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B cell clonal elimination induced by membrane-bound self-antigen may require repeated antigen encounter or cell competition

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Abstract

Transgenic mouse experiments indicate that autoreactive B cells are eliminated upon encounter with membrane self-antigen. In this study we tested how B cell tolerance to MHC class I antigens is affected by altering the frequency of antigen-carrying cells in mixed bone marrow (BM) chimeras. When antigen-bearing cells are present at low frequency, the reactive B cells and their antigens may coexist in the peripheral lymphoid organs, but under these conditions the B cells are functionally anergic and have a shortened lifespan. Such putative anergic cells are strongly deleted in the presence of additional, non-antigen-bearing, non-transgenic B cells. Since the antigen concentration on the surface of each antigen-bearing cell should be high, these results suggest that for efficient deletion of auto-reactive B cells multiple antigen encounters may be required, particularly when cellular competition is weak. These results have implications for the therapeutic use of BM chimerism to induce B cell tolerance to grafts.

Keywords

B lymphocyte; Immune tolerance; Antigen dose; Receptor editing; Anergy

1 Introduction

Tolerance induction in immature, autoreactive B cells has been shown to occur through several mechanisms including receptor editing [1–5], deletion [6–9], and functional inactivation [10, 11]. The particular mechanism invoked has been attributed to the nature of the antigenic stimulus, whereby multivalent antigens generally induce receptor editing and deletion [1–3, 7], and low-valency antigens cause functional inactivation [10, 11]. BCR affinity for Ag is also presumed to contribute to the tolerogenic signal, and recent studies have shown that receptor editing and deletion of autoreactive B cells is extremely efficient even with very low affinity, multivalent Ag [12, 13]. Anti-Ig treatment *in vivo* can induce either B cell deletion or anergy, depending upon the antibodies used [14–17]. In addition, previous *in vivo* and *in vitro* studies have suggested that high anti-Ig concentrations induce deletion of B cells while low concentrations induce anergy (reviewed in [18]), but these conclusions are difficult to generalize due to the possible inhibitory effect of FcR on BCR signaling [19–22].

In 3–83 (anti-H-2^{k, b}) Ig transgenic (Tg) mice, the class I auto-antigen can be expressed on virtually all cells at significantly high levels. Thus the ubiquitous expression of high-avidity Ag may allow deletion of cells with low-affinity BCR, whereas the same Ag at lower density or dose may alter the mechanism of tolerance induction or lead to a complete loss of tolerance. To study the effect of the Ag dose on B-cell tolerance using 3–83 μ Tg mice, we prepared mixed bone marrow (BM) chimeras in which the number of Ag-bearing cells, rather than the density of Ag per cell, was titrated. This approach essentially tests the role of the frequency of Ag encounter on B-cell tolerance induction, as opposed to altering the strength of the BCR signal as occurs when varying Ag valency or affinity. In addition, we probed tolerance induction in the context of a polyclonal B cell repertoire. These experiments allow an analysis of B cell tolerance mechanisms to antigens borne by a subset of BM cells, an area of research with potential clinical importance.

2 Results

2.1 The number of idiotype (Id)-positive cells in peripheral lymphoid organs decreases with Ag dose

To investigate the role of Ag dose on B cell tolerance induction, we prepared mixed chimeras in which Ag-deficient recipient mice were reconstituted with various ratios of 3–83 μ BM cells from mice that were Ag-carrying (K^k or D^k) and Ag-free (H-2^d) (illustrated in Fig. 1). This generates a situation in which all B cells carry the 3–83 transgenes, but the proportion of antigen-carrying hematopoietic cells varies. In the recipients, K^k- and D^k-positive cells were detected with anti-class I Ab Y3 (Fig. 2 A, top panel), whereas B cells carrying the Tg-encoded receptor were identified by co-staining with an anti-3–83 idiotype (54.1) and a polyclonal anti-IgM Ab (Fig. 2 A, lower panels). A total of 53 mice were analyzed from 4–18 weeks post reconstitution. As the timepoints of analysis did not appear to affect the results significantly, data from these various experiments were pooled.

In mixed chimeras the frequency of 3–83⁺ cells recovered from spleens and lymph nodes was found to diminish sharply with increasing Ag dose (Fig. 2 A, lower panels; Fig. 2 B). Deletion was complete in all peripheral lymphoid organs investigated when approximately 1/3 of the cells or more expressed Ag (Fig. 2 B). In mice expressing few Ag-bearing cells, deletion from the lymph nodes was always more complete than deletion from the spleen. Only about 1 % antigen-carrying cells present in the lymph nodes was apparently necessary to delete virtually all reactive B cells (Fig. 2 B, squares). Moreover, partial deletion relative to antigen-free controls was even observed in chimeric mice with frequencies of antigen-bearing cells below the level of detection, 0.1 % (Fig. 2 B). In recipients with incomplete deletion (“partial-deletors”) the majority of the remaining Id⁺ cells expressed reduced levels of surface BCR compared to H-2^d controls (mean fluorescence intensity of Id⁺ cells was 36 $\pm$ 6 vs. 61 $\pm$ 8 in LN; and 34 $\pm$ 5 vs. 48 $\pm$ 7 in spleen), suggesting that these cells had encountered Ag.

2.2 Percentage of receptor-edited cells increases with Ag dose

To determine whether limiting Ag dose would influence the receptor editing process, we looked for cells that had undergone receptor editing among the reconstituted mixed chimeras by staining the peripheral lymphoid organs with anti-IgD^a (Tg H-chain allotype) and anti-(endogenous light chain) antibodies. We have previously shown a significant increase in the number of IgD^a, ⁺ peripheral B cells in 3–83 Tg mice expressing Ag in the BM, with these double-positive cells constituting approximately 50 % of the peripheral B cells [3, 13, 23]. In the reconstituted mice, the percentage and absolute number of “receptor-edited” IgD^a, ⁺ cells increased with Ag dose (Fig. 3). It is noteworthy that in these chimeric mice, the IgD^a, ⁺ population contained both cells that had edited to an endogenous kappa light chain and, in

cases of low Ag dose, Id⁺ cells that had down-regulated surface Ig (sIg) expression (Fig. 3 A).

2.3 Remaining Id⁺ cells in partial deletors exhibit reduced functionality *in vivo* and *in vitro*

To characterize the state of tolerance in the mixed BM chimeric mice with either no, limited, or substantial numbers of Ag-carrying cells, we assessed B cell functionality by measuring levels of Id⁺ serum antibodies (Fig. 4 A) and the amount of Id IgM secreted per Id⁺ cell in LPS-stimulated B-cell cultures (Fig. 4 C). Whereas Ag-free mice had substantial amounts of Id⁺ antibodies in both their sera (Fig. 4 A) and LPS culture supernatants (Fig. 4 C), chimeric mice exhibiting incomplete deletion of Id⁺ cells secreted greatly reduced amounts of Id⁺ antibodies in LPS culture supernatants and in sera (Fig. 4 A, C). While the reduced serum idiotype concentration may reflect binding of antibody to antigen-bearing cells *in vivo*, the decrease in Id⁺ antibody secretion in LPS cultures of B cells derived from antigen-carrying mice was probably due to reduced functionality of the remaining B cells. Moreover, mice lacking transgenic B cells in the peripheral lymphoid organs also lacked Id⁺ antibodies in both sera and LPS supernatants (Fig. 4 A, C). Tolerance was specific to Id⁺ B cells, as all sera and most LPS cultures contained significantly more Id⁻ than Id⁺ IgM (Fig. 4 B, D), particularly in the case of complete deletion in which more receptor editing occurred. The serum Id⁺ antibodies detected retained their specificity to class I Ag as they stained H-2^k but not H-2^d expressing tumors (data not shown). Thus in chimeric mice with incomplete deletion of the Tg B cells, the remaining Id⁺ cells had reduced Ab secretion both *in vivo* and *in vitro*.

2.4 B cells in partial deleting chimeric mice have shortened lifespans

To characterize further the undeleted Id⁺ B cells in the chimeric mice demonstrating partial deletion, we examined the ability to these cells to take up 5-bromo-2'-deoxyuridine (BrdU) compared to B cells from mice either lacking Ag or showing complete deletion. After treating mice for 13 days with BrdU, ~20–30 % of the B220⁺ cells in the lymph nodes and 25–35 % of the B220⁺ cells in the spleens of reconstituted mice showing either no or complete deletion had incorporated BrdU (Fig. 5), a finding consistent with previous data reporting relatively long peripheral B cell lifespans in both Ig Tg [23–25] and normal mice [26]. In contrast, 45–85 % of B220⁺ cells in both the lymph nodes and spleens of the partial deletors had labeled with BrdU during the same time period (Fig. 5), indicating a much faster rate of turnover of these cells. Since the B220⁺ population in the partial-deleting recipients consisted of both “receptor edited” cells (Fig. 3), which are not self reactive and have a long lifespan, and the undeleted Id⁺ cells, the measured turnover (50–80 % of B220⁺ cells) in this BrdU assay is an underestimate of the true turnover of the remaining Id⁺ population. This high rate of peripheral B cell turnover has been reported in other mice exhibiting B cell anergy induction [23, 25].

2.5 In the presence of non-Tg competitive B cells, deletion of autoreactive B cells with even very small doses of low-affinity Ag is extremely efficient

Since anergic B cells have been shown to have a survival disadvantage when in competition with non-anergic B cells [24, 27, 28], we tested the effect of non-Tg B cells in our mixed BM chimeric mice, under conditions that, in the absence of such cells, manifested incomplete deletion and B cells of an anergic phenotype. Mixed BM chimeras were generated in which 25 % of the donor BM was derived from non-Tg mice and 75 % was from a mixture of Tg donors that lacked or expressed antigen (illustrated in Fig. 6 A). This 75 % Tg:25 % non-Tg ratio resulted in approximately 25 % of the B cells bearing the Tg receptor (Fig. 6 B, compare no Ag 100 % Tg to Tg:non-Tg), a result similar to that reported in a different BCR Tg mouse system [27]. As reported earlier in this study, reconstituted mice derived from monoclonal B cell mice receiving limiting amounts of Ag (< 1%) showed

incomplete deletion of the reactive B cells from the peripheral lymph organs with approximately one-third of the Id⁺ lymph node cells remaining (Fig. 6B, “100% Tg/< 1% Ag”). However, in the more physiological scenario of a heterogeneous B cell repertoire, all of the Id⁺ cells were deleted from the lymph node and spleen with similar amounts of limiting Ag (< 1%) (Fig. 6B, “75% Tg:25% Non-Tg/< 1% Ag”), suggesting that the putatively anergic B cells were unable to compete with the non-Tg B cells.

3 Discussion

A major conclusion to be drawn from this study is that self tolerance responses to a highly cross-linking, membrane-bound Ag can occur through several mechanisms including anergy, clonal elimination, and receptor editing, depending upon antigen availability and the frequency of antigen-bearing cells. Low frequencies of antigen-bearing cells could not completely delete reactive B cells, but induced partial deletion and anergy. Higher doses of antigen induced clonal elimination and the appearance of B cells that had apparently undergone receptor editing. Since each Ag-bearing cell expresses high levels of MHC class I antigen, increasing the frequency of Ag-bearing cells should not greatly affect the strength of signal caused by any particular encounter between 3–83 B cells and Ag-bearing cells. Therefore the ability of higher frequencies of Ag-bearing cells to mediate more efficient deletion is likely to be the result of an increased frequency of B cell:antigen encounter. This may indicate the need for sustained signaling in B cell tolerance. An alternative interpretation is that, because in our mice manifesting B cell anergy some serum Id was detected, these antibodies cover up Ag, reducing the effective density of antigen and preventing deletion by weakening the BCR signal. We do not favor this latter interpretation, however, because such antibody production should only occur late in B cell development.

The anergy in the Tg B cells induced by a low dose of Ag-bearing cells was indicated by several markers including down-regulated IgM receptor expression, reduced antibody secretion both *in vivo* and *in vitro*, and shortened lifespan. These characteristics have previously been associated with anergy in HEL- and DNA-specific BCR Tg mice [10, 11]. Our data is also consistent with data from Okamoto and colleagues in which a variety of tolerance mechanisms were induced in anti-RBC membrane protein Ig Tg mice [29]. It is noteworthy that in mice where functional inactivation was seen, there was some degree of deletion of 3–83 B cells as well. This was also seen, but not emphasized, in the studies of other groups studying anergy [10, 15, 30]. This may reflect the shorter lifespan of the inactivated cells [25, 27].

Although the chimera studies using 3–83 μ Tg donor BM exclusively were important in identifying a change in tolerance phenotype (from receptor editing to anergy) with low Ag dose, the experiments in which the 3–83 μ Tg cells existed within a polyclonal repertoire did not reveal this distinction in tolerance mechanisms. In fact, the mixed chimera experiments in which both 3–83 μ Tg cells and Ag-bearing cells were titrated showed that deletion of Id⁺ cells is extremely efficient even with very low Ag doses (< 1 %) of low-affinity Ag (D^k). One possible interpretation of these experiments is that the non-Tg cells have a competitive advantage over anergic, Id⁺ cells, as previously described [24, 27, 28]. This suggests that in a normal, polyclonal repertoire the anergic B cells would be even shorter-lived and anergy would be essentially indistinguishable from deletion.

The question of the mechanism of B cell tolerance to high-avidity BM-derived antigens is pertinent to possible new approaches to xenograft transplantation. Humans and other primates have large quantities of natural anti-Gal 1-3Gal 1-4GlcNAc-R (Gal) antibodies in sera directed against glycoproteins present on most non-primate mammalian cells. These antibodies are largely responsible for the hyperacute rejection of xenografts [31–33]. Recent

studies have shown that Gal-specific antibody responses of 1,3-galactosyltransferase-deficient mice can be tolerized in BM chimeras in the presence of Gal-bearing cells [34]. It will be of interest in the Gal experimental system to determine mechanisms of tolerance to this widely expressed membrane-bound Ag. The factors that lead to deletion rather than anergy are of more than academic interest, as anergy, unlike deletion, is potentially reversible. The findings of the present study may therefore be important in defining procedures for inducing tolerance to achieve successful long-term xenograft transplantation, which could solve the ever present problem of graft shortage in the transplantation field.

4 Materials and methods

4.1 Mice

3–83 μ Ig Tg mice [35], which express IgM and IgD forms of the 3–83 (anti-H-2^{k, b}) antibody, were maintained under specific pathogen-free conditions at the National Jewish Medical and Research Center (NJMRC). 3–83 μ mice were backcrossed a minimum of ten times onto the B10.D2 background and subsequently bred one generation with B10.BR (H-2^k), B10.D2/nSnJ (H-2^d), or C3H-H-2o2/SfSn (C3H.OH: K^dD^k), C57BL/6 (H-2^b) mice (all purchased from Jackson Laboratory, Bar Harbor, ME).

4.2 Mixed bone marrow chimeras

BM cell suspensions were prepared from donor mice (B10.D2, B10.BR, and C3H.OH or F1 mice resulting from breeding of these mice with 3–83 μ Tg mice) as described [6]. After depletion of RBC by ammonium chloride lysis [36], T, B, and natural killer (NK) cells were depleted by incubation of the cell suspensions in an antibody cocktail containing T24/40 [37] and H013.49 [38] anti-Thy-1 supernatants, 2.4G2 [39] anti-FcR II supernatant, and RA3/6A1 [40] anti-B220 antibody (9 μ g/ml) followed by “panning” on rabbit anti-mouse Ig coated plates [41]. BM cell preparations from the various donor mice were mixed to achieve the desired ratios of Ag-bearing:H-2^d BM as well as desired ratios of Tg:non-Tg donor BM cells. Approximately 100 μ l/mouse (4×10^6 cells) was injected into the tail vein of lethally irradiated 6–8-month-old B10.D2 female recipients. Groups of mice were analyzed at 4, 6, 10, 16, or 18 weeks after BM reconstitution, all with similar results.

4.3 Antibodies, immunofluorescence and cell lines

Preparation of lymphoid cells, Ab reagents, fluorochrome conjugation, and flow cytometry analysis were as described previously [13]. 4549 (H-2K^k) and Sp2/0 (H-2^d) cell lines were also previously described [13]. To test binding of serum antibodies to Ag-bearing hybridoma cells, 5×10^5 4549 or Sp2/0 hybridoma cells were incubated in 100 μ l of a 1:20 dilution of mouse serum for 30 minutes on ice. After two washes, the bound antibodies were detected with FITC-labeled goat anti-rat IgM antibody (Southern Biotechnology Associates, Inc., Birmingham, AL). Cells were analyzed on an FACS Profile (Coulter Electronics Inc., Hialeah, FL).

4.4 BrdU incorporation assay

BrdU (Sigma Chemical Co., St. Louis, MO) was provided to mice in filtered, deionized drinking water at a concentration of 1 mg/ml for 13 days. Staining for the incorporation of BrdU was described previously [42].

4.5 LPS cultures and serum antibody analysis

Spleen and lymph node cells were stimulated with LPS (Sigma Chemical Co.) as described previously [23]. The cell concentration in the cultures was adjusted depending on the number of 3–83 B cells in the organ. The lymph node cells from chimeric mice lacking Ag

(H-2^d) were cultured at 1×10^6 cells/ml, those from mice containing a mixture of H-2^d and Ag-bearing cells were cultured at 2×10^6 /ml, and lymph node cells from mice containing all Ag-bearing cells were cultured at 3×10^6 cells/ml. Spleen cells were cultured at the following concentrations depending on the amount of Ag: < 10 % Ag, 1×10^6 cells/ml; 15–50 % Ag, 2×10^6 cells/ml; and 100 % Ag at 5×10^6 cells/ml. Supernatants were collected on days 3 and 7. Ig concentrations in culture supernatants and sera were determined by ELISA as described previously [35].

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Fig. 1.

Preparation of mixed BM chimeric mice. BM cells from Ag-free, H-2^d 3-83 μ mice were mixed at various ratios with BM cells from K^k or D^k (antigen-bearing) 3-83 μ mice, and injected into lethally irradiated, Ag-free B10.D2 recipients. Lymphoid tissues of chimeric mice were analyzed 4–18 weeks after reconstitution for their abundance of K^k or D^k-bearing cells and for B cell tolerance.

Fig. 2.

In mixed BM chimeras, deletion of Id⁺ B cells from peripheral lymphoid organs increases with Ag dose. (A) Lymph node (LN) cells from chimeras were stained for K^k- or D^k-bearing cells with Y3 Ab (top row of panels) and for 3–83⁺ B cells in LN (middle row) and spleen (lower) using 54.1 (anti-Id) and anti-IgM antibodies. (B) The percentage of Id⁺ cells in experimental chimeras relative to Ag-free controls was calculated and plotted as a function of the percentage of Ag-bearing cells detected in LN as assessed with Y3 antibody. Each point indicates the value obtained in an individual mouse. Squares, spleen; circles, LN.

Fig. 3.

The frequency of receptor-edited cells increases with Ag dose. LN and spleen cells from mixed BM chimeras were double-stained with IgD^a and λ antibodies to detect cells that had undergone receptor editing. (A) Representative FACS data of splenic B cells, with the Ag percentages representing the amount of Ag in the donor BM inoculum. (B) Combined data from three such experiments showing the percentage of IgD^{a+}, λ ⁺ cells vs. percentage of Ag-bearing cells. Each point represents the value obtained with an individual mouse.

Serum Antibodies

In Vitro LPS-stimulated Antibody Secretion

Fig. 4.

Id⁺ B cells from Ag-containing chimeras secrete less antibody *in vivo* and *in vitro*. Mice from four experiments were grouped by tolerance phenotypes as follows: -, no deletion; +/- -, partial deletion in lymph node and spleen; +(LN), partial deletion from spleen/complete deletion from lymph nodes; +: virtually complete deletion. (A, B) Chimera sera were tested for Id⁺ (A) and IgM (B) antibodies by ELISA. In (A) numbers within graph show mice analyzed/group. (C, D) Lymphoid cells from chimeras were cultured with LPS for 3 days and antibodies in supernatants quantitated by ELISA: (C) Id⁺; (D) IgM. To compare cultures, the amount of Id⁺ and IgM antibodies secreted per Id⁺ or IgD⁺ cell was calculated.

Fig. 5. Remaining Id⁺ B cells in partial-deleting mixed BM chimeras have shortened lifespans. Reconstituted mice were given BrdU water for 13 days and the percentage of B220⁺ LN and spleen cells incorporating BrdU was measured. Chimeras were categorized for tolerance phenotypes as described in Fig. 4.

Fig. 6.

In a polyclonal B cell repertoire low doses of ultra-low affinity Ag delete autoreactive B cells efficiently. (A) Experimental strategy. The upper section is the strategy described in Fig. 1. Below is shown the strategy for producing a “polyclonal mouse” by reconstituting antigen-free recipients with a BM preparation including 25 % B10.D2 and 75 % 3–83 μ Tg cells. The Tg fraction was made up of Ag-free (H-2^d) and antigen bearing (D^k) cells at various ratios. (B, C) Lymph nodes cells from recipients were stained to detect specific B cells (B) or Ag-bearing cells (C). Data are representative of three similar experiments.