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Antibodies to Surface IgM Can Accelerate Apoptosis of Mature B-Lymphocyes at Sub-Stimulatory Concentrations†

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Abstract

Antibody to B-cell surface immunoglobulin D (IgD) or surface IgM results in crosslinking of Ig molecules and signal transduction. The function of these surface immunoglobulins has traditionally been investigated by extensive crosslinking experiments and interest has been focused on activation assays. We investigated the effects on apoptosis of culture with anti-μ antibody (anti-μ) concentrations ranging from 0.001 μg mL^{-1} to 50 μg mL^{-1}. Previous experiments have shown that weak dose anti-δ antibody (anti-δ) increases mature B-cell apoptosis at both 16- and 64-hour time points, while greater dose anti-δ results in cell cycle entry at 64 hours. The question addressed is whether anti-μ induces the same biphasic response. After 16 hours of culture, both a monoclonal and a polyclonal anti-μ at weak concentrations caused much less of an increase in apoptosis than anti-δ, although cell cycle entry at 64 hours was similar. Together, these results suggest a mechanism for low-zone B-cell tolerance induction, a process that was previously thought only to occur in T-cells.

Introduction

The transition of B-lymphocytes from immature to mature cells involves a change in expressed surface immunoglobulin (sIg). Immature B-cells possess only the IgM form (Vitetta et al., 1975) and, as they mature, the ratio of sIgM to sIgD steadily increases to between 1:5 and 1:10 (Vitetta et al., 1975; Yuan and Vitetta, 1978; Havran et al., 1984). It remains unclear why these molecules are coexpressed in mature B-cells, but it is thought that the changes in ratio affect the cell's functional responsiveness (Monroe, 1996). Extensive attempts have been made to reveal differences in the functions of IgM and IgD, but to date the results have been contradictory. For example, in the cell line WEHI-231, a model of immature B-cells, crosslinking of sIgM with anti-μ (against the heavy chain of IgM) resulted in B-cell death (Benhamou et al., 1990; Hasbold and Klaus, 1990). This was not observed when sIgD was crosslinked on δ-chain transfectants of these cells (Ales-Martines et al., 1988; Tisch et al., 1988). However, in signaling experiments using CH33 cells transfected with the μ or δ chain, Webb et al. (1989) observed signaling to occur in a similar manner through both types of sIg.

According to the widely-accepted model developed by Bretscher and Cohn (1968), B-cell activation occurs as a result of two signaling events. Signal 1 involves the binding of antigen to sIg. Signal 2 results from the interaction of a T-helper-cell or its products with the B-cell. There is evidence that, when a B-cell receives signal 1 without adequate signal 2, apoptosis results. For example, in collaboration with Dr. F. Finkelman, we have recently shown that when anti-δ is injected into a mouse under conditions where T-cell help has been depleted, B-cells undergo apoptosis (Peckham et al., unpublished). This was further investigated *in vitro* by incubating mature splenic murine B-lymphocytes with the soluble mouse IgG2b monoclonal antibody H.δa/1 against sIgD (gift of F. Finkelman). A weak dose of this antibody (0.1 to 0.5 μg mL^{-1}) increased apoptosis, whereas a dose of 10 to 100 μg antibody mL^{-1} triggered a little RNA and DNA synthesis with less apoptosis (Peckham et al., unpublished). This cell cycle entry may have been dependent on a small amount of help from residual T-cells in the preparation (about 2%). These results support the hypothesis that a minor amount of B-cell receptor engagement may produce low-dose tolerance in B-cells via apoptosis induction.

Approaches taken to study sIgD and sIgM functioning have traditionally involved crosslinking these receptors with antibodies to the Ig heavy-chain constant regions (for example, Goroff et al., 1986). Because much of the research on surface Ig signaling was carried out before the emergence of interest in apoptosis, most studies focus on activation assays and use the greater anti-Ig concentrations that cause activation. In addition, apoptosis induction in mature B-cells by anti-μ or anti-δ has been thought to require very extensive crosslinking of the sIg (Parry et al., 1994).

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We describe the results of crosslinking sIgM in mature splenic B-lymphocytes with a range of concentrations of two anti-μ. We sought to determine whether weak doses of anti-μ induce apoptosis and greater doses induce cell cycling, a pattern similar to that seen after crosslinking of sIgD. Our results showed that polyclonal and monoclonal anti-μ were much less effective in driving early apoptosis at 0.1 to 0.5 μg mL^{-1} than equal concentrations of anti-δ had been (Peckham et al., unpublished).

Materials and Methods

Mice: Female BALB/c mice, ages 6 to 10 weeks (The Jackson Laboratory, Bar Harbor, ME), were housed in a pathogen-free facility with high-efficiency particle-free filtered air, autoclaved food and water, limited personnel access, and periodic viral antibody (Ab) screening.

B-lymphocyte preparation: Spleens were disrupted between sterile ground-glass slides and cells were gently resuspended. They were treated with RBC-lysing buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.0001 M EDTA) and panned in the presence of 3% BSA (Severson et al., 1987). Residual T-lymphocytes were lysed with anti-Thy 1.2 (Accurate Scientific, Westbury, NY) plus rabbit complement (Rockland, Gilbertsville, PA) at a ratio of 1:18 complement:cell suspension, with cells at a concentration of 20 x 10^6 cells mL^{-1}. Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation was used to separate cells. Cells from the 76%/49% Percoll interface (ρ = 1.038 to 1.081) were obtained which are > 95% $B220^+Ig^+$, 2% $Thy\text{-}1.2^+$, highly homogenous in forward and orthogonal scatter by flow cytometry, low in transferrin receptor (Vora et al., 1990) and IL-2R expression, and restricted to the G_0 phase of the cell cycle (Futran et al., 1989). Cells were either assayed directly (T_0 samples) or placed into culture media.

Cell culture: Cells were cultured in complete RPMI 1640 medium (GIBCO BRL Laboratories, Grand Island, NY), supplemented with 5% FCS, 1 mM Na-pyruvate, 10 mM HEPES, 0.1 mM nonessential amino acids, and 1 mM L-glutamine. They were cultured in 96-well Costar plates in 0.2 ml of culture medium at a concentration of 2 x 10^6 cells mL^{-1} and were incubated at 37° C in a water-saturated atmosphere containing 5% CO_2.

Antibodies: IgG1 B.7.6 rat anti-Cμ2 monoclonal antibody (a gift from T. Waldschmidt; Leptin et al., 1984) and polyclonal goat IgG anti-μ Ab from Jackson Immunoresearch Laboratories (West Grove, PA) were used at a range of concentrations.

Flow cytometric analysis: Analyses were performed on a Coulter EPICS 753 Flow Cytometer (Coulter Corp., Hialeah, FL).

1) Analysis of cell cycle and apoptosis by acridine orange (AO):AO binds DNA and RNA metachromatically. Cell cycle and apoptosis were measured simultaneously by AO staining according to the method of Traganos et al. (1977). Buffer 1 consisting of citrate-phosphate buffer (0.02 M phosphate which contained 0.1 % Triton X-100, 0.2 M sucrose, and 100 μM Na_2EDTA, at pH 3.5) was added to permeabilize cells. One minute later, citrate-phosphate buffer 2 was added. It consisted of 0.01 M phosphate containing 0.1 M NaCl and 20 μg mL^{-1} AO (Polysciences, Warrington, PA) at pH 3.8. Cells were analyzed by flow cytometry using a 488-nm Argon laser excitatory band, a 525-nm band-pass filter (green = DNA) and a 635-nm band-pass filter (red = RNA). Cells in G_0 isolated from a density gradient had a narrow range of RNA and DNA content by flow cytometric analysis. A gate around this population established the G_0/G_1 and the G_0 G_0/hypodiploid interfaces. The S-phase cells contained greater than 1X DNA and G_2 + M phase cells contained 2X DNA. Hypodiploid cells contained less DNA than G_0 cells, but more than cellular debris. Minimum and maximum staining of hypodiploid cells was identified from a forward scatter against a DNA histogram of AO-stained cells from unstimulated 16-hour cultures (T'raganos et al., 1977).

2) Merocyanine (MC) 540 staining: B-cell apoptosis may be detected by staining with a lipophilic dye such as Merocyanine 540 (MC540; Sigma; Mower et al., 1994). About 4 hours before apoptotic B-cells cleave their DNA, their plasma membrane becomes symmetrical (due to the loss of function of aminophospholipid translocase) which increases membrane propensity for MC540 binding (Ashman et al., 1996). Dye was added to the cells for a final concentration of 5 μg mL^{-1} directly before FACS analysis. Cells were analyzed by flow cytometry using an argon laser with a 488-nm excitatory band. Emissions were split with a 600-nm short pass dichroic mirror. A band pass filter (575-mn) was placed in front of the photomultiplier tube to measure emissions.

Thymidine Incorporation: After 64 hours of culture, B-lymphocytes were pulsed for 8 hours with 0.5 μCi [^{3}H]TdR (Amersham) per well. A Packard Filtermate 196-cell harvester (Packard Instrument Co., Meriden, CT) was used to harvest cells onto glass fiber filters. The filters were washed with water to lyse cells and remove unincorporated TdR, and were counted in a Packard Matrix 96 Direct Beta Counter.

Statistical analyses: In order to compare B-lymphocyte responses to anti-μ with previous results

with anti-δ, mature murine splenic B-cells were cultured for 16 and 64 hours with both monoclonal and polyclonal anti-μ over a range of concentrations. After 16 or 64 hours, B-cells were stained with AO and analyzed by flow cytometry. Statistical analysis of the data was performed using an analysis of variance (ANOVA) with arcsine transformed percentages of cells in apoptosis (Zar, 1984).

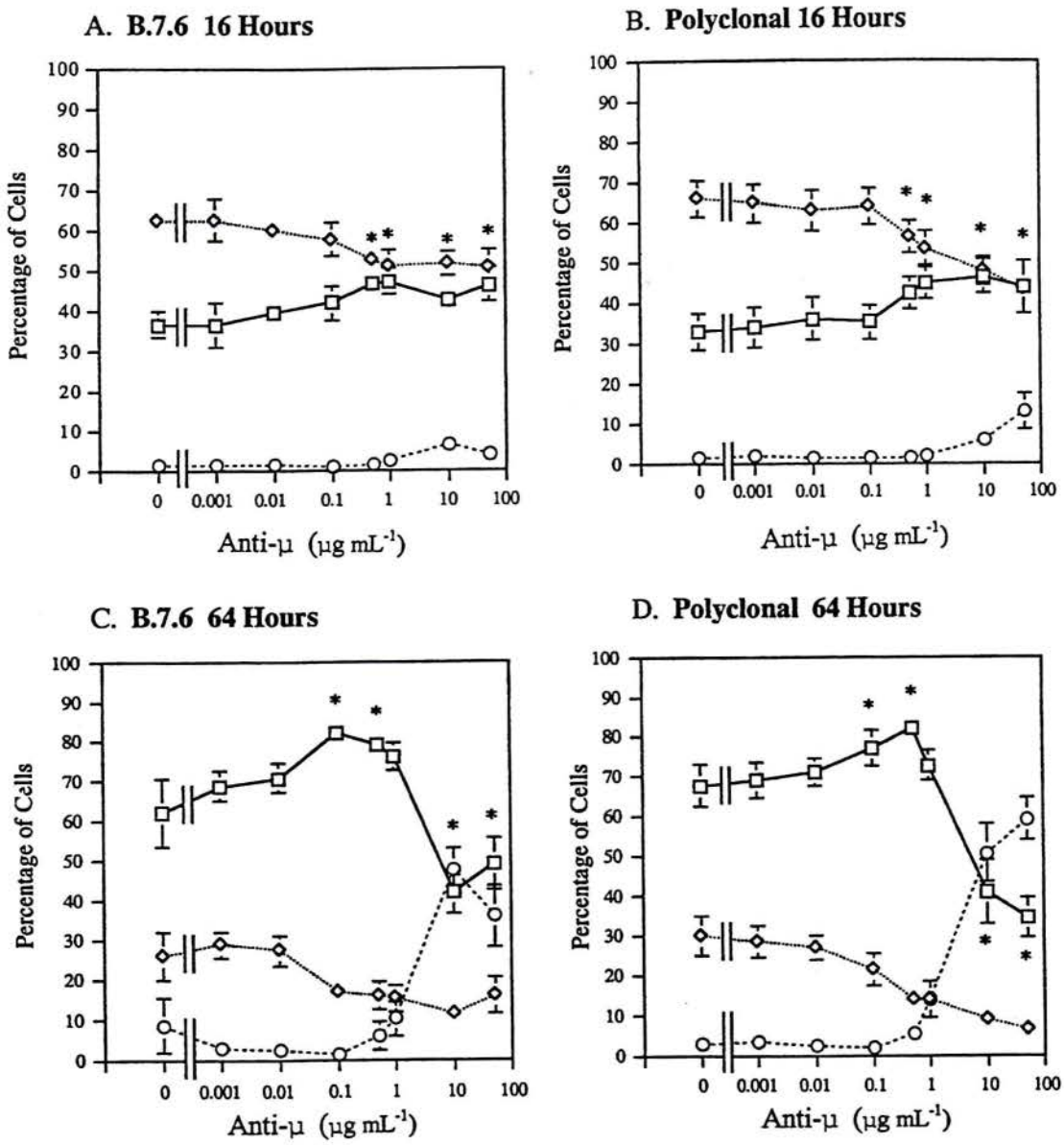

Figure 1. Acridine orange staining of DNA and RNA of B-cells cultured with a monoclonal or a polyclonal anti-μ preparation. Mature splenic B-cells were cultured for 16 or 64 hours with concentrations of monoclonal (B.7.6) or polyclonal anti-μ from 0.001 to 50 μg mL^{-1}. Cells were stained with acridine orange (AO) and analysis was performed by flow cytometry, —□— = cells in apoptosis, —◇— = cells in G_0, —○— = cells in cycle. Symbols represent means +/- SEM of four complete experiments. Statistical analysis of the data was performed using an analysis of variance (ANOVA) with arcsine transformed percentages of cells in apoptosis (Zar, 1984). Statistically significant points are marked with *. Effects of the monoclonal and polyclonal antibodies were the same at both time points (ANOVA). Panels A and B: Antibody concentrations of 0.5, 1, 10, and 50 μg mL^{-1} increased apoptosis after 16 hours of culture (Dunnett's method. $p < 0.05$). Panels C and D: Antibody concentrations of 0.1 and 0.5 μg mL^{-1} increased apoptosis whereas antibody concentrations of 10 and 50 μg mL^{-1} decreased apoptosis after 64 hours of culture (Dunnett's method, $p < 0.05$).

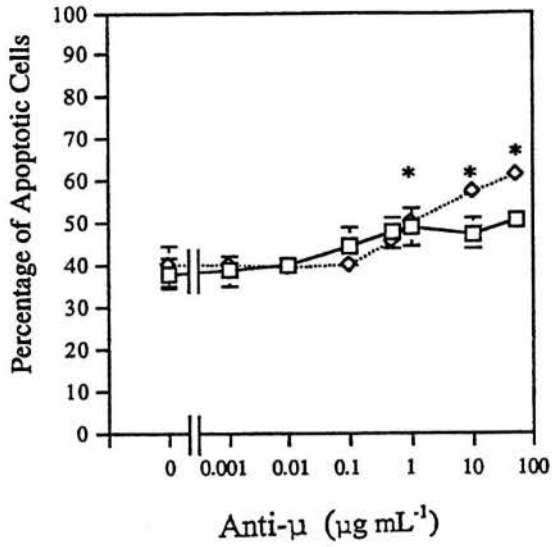

Figure 2. Plasma membrane unpacking of B-cells cultured with a monoclonal or a polyclonal anti-μ preparation. Mature splenic B-cells were cultured for 16 hours with concentrations of monoclonal (B.7.6) or polyclonal anti-μ from 0.001 to 50 μg mL^{-1}. Cells were stained with MC540 and analyzed by flow cytometry, —□— = B.7.6, —◇— = polyclonal antibody. Symbols represent means +/- SEM of four complete experiments. Statistical analysis of the data was performed using an analysis of variance (ANOVA) with arcsine transformed percentages of cells in apoptosis (Zar, 1984). Statistically significant points are marked with *. Effects of the monoclonal and polyclonal antibodies were the same at both time points (ANOVA). Antibody concentrations of 1, 10, and 50 μg mL^{-1} increased cell staining which is strongly associated with apoptosis in murine B-cells (Dunnett's method, $p < 0.05$).

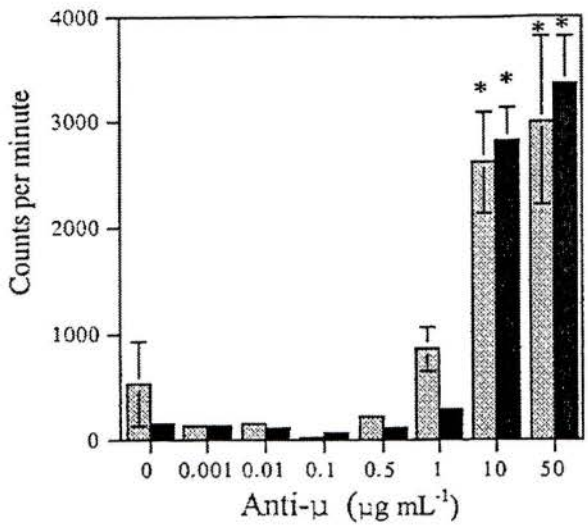

Figure 3: Tritiated thymidine incorporation of B-cells cultured with a monoclonal or a polyclonal anti-μ preparation. Mature splenic B-cells were cultured for 64 hours with concentrations of monoclonal (B.7.6) or polyclonal anti-μ from 0.001 to 50 μg mL^{-1}. Cells were pulsed for 8 hours with [^{3}H]TdR and they were harvested onto glass fiber filters. Filters were washed and counted in a Direct Beta Counter. Symbols represent means +/- SEM for 3 experiments with 3 replicate wells for each Ab concentration. □ = B.7.6 mean CPM, ■ = polyclonal mean CPM. Statistical analysis of the data was performed using an analysis of variance (ANOVA) with arcsine transformed percentages of cells in apoptosis (Zar, 1984). Antibody concentrations of 10 and 50 μg mL^{-1} significantly increased [^{3}H]TdR uptake and are marked with *.

Results

Effects of the monoclonal and polyclonal antibodies were the same at both 16- or 64-hour time points. At 16 hours, relative to B-cells cultured in medium alone, the two anti-μ preparations exhibited increased levels of apoptosis at 0.5, 1, 10, and 50 μg mL^{-1} (Dunnett's Method, $p < 0.05$); (Figures 1A and 1B). After 64 hours of culture, doses of 0.1 and 0.5 μg mL^{-1} increased levels of apoptosis (Dunnett's Method, $p < 0.05$) whereas doses of 10 and 50 μg mL^{-1} decreased levels of apoptosis (Dunnett's Method, $p < 0.05$; Figs. 1C and 1D).

Two methods were used to confirm the AO data. Cell membrane unpacking was measured at 16 hours by MC540 staining followed by flow cytometry and showed an increase in apoptosis at anti-μ concentrations of 1, 10, and 50 μg mL^{-1} (Dunnett's Method, $p < 0.05$; Fig. 2). This paralleled the AO data in that greater concentrations of anti-μ increased apoptosis at the 16-hour time point. Incubation with [^{3}H]TdR after 64 hours resulted in increases of [^{3}H]TdR uptake at anti-μ concentrations of 10 and 50 μg mL^{-1} (Dunnett's Method, $p < 0.05$; Fig. 3), which confirmed the AO observation that cells cultured with greater concentrations of anti-μ began to go into cycle after 64 hours.

Discussion

We investigated the effects of incubation with decreasing concentrations of a monoclonal and a polyclonal soluble anti-μ on splenic B-lymphocytes. After 16 hours of culture, anti-μ did not produce an observable effect at the lesser concentrations (0.001, 0.01, or 0.1 μg mL^{-1}); these B-cells remained in G_0. At greater concentrations, however, anti-μ did cause increased entry into apoptosis. We found that, after 64 hours of culture, a weak dose of anti-μ (0.1 and 0.5 μg mL^{-1}) substantially increases the rate of cell

progression from G_0 to apoptosis without evidence for G_1 entry, whereas doses of 10 and 50 $\mu g\ mL^{-1}$ drive RNA and DNA synthesis, accompanied by protection from apoptosis. Data obtained with the monoclonal and the polyclonal antibody approaches show a similar effect over the range of concentrations used. From this we conclude that the difference in degree of Ig clustering on the surface of the B-cell produced by these two preparations did not affect the cell's response.

These experiments are an investigation into the Signal 1/Signal 2 model developed by Bretscher and Cohn (1968). By purifying the B-cells, we obtain a suspension that is > 95% $B220^+Ig^+$ and about 2% Thy-positive (Ashman et al., 1996). It is presumed that such B-cells will not receive an optimal T-helper second signal from the small number of T-cells present after crosslinking of their surface Ig has occurred (although by further sort-purifying them, this assumption could be tested). In our previous results, lesser doses of anti-δ resulted in a level of crosslinking that required a second signal (IL-4 or anti-CD40) to rescue the B-cell from apoptosis (Peckham unpublished). At doses $\geq$ 10 μg anti-δ mL^{-1}, some cell cycle entry is seen without added signal 2 (Figures 1C and 1D) but the addition of anti-CD40 greatly increased cell cycle entry (Peckham unpublished). This observation is in accord with our finding that addition of IL-4 and/or anti-CD40 can also reduce B-cell entry into spontaneous apoptosis (Illera et al., 1995; Peckham et al., unpublished).

The classic study by Weigle et al. (1973) suggested that serum proteins could induce both T- and B-cell tolerance, but that it took more time and greater antigen (Ag) concentrations for this to occur in B-cells. They also promoted the idea that only T-cells were sensitive to low-dose tolerance induction. The evidence in this paper suggests that B-cells may also be sensitive to low-zone tolerance.

Two studies by Parry et al. (1994a, 1994b) are especially relevant to our findings. They investigated the ability of plastic-immobilized anti-μ and anti-δ monoclonal Abs to activate murine B-lymphocytes (1994b). Cells were lysed after incubation and nuclei were stained with propidium iodide. At doses typically used in soluble form to stimulate B-cells, immobilized Abs were instead found both to cause increases in the numbers of apoptotic nuclei and to cause precipitous decreases in [^{3}H]TdR incorporation. While this may indeed represent effects of elevated crosslinking, we speculate otherwise. When wells are coated with Ab, less than 5% of the Ab adheres to the plastic. In addition, much of this Ab is non-functional (that is, unable to make contact with any sIg on cells added to the well) or functionally limited (that is, unable to crosslink any sIg; Dr. J. Butler, personal communication). These considerations suggest that the apoptosis-inducing effects of immobilized Abs may actually be due to weak-dose soluble Ab, which would reconcile these observations by Parry et al. with our present study.

Parry et al. (1994a) found that when T-cell help is unavailable to a mature B-cell, the cell will either enter the cell cycle or undergo apoptosis depending upon the extent of the receptor crosslinking. These experiments were used to investigate the effect of hypercrosslinking of B-cell receptors with biotin-anti-μ or biotin-anti-δ in combination with avidin. Hypercrosslinking caused early (12 to 16 hour) entry into apoptosis. Comparing the apoptotic effects of hypercrosslinking to our findings that small amounts of crosslinking also cause B-cells to become apoptotic in the absence of T-cell help suggests that there may be a window of "optimal" crosslinking which causes activation of the B-cell above which and below which the cell is deleted. Parry et al. (1994a) suggested that large molecular weight polymers and perhaps cell surface Ags might represent hypercrosslinking situations in the body. The weak-dose results suggest a mechanism for low-zone tolerance of peripheral B-cells. Similar conclusions regarding extensive sIg crosslinking were reached by Watanabe et al. (1998) who observed B-cell apoptosis upon exposure to anti-Ig-coated erythrocytes.

These findings may provide evidence of a mechanism for the induction of B-cell tolerance in the periphery. For example, at the end of an immune response when much of the Ag has been cleared, the weak doses of Ag present would induce apoptosis of reactive B-cells that had encountered the Ag late in the immune response. Another case might be tolerance to self-antigens. Because negative selection is not perfect, there exist low-affinity B-cell clones that emerge from the bone marrow and are cross-reactive to self-Ags (Paul, 1989). Encounters of these clones with soluble self-Ag would lead to low levels of binding, so there would be an effective low dose seen by the B-cell which would lead to apoptosis in the absence of T-cell help.

In summary, our results suggest that, like T-cells, mature B-cells may exhibit low-zone tolerance which might be mediated through low levels of surface Ig crosslinking.

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