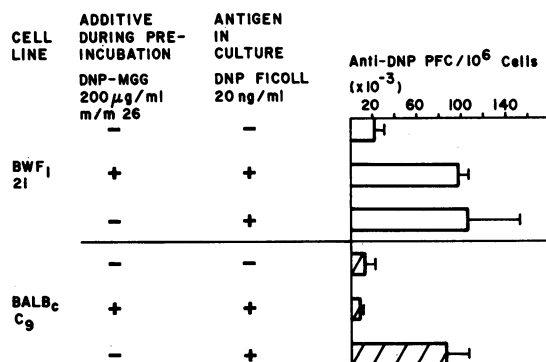


Figure 3. BWF₁ cell line lymphocytes are resistant to tolerance induction by DNP-MGG. A 48-h preincubation of BALB/c cell line cells with DNP-MGG before culture with antigen DNP-Ficoll suppressed their PFC response compared with their control ($P < 0.01$). Similar treatment of BWF₁ cell line did not suppress the anti-DNP response ($P > 0.10$).

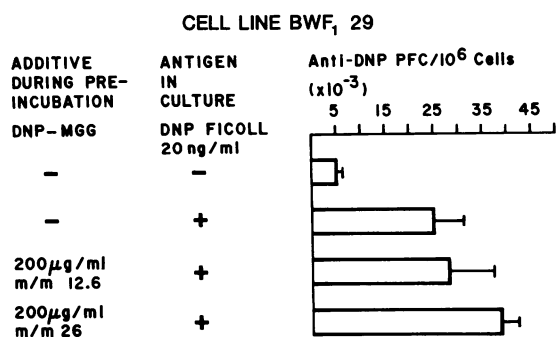


Figure 4. Effect of varying the hapten carrier ratio on tolerance induction by DNP-MGG in BWF₁ cell lines. A 48-h preincubation of BWF₁ cell line lymphocytes with either moderate or highly substituted DNP-MGG conjugates was followed by culture with antigen. Neither DNP_{12.6} MGG or DNP₂₆ MGG suppressed the immune response of BWF₁ 29 cell line.

μg/ml DNP-MGG. Thus, at least 500 μg/ml DNP-MGG is needed to induce tolerance in BWF₁ B cells, which indicates that BWF₁ B cell lines have a 50-fold difference in sensitivity to tolerance induction with DNP-MGG when compared with the BALB/c cell lines.

Similar studies were done using the tolerogen TNP-dGL, which induces B cell tolerance without affecting Fc receptors. TNP-dGL (100 μg/ml) of varying epitope densities was used in each 48-h preincubation. The results (Fig. 6) show that TNP₇ dGL did not induce tolerance in either BALB/c or BWF₁ cell lines. TNP₂₇ dGL induced tolerance in nonautoimmune BALB/c cell lines ($P < 0.05$) but not in the BWF₁ cell lines, while TNP₁₃₃ dGL induced tolerance in both BWF₁ and BALB/c cell line lymphocytes.

We have previously shown that BALB/c cell lines can grow in the presence of DNP-MGG, and become tolerant. This tolerance has many characteristics that are predicted for the theoretical clonal anergy (23, 24). Thus, we tested whether B cell lines from autoimmune mice can grow in the presence of DNP-MGG, and whether they become tolerant. Several cell lines were propagated in the presence of 200 μg/ml DNP-MGG and tested for tolerance as described in Methods. The BWF₁ and BALB/c cell lines were tolerant at the end of 30 d (Fig. 7). Like the BALB/c cell lines, the BWF₁ cell lines lost tolerance soon after removal of DNP-MGG.

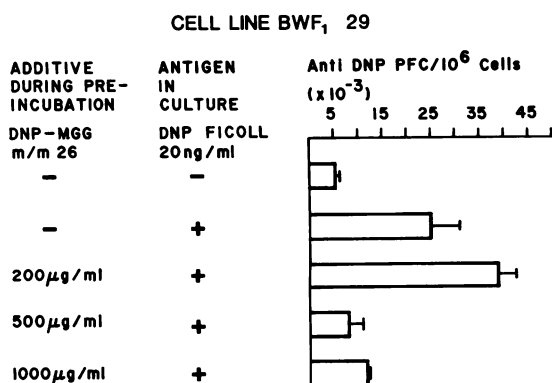


Figure 5. Effect of dose of DNP-MGG on tolerance induction. BWF₁ cell line lymphocytes were preincubated with varying doses of DNP-MGG, and then cultured with antigen. While 200 μg of DNP-MGG did not suppress the PFC response of BWF₁ 29 ($P > 0.05$), both 500 μg/ml and 1 mg/ml inhibit the anti-DNP response ($P < 0.05$).

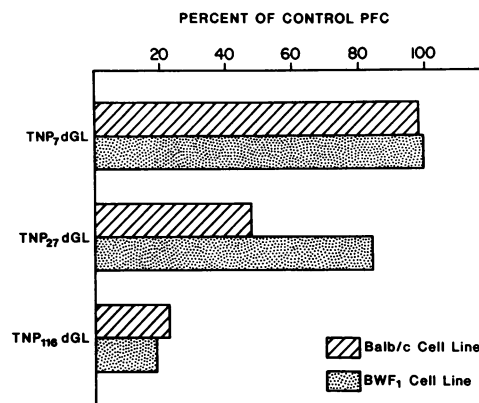


Figure 6. Effect of varying the hapten carrier ratio on the induction of tolerance by TNP-dGL in BALB/c and BWF₁ cell lines. Cell line lymphocytes were preincubated with TNP-dGL of varying substitution ratios for 48 h, washed, and their anti-TNP response compared with the response of controls incubated with DNP-Ficoll alone. The results are expressed as percent of the control (DNP-Ficoll) response. TNP₂₇ dGL suppressed the response of BALB/c cells ($P < 0.05$) but not BWF₁ cells ($P > 0.10$). TNP₁₁₆ dGL suppressed both cell lines ($P < 0.01$).

Discussion

The availability of pure B cell lines that have been outside of the host regulatory influence for a period of time allows us to explore possible defects of B cell response that may be intrinsic to the B cell itself. Although these B cell lines are not entirely "normal" due to their prolonged in vitro growth, they are non-malignant and have many characteristics that are similar to normal splenic B cells (11). Since the BWF₁ cell lines are being compared with nonautoimmune BALB/c cell lines, any abnormalities of the cells that could be due to prolonged in vitro growth would be controlled, since they should also exist in BALB/c lines. Tolerance induction in these cell lines is similar to that seen in splenic B cells in vitro: it is antigen specific, dose dependent, and not associated with cell death (11). Thus, this is a suitable model for the study of the tolerance defect in the BWF₁ B cell.

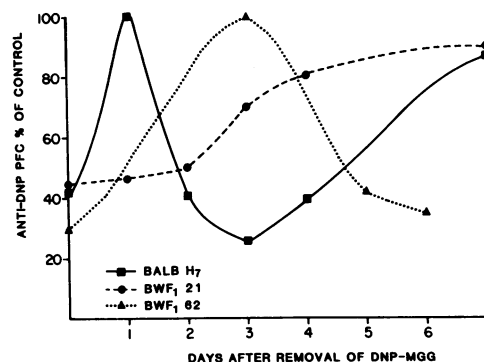


Figure 7. Propagation of BWF₁ cell lines in the presence of DNP-MGG for 30 d suppresses their response to DNP-Ficoll. Both BWF₁ and BALB/c cell line cells were propagated in the presence of 200 μg/ml DNP-MGG for 30 d. The cells were then washed free of DNP-MGG and placed back in culture. At varying times after removal of DNP-MGG, some of the cells were stimulated with antigen and their PFC response compared with controls. BWF₁ cell lines were significantly suppressed on day zero but lost tolerance soon after removal of DNP-MGG. This is similar to the response of BALB/c cell line lymphocytes.

These studies indicate that the BWF₁ B cell line lymphocytes are no different than the normal BALB/c cell lines or normal spleen cells in the kinetics of the immune response produced by antigen-specific stimulation with DNP-Ficoll. These BWF₁ cell lines are also similar to BALB/c cells in their requirement for filler cells and nonspecific T cell-derived factors to produce an immune response upon antigen stimulation. These studies do not address the question of whether autoimmune B cell lines produce more antibody or greater numbers of PFC after stimulation, nor do they rule out the possibility that the cells may require significantly lower concentrations of T cell-derived factors to initiate and sustain an immune response. These questions are being addressed in other studies. However, we have demonstrated that B cells from BWF₁ mice do have a resistance to tolerance that is intrinsic and not dependent on T cells.

Our studies do not rule out the possibility that there is a concomitant defect in the T cells themselves or in the T cell-derived growth factors that may also play a role in the abnormal antigen-specific tolerance seen in BWF₁ mice. Nor can these experiments be extrapolated to tolerance of nucleoside or DNA antigens or to other strains of autoimmune mice. Most of the studies showing tolerance defects have used heterologous antigens, and indeed this study also uses the heterologous determinant DNP. However, Lewis et al. was able to induce tolerance to the self antigen, nucleosides, in BWF₁ mice (25). Thus, these studies do not address mechanisms of self-tolerance directly. However, we now have the ability using these same methods to study antigen-specific tolerance in other mouse strains, and with self-antigenic determinants that may be more pertinent to the autoimmune process.

The resistance to tolerance in the BWF₁ B cell is not absolute, but is due to a decrease in sensitivity of the cell to tolerance induction. Tolerance to DNP-MGG requires 50 times more tolerogen than needed for BALB/c B cells. TNP-dGL requires fivefold increase in the density of hapten on carrier. Goldings has previously shown that the tolerance defect in NZB mice in vivo is not seen with high hapten carrier ratios (3). Thus, these in vitro studies are in agreement with previous in vivo data. The exact defect responsible for the resistance to tolerance induction seen in the BWF₁ cell lines is not yet clearly understood. There are a number of possible explanations for this. It is unlikely that Fc receptors play a role, since there is also resistance to tolerance with TNP-dGL. Perhaps there are fewer receptors by which the DNP-MGG initiates tolerance. Alternatively, the receptor affinity for DNP-MGG may be lower than that on the normal B cells. That higher doses of DNP-MGG and higher epitope density of TNP-dGL can induce tolerance is compatible with these possibilities. However, it may also be possible that the receptors themselves are normal, but the biochemical events that follow receptor tolerogen interaction are abnormal. It is obvious that further studies are required to determine what changes occur in these cells once they are exposed to tolerogen and what differences there are in these changes between the B cell lines from normal and autoimmune mice.

There is a great deal of evidence that propagating the B cell lines in the presence of DNP-MGG is a model of clonal anergy (23, 24). B cells grown in the presence of DNP-MGG grow at the same rate as controls and continue to develop and express surface receptors to the same extent as controls, but they become unresponsive to antigen. That BWF₁ cells grown in DNP-MGG also become unresponsive to the same extent as controls suggests that immature B cells from this strain are not as resistant to tolerance as the mature B cells. An alternative explanation is

that longer exposure of mature cells to the tolerogen may allow tolerance induction. These alternatives are being explored. However, our findings of normal tolerance induction in immature BWF₁ B cells and defects in tolerance induction in mature B cells are compatible with the course of events in the BWF₁ mouse, in which the animal is tolerant to self after birth but acquires autoimmunity as it gets older.

Acknowledgments

The authors wish to thank Linda Pratt for her expert technical assistance, and Donna Faulkner for her assistance in preparing the manuscript.

This work was supported by U. S. Public Health Service grants AI-21066, AM-7448, and AM-20582, by an Arthritis Foundation Center grant, and by a grant from the Grace M. Showalter Trust.

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