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Negligible Class II MHC Presentation of B Cell Receptor-Derived Peptides by High Density Resting B Cells

Christopher M. Snyder, Xianghua Zhang, and Lawrence J. Wysocki

Resting B lymphocytes have been credited with inducing T cell tolerance to Ig-derived and monovalent self-Ags that are internalized via the B cell receptor (BCR). These conclusions are predicated upon the assumptions that resting B cells display BCR-associated peptides in class II MHC and that the cells remain quiescent during the course of experimental manipulation. To determine whether resting B cells display BCR-associated epitopes in class II MHC, we devised a sensitive assay that averted potential activation of B cells by Ag and minimized activation by prolonged culture. Ex vivo, Percoll-fractionated B cells expressing a κ transgene encoding a T cell epitope were cultured with a reactive T cell hybridoma for 12 h. Whereas low density, LPS-activated, and BCR-activated B cells elicited significant IL-2 from the T cell hybridoma, resting high density B cells did not. Parallel results were obtained with normal B cells expressing a second epitope encoded by an endogenous Vκ gene. Anergic B cells, which are uniformly low density, also significantly stimulated the T cell hybridoma. Finally, longer culture periods with normal B cells resulted in a higher degree of B cell activation and significant stimulation of reactive T cell hybridomas. Our results provide evidence that activation of B cells profoundly enhances the processing and presentation of BCR-associated Ags. The Journal of Immunology, 2002, 168: 3865–3873.

A substantial body of evidence supports the idea that a productive, Ag-specific collaboration between B and T lymphocytes requires aggregation of the receptor Ab on the B cell surface. Although signaling through the B cell receptor (BCR) has direct autonomous effects on the physiological state of the B cell, it also exerts indirect effects by inducing expression of costimulatory surface molecules that mediate communication with T cells. Signaling induced by BCR cross-linking also enhances processing and presentation of MHC class II presentation of Ag by B cell lines (1–9). However, it is presently unclear whether normal B cells must be activated to induce processing and presentation of BCR-associated Ag. Early studies involving relatively insensitive non-BCR-mediated uptake of Ag produced conflicting results regarding this question (10–13). However, results of more recent studies have led to a currently held view that resting B cells cycle their BCR through Ag-processing compartments and are able to process and present BCR-associated Ags in the context of class II MHC. This conclusion is largely inferred from three avenues of investigation. B lymphomas can shuttle Ig into MHC II-containing compartments in the absence of BCR ligation (2, 14, 15). Ex vivo isolated B cells can activate T cell lines that are specific for Ig allotypic epitopes located in both H and L chains (16–21). Normal B cells treated with monovalent Ags, such as Fab anti-Igδ, are able to stimulate T cell lines reactive with the Fab-derived peptides (22–24). The physiological relevance of these in vitro results is supported by corresponding in vivo studies suggesting that resting B cells can induce tolerance in Th cells to BCR-derived or monovalent Ags (25–27). These data are consistent with an extensive literature concluding that, in the absence of costimulation, TCR engagement of peptide-MHC may lead to T cell tolerance in vitro and vivo (28–36).

Important complicating issues have obscured interpretations of these B cell presentation and tolerance studies. Evidently, B cells become activated on prolonged culture with specific T cell lines and clones (12, 24, 37–39). It is unclear whether this activation requires cognate T-B interactions or whether it results from undefined stimulatory events associated with B cell isolation and culture. Monovalent Ags should not stimulate B cells. In some cases, however, Fab anti-Igδ evidently increased the rate of BCR turnover (15). The reason for this is unclear, but it could be due to limited aggregation of the Fab reagent or to trace contamination by whole anti-Igδ. The use of T cell lines and clones to assess class II MHC-restricted peptide display also introduces complications because of potential costimulatory requirements for T cell activation (40, 41). Finally, the idea that resting B cells present BCR-associated Ags for T cell tolerance is challenged by more recent data suggesting that T cell tolerance in vivo requires costimulation and is associated with T cell proliferation (42–46).

To more accurately assess the potential of resting B cells to present BCR-associated epitopes in class II MHC, we made use of the fact that peptides derived from Ab V regions can serve as Ags for T cells (18, 47–52). The use of B cells expressing Abs with such epitopes averts potential BCR aggregation problems that might occur upon treatment with monovalent Ags. Furthermore, we used T cell hybridomas without costimulatory requirements to assess peptide display in class II MHC. We shortened the T-B coculture period to limit the period during which B cells could become activated. Finally, we used transgenic B cells expressing a functional κ L chain containing an antigenic T cell epitope to maximize sensitivity. Our results indicate that only activated or anergic B cells present significant levels of BCR-derived peptides to a T cell.
cell hybridoma. High density resting B cells show no demonstrable evidence of such presentation, despite a high level of BCR molecules.

Materials and Methods

Mice and T cell hybridomas

T cell hybridomas specific for Ig V region epitopes were generated and described previously (51, 52). Homozygous B6 α-transgenic mice were crossed once with A/J mice, and F1 offspring were used for all experiments involving the Vx epitope except that illustrated in Fig. 5, which made use of α-transgenic mice resulting from five backcrosses to the A/J strain. Mice carrying p-azophenylarsonate-specific canonical H chain and L chain transgenes were generated and characterized in an earlier study (53). Ads/AI (H + α) transgenic mice were derived from a cross of H chain-transgenic mice (crossed five times with A/J) with homozygous Vx-transgenic mice on a B6 background. Nontransgenic APC were derived from A/J mice.

Cell purification

For all assays, splenocytes from 4- to 8-wk-old mice were harvested by gently perfusing spleens through a 70-μm pore size cell strainer (BD Falcon, Franklin Lakes, NJ) and immediately washing in T cell medium (RPMI 1640 (Sigma-Aldrich), St. Louis, MO), supplemented with 2-ΜE (50 μM), gentamicin sulfate (50 μg/mL; Gemini Bio-Products, Woodland, CA), penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL) (both from Sigma-Aldrich), 1-glutamine (2 mm), HEPEs (7.5 mM), sodium bicarbonate, 1% concentrations of MEM essential amino acids, nonessential amino acids, and sodium pyruvate (Life Technologies, Gaithersburg, MD), and 10% FCS (Gemini Bio-Products). RBC were removed by lysis with ammonium chloride, and splenocytes were washed in T cell medium and used without further manipulation unless otherwise indicated.

For some assays, cells were separated on the basis of buoyant density. For this procedure, splenocytes were washed again and resuspended in Percoll (ρ = 1.079) (Sigma-Aldrich). The Percoll layer (2.5 ml/spleen) was overlaid with balanced salt solution (2 ml/spleen) in a 15-ml conical tube. Cells were centrifuged in Percoll for 30 min at 4°C at 1500 rpm (4700 × g) in a Sorvall T6000 tabletop centrifuge (Kendro Laboratory Products, New- ton, CT). High density cells were collected as a pellet at the bottom of the tube. Cells above the pellet in Percoll and medium were defined as low density. High density and low density cells were washed three times in T cell medium before use. Generally, the high density fraction contained between 2 and 5% large cells as revealed by flow cytometry.

For one experiment (Fig. 3D), A/J nontransgenic B cells were isolated by negative selection following the StemSep magnetic separation protocol for the enrichment of murine B cells (StemCell Technologies, Vancouver, Canada). Briefly, freshly isolated splenocytes were suspended in 1 ml of PBS + 2.5% FCS and blocked with 5% normal rat serum for 15 min at 4°C. The cells were then incubated for 15 min at 4°C with 10 μl of the murine B cell enrichment mixture. This mixture includes biotinylated Abs against CD4, CD8, CD11b, TRAIL119 (erythrocytes), and Gr-1 (myeloid cells) (11.5.2) (all from BD Pharmingen). Biotinylated Abs were visualized with streptavidin-PE in most cases, but occasionally with streptavidin-europium conjugate. Europium fluorophore (at 0.175 pmol/ml) was used as an internal standard.

Flow cytometry

Cells were incubated with various Abs in staining buffer (PBS + 2% FCS + 0.01% sodium azide) for 20 min. Abs were: anti-B220-CyChrome (BD Pharmingen) or anti-B220-PE (Southern Biotechnology Associates, Bir- mingham, AL) (clone: RA3-6B2 in both cases), anti-mouse CD86-FITC (GL-1), anti-mouse CD69-PE (H1.2F3), and anti-mouse IAα-biotin (11.5.2) (all from BD Pharmingen). Biotinylated Abs were visualized with streptavidin-europium in most cases, but occasionally with streptavidin-PE (Biolegend, San Diego, CA), which was used as an internal standard. Cells were incubated in a 1:5 dilution with a blocking buffer (2% BSA, 1% gelatin in PBS) for 20 min at 4°C, and incubated for 30 min with a primary Ab, which was removed by washing three times in staining buffer before addition of the next staining reagent or analysis by Coulter Epics XL or BD FACSCalibur flow cytometers.

Results

Self-presentation of an endogenously VH epitope by B cells in normal mice

Initially, we examined class II MHC-restricted presentation of a natural epitope presented in complementarity-determining region (CDR)2 of H chains encoded by the Vγδκδε gene. The Vγδκδε epitope gene encodes the H chain V domain of a predominant canonical Ab species elicited in the A/J immune response to p-azophenylarsonate. T cell hybridomas specific for this epitope were derived and characterized previously (52). They react by producing IL-2 in an IE-3-restricted manner to APC that are fed either whole canonical Abs or synthetic peptides spanning CDR2 residues 46–61. The hybridomas also produce IL-2 in response to whole untreated splenocytes derived from mice that carry I-Ek and VH Id CR genes (51).

To identify the stimulatory APC among normal A/J splenocytes, we subjected them to a depletion panning procedure using anti-B220 to remove B cells (Fig. 1A). B cell-depleted cultures failed to stimulate a T cell hybridoma (C58-H7) that was specific for the CDR2 epitope (Fig. 1A). Similar results were obtained on deple- tion panning with anti-Id (data not shown). Panning with control Abs had no effect. Loss of activity was not due to cellular damage inflicted by the procedure because depleted splenocytes stimulated an IL-2 response by C58-H7 when they were fed with the whole canonical mAb 36-65. These results suggested that B cells were the primary natural APC for this Vγδκδε epitope.
To determine whether B cells synthesizing the canonical V_H peptide were the same cells that stimulated our T cell hybridoma, we performed a depletion panning experiment with mAb AD8. This Ab recognizes a determinant in the canonical V_H domain, irrespective of D_H, J_H, or L chain sequences. Splenocytes depleted with AD8 lost most of their capacity to stimulate C58-H7 (Fig. 1B). Moreover, the recovered AD8-adherent population was ~30 times more active in stimulating C58-H7 than was a population of untreated splenocytes (data not shown). This degree of enrichment is consistent with the level of nonspecific adherence of cells (2–5%) that occurs in the panning procedure. Although we could not formally exclude the possibility that professional APC were presenting Ig derived from the culture supernatant or ingested B cells, the results strongly suggested that B cells were self-presenting the VHCDR2 epitope. This is supported by experiments described later in the \( \kappa \)-transgenic system (Fig. 5B).

It was tempting to conclude from these results that resting B cells self-present peptides from their V regions in the context of class II MHC, particularly in light of literature claims that resting B cells are proficient APC. However, we observed quantitative inconsistencies from mouse to mouse in splenic APC activity for the VHCDR2 epitope (data not shown). Moreover, peritoneal cells, which are naturally enriched for B1 cells, were more proficient at stimulating the C58-H7 hybridoma than were splenocytes (Fig. 1C). Because B1 cells are often activated, we considered the possibility that activated B cells, presenting the VHCDR2 epitope, might be responsible for the observed stimulation of our T cell hybridomas.

To test this idea, we fractionated A/J splenocytes into low density (active) and high density (\( \rho > 1.079 \) resting) populations using Percoll. To preclude possible activation of cells, we neither eliminated T cells via complement-mediated lysis nor attempted any additional purification steps. Fractionated cells were analyzed by flow cytometry for B220 expression. Fig. 2 shows that while low density A/J splenocytes effectively stimulated our hybridoma C58-H7, high density splenocytes failed to do so above background levels.

**A transgenic mouse in which B cells express a defined Vκ epitope**

Our data provide the first direct evidence that normal, nontransgenic, B cells self-present V region-derived peptides in class II MHC. They also suggest that high density resting B cells are poor at, or unable to, present BCR-derived peptides. However, only ~1 in 300 B cells express the V\( _\kappa \)D\( _\kappa \)K\( ^{\kappa} \) epitope (56), so it was not possible to quantitatively determine how poor resting B cells were at presenting BCR-derived peptides. For this reason, we developed a more sensitive system involving a mouse containing large numbers of B cells expressing a \( \kappa \) transgene. The transgene contains a pair of somatic mutations at codons 7 and 8 in framework region (FR) 1 that produce an I-A\( ^{\kappa} \)–restricted epitope in the \( \kappa \) polypeptide (52). The B cells of this mouse are functionally normal, phenotypically naive, and highly diverse due to normal endogenous H chain gene rearrangements (53). A reactive T cell hybridoma (T17-38) with no costimulation requirements for IL-2 production was available to assess the levels of peptide displayed by I-A\( ^{\kappa} \). With such an experimental system, it was possible to more clearly define APC actively presenting BCR-derived epitopes.

![FIGURE 1. B cells self-present a V_H region peptide. A, Splenocytes from A/J mice were depletion panned with anti-B220 or BSA (mock panned) as a control and cultured for 20 h with T cell hybridomas reactive with a CD2R peptide encoded by an endogenous VH gene (V_H{\kappa}K\( ^{\kappa} \)). mAb 36-65 was added as a positive control to demonstrate APC competence. “Negative” indicates T cells incubated without APC. IL-2 responses by the T cell hybridomas was measured using HT-2 cells in an MTT assay. Three different T cell hybridomas were used for this analysis: C58-H7; D, C58-H4; and E, C58-H3. B, A/J splenocytes were depletion panned with AD8, normal rat Ig (NratIg), or BSA (mock panned) and cultured as above. C, APC from the indicated tissues were compared for their ability to stimulate the VHCDR2-specific T cell hybridomas. In all experiments, \( 3 \times 10^3 \) APC were incubated with \( 10^5 \) T cell hybridomas. IL-2 production was measured in an MTT assay with HT-2 cells.](http://www.jimmunol.org/)

![FIGURE 2. Poor presentation of the VHCDR2 epitope by high density splenocytes. High density (\( \rho > 1.079 \)) and low density (\( \rho < 1.079 \)) A/J splenocytes were isolated and incubated at varying numbers with \( 10^5 \) C58-H7 cells for 20 h before quantifying IL-2 in culture supernatants using a time-resolved fluorimunometric assay (see Materials and Methods). Background IL-2 measurements for this experiment are ~200 pg/ml IL-2 as revealed by wells containing APC only.](http://www.jimmunol.org/)
FIGURE 3. κ-Transgenic splenocytes, unless stimulated in some way, poorly stimulate a T cell hybridoma reactive with a Vxκ FR1 peptide. A, κ-transgenic splenocytes were isolated, activated or not with goat anti-mouse κ (GaMK), and cultured at the indicated numbers with 10^6 T17-38 cells for 12 h. B, κ-transgenic splenocytes were isolated, incubated overnight with or without LPS, and then cultured at the indicated numbers with T17-38 cells for 12 h as in A. C, Vxκ-transgenic splenocytes were fractionated according to density in Percoll. High density cells, goat anti-mouse κ cross-linked high density cells, and low density cells were incubated with T17-38 for 12 h. Shown are stimulation curves with respect to the number of B cells per well. D, High and low density B cells are similar in their capacity to present the exogenous κ peptide to T17-38. B cells from A/J spleen unfractionated splenocytes varied from experiment to experiment. Furthermore, we noticed that the level of stimulation induced by unfractionated splenocytes varied from experiment to experiment (not shown). We tested high density (resting) and low density (activated) cell fractions isolated via a single layer of Percoll (Fig. 3, A and B). However, the response seemed weak considering the large number of potential APC, the tight allelic exclusion (53), and the fact that the transgenic B cells express a large number (~4 x 10^5) of receptors. Furthermore, we noticed that the level of stimulation induced by unfractionated splenocytes varied from experiment to experiment (not shown). We tested high density (resting) and low density (activated) cell fractions isolated via a single layer of Percoll (ρ = 1.079). Cells in the high density fraction were consistently small, were of uniform size, were IgD high and IgM low, and included cells with a broad range of sizes and with an increased frequency of class II MHC-positive, B220-negative cells relative to unfractionated splenocytes (Fig. 3C). The Percoll separation did not compromise presentation by high density B cells because after their activation with a goat anti-κ Ab, they proficiently induced IL-2 production by T17-38 (Fig. 3C). To demonstrate that T17-38 could efficiently interact with high density B cells, we titrated the VxκFR1 peptide on a fixed number (1.5 x 10^5) of low and high density A/J B cells. As shown in Fig. 3D, the exogenous peptide was efficiently presented down to a concentration of 3–6 nM by both populations.

To define potential APC in the cultures, high density and low density cell fractions were panned with anti-B220 Abs to remove B cells. Depletion panning left high density fractions with 1.6% B220-positive cells and low density fractions with less than 4.8% B220-positive cells. The panned high density cells were unable to stimulate T17-38, even when a synthetic peptide corresponding to the κ FR1 epitope was added to the culture (Fig. 4A). This confirmed the results of our FACS analysis showing few MHC-positive B220-negative cells in the high density fraction. Conversely, anti-B220 panned, low density cells efficiently stimulated T17-38 when the FR1 peptide was added to the culture, apparently due to the presence of non-B APC in this fraction.

Because other APC were present in the low density fraction, it was possible that they ingested secreted Ig or apoptotic B cells and were responsible for the observed stimulation of T17-38. To test this idea, we performed a mixed APC experiment in which T cells depleted high density or low density cells from κ-transgenic B6 mice were incubated at varying ratios with nontransgenic A/J × B6 low density splenocytes. The transgenic B cells lack the appropriate I-A\(^k\) molecule, whereas the nontransgenic APC express I-A\(^k\) but not the κ FR1 epitope. Therefore, any stimulation of T17-38 in these cultures would have to be due to the uptake of the κ epitope by nontransgenic APC in the low density fraction. We observed no stimulation of T17-38 at any concentration of cells unless exogenous Vκ FR1 peptide was added to the culture (Fig. 4B). This indicated that the B cells were self-presenting the Vκ FR1 epitope and thus were directly responsible for observed stimulation of T17-38, even in cultures with diverse populations of low density APC.

Negligible presentation of the Vκ epitope by high density B cells

In Fig. 5A, it can be seen that addition of low density cells to the high density APC “reconstituted” the ability of the culture to stimulate T17-38. This mixing result led us to analyze the data in a different way. We reasoned that should active B cells be responsible for the observed stimulation, then the stimulatory capacity of a culture should depend only on the total number of these cells. To test this idea, we mixed high density cells with low density cells at varying ratios. In Fig. 5B, it can be seen that by increasing the percentage of low density cells, the stimulatory capacity of the culture as a whole was increased. High density B cells could not account for this change in stimulation, because the response curves did not align when normalized to high density B cell numbers. However, Fig. 5C shows that as the ratio of low to high density cells changed, the number of low density B cells per culture could...
account for the stimulation. Thus, we can conclude that the stimulatory B cells reside within the low density population. B cells become activated during culture

To better understand activation dynamics, we analyzed high density and low density populations of B cells by flow cytometry after culture. In all cases, cells were cultured for 12 h at the highest concentrations used in our previous assays (10⁶ APC, 10⁵ T17-38). B220⁺ cells were analyzed for expression of the early activation markers CD69, CD86 (B7-2), and I-Aκ. Fig. 6A shows that starting populations of both high density and low density B cells expressed similar levels of these molecules and that after 12 h in culture their levels were increased on each population. However, low density B cells expressed significantly higher levels of each marker at 12 h than did the high density population.

To determine whether B cell activation in culture was due to cognate interactions with T17-38, we examined activation markers on splenocytes cultured without T17-38. In this situation, there was significant up-regulation of CD86 and class II MHC by low density (not shown) and high density B cells, although not to the same level as that seen when T cells were present (Fig. 6B). In contrast, little or no up-regulation of CD69 was detected on either low or high density B cells in the absence of T17-38. To verify the influence of T cells on B cell activation, we added the VγFR1 peptide to the high density culture in the presence of T17-38. In this case, high density resting B cells up-regulated activation markers to a higher level than when peptide was excluded (Fig. 6C). It appears that culturing splenocytes alone induces B cell activation, but cognate interactions with T cells result in a greater degree of activation as assessed with activation markers.
Prolonged culture leads to stimulation of T cells by resting B cells

These results suggested that high density B cells might stimulate a significant IL-2 response by T17-38 if the culture period was extended. We tested this by culturing the high density fraction of transgenic splenocytes with T17-38 for 24 h. Fig. 7 shows that although high density splenocytes elicited no IL-2 from T17-38 during 12 h of culture, significant IL-2 was produced after 24 h of culture. Because other studies demonstrated that LPS could activate B cells to self-present BCR peptides (Fig. 3), we tested the idea that endotoxin in the cultures might be responsible for the elevated IL-2 response in the 24-h cultures. However, the addition of polymyxin B to these cultures did not reduce the levels of IL-2 (data not shown). Thus, LPS was probably not responsible for the elevated IL-2 production seen in the longer cultures. At most, we might have expected a 2-fold increase by doubling the culture period unless a positive feedback loop was in effect or the B cells improved their proficiency at presentation during culture. Collectively, these results suggest that as the culture period with T cells is lengthened, quantitative analyses of APC activity by B cells become less accurate.

Phenotypically anergic B cells present endogenous Vκ epitopes

We tested the capacity of anergic B cells to present the VκFR1 epitope by making use of a recently generated transgenic model of tolerance. B cells in this mouse (Ars/A1) express the canonical κ transgene encoding the VκFR1 epitope. They also express a H chain transgene that together with the κ transgene encodes a BCR with specificity for an undefined autoantigen that induces an anergic state by a receptor desensitization mechanism (53). The transgenic cells express activation markers and fail to produce a significant Ca2⁺ flux upon receptor cross-linking. Splenic B cells from this animal were uniformly low density (not shown) and appeared to be uniform in phenotype (53). As shown in Fig. 8, the stimulation induced by Ars/A1 splenocytes was indistinguishable from that induced by Vκ transgenic low density splenocytes but several fold greater than the stimulation induced by whole unfractionated splenocytes, when normalized for B cell numbers. Furthermore, high density Vκ transgenic splenocytes that were activated with goat anti-κ stimulated T17-38 equivalently to the low density and Ars/A1 splenocytes (not shown). However, as seen before, unstimulated high density splenocytes induced no detectable IL-2 response from T17-38.

Discussion

In this study, we evaluated the potential of high density resting B cells to present BCR-derived epitopes in class II MHC. These experiments may provide insights into the ability of resting B cells to influence the T cell repertoire. By using κ-transgenic B cells synthesizing a V region epitope at high levels, a T cell hybridoma with no costimulatory requirements, a short culture period to minimize nonspecific activation of B cells, and a sensitive fluoroimmunometric assay, we have circumvented many of the lingering questions surrounding the ability of resting B cells to act as efficient presenters of BCR-associated epitopes.

FIGURE 6. Activation of B cells in culture with and without a reactive T cell hybridoma. High and low density splenocytes cells were isolated as described and incubated (10⁶ well) with or without T17-38 (10⁵ well). A, Up-regulation of early activation markers in the presence of T17-38. CD69 and CD86 and I-Ak levels were analyzed on high and low density B cells before and after 12 h of culture. Shown are separate curves for high and low density cells gated for B220 expression. B, High density cells were incubated without T17-38 cells and analyzed as above. C, High density cells were incubated with T17-38 cells and cognate peptide (725 nM) and analyzed as above.

FIGURE 7. Lengthening the culture period markedly enhances stimulation by high density B cells. Isolated high and low density splenocytes were incubated at varying numbers with 10⁵ T17-38 cells for 12 or 24 h. Responses were normalized to the number of B cells per well.

FIGURE 8. Anergic B cells present the κ FR1 peptide. Ars/A1 splenocytes (Vκ/VHdκ transgenic, anergic) were compared with unfractionated, high density, and low density Vκ-transgenic splenic B cells for their capacity to stimulate an IL-2 response from T17-38 T cells (10⁵) in a 12 h assay.
A κ-transgenic mouse provided us with a virtually normal high density, ex vivo B cell population in which the presentation of an endogenously synthesized, rather than exogenously supplied, BCR-associated epitope could be quantitatively assessed. This system precluded BCR aggregation that might otherwise have occurred if an exogenous epitope were targeted to the BCR. Our results revealed that high density, resting B cells were unable to present a class II MHC-restricted T cell epitope from the FR1 of the Vκ region, despite their competence with respect to the exogenously supplied peptide correlate. In contrast, activated B cells were efficient self-presenters of the endogenously generated FR1 κ peptide. The sensitivity of our experimental system imparts power to this conclusion. Even $7 \times 10^5$ κ-transgenic cells in the high density (resting) fraction were unable to elicit a significant response by a reactive T cell hybridoma, T17-38, that responded to high density B cells treated with concentrations of antigenic κ peptide as little as 6 nM (Fig. 3, C and D). Assuming that our peptide binds to I-A$^k$ within the normal range of affinities ($10^{-5}$–$10^{-6}$M), we estimate that the T17-38 hybridoma requires only 60–600 MHC-peptide complexes to produce detectable IL-2. This is in line with the published range of MHC-peptide complexes necessary to elicit a productive T cell response (58–60).

Due to the stringent nature of our selection procedure for high density of lymphocytes, the low density population was less well defined. It consisted of cells on top of the ρ = 1.079 Percoll layer and also cells within this layer. Undoubtedly, this population is heterogeneous in density. Consequently, it is presently unclear whether all or only some of the low density B cells can stimulate IL-2 production from T17-38. However, within the first 12 h, all of the stimulatory activity resides within the low density fraction of cells (Fig. 5). Thus it is likely that certain phenotypic changes in high density B cells must occur before BCR-derived peptides can be presented.

Prevailing views in the literature appear to run counter to our primary conclusion. We know of only one other study supporting the view that resting B cells do not present BCR-associated epitopes. Bartnies and Hannestad (21) found no evidence of class II MHC-restricted presentation of an IgG2a peptide by splenocytes to a reactive T cell hybridoma. Their results support the idea that resting, memory B cells may also be deficient in presentation of BCR-derived peptides. Divergent conclusions from other prior studies can be largely reconciled by differences in experimental procedures. T17-38 hybridomas produced strong IL-2 responses after only 10–12 h of stimulation, enabling us to study presentation by B cells before changes in their physiology significantly impacted T cell stimulation. FACS analysis revealed that even within 12 h, high density B cells began to up-regulate activation markers and that some of the activation was B cell autonomous, as revealed in cultures lacking T17-38 cells. In analogous preceding studies, cells were cultured for a minimum of 24 h and generally for 3–4 days.

It is clear that some degree of B cell activation was also due to the presence of, and possible cognate interactions with, T17-38. By 24 h of coculture, disproportionately more IL-2 was made than at 12 h. Apparently, this was not a simple situation in which a few stimulated hybridomas produced more IL-2 with time. If this were true, the amount of IL-2 should increase proportionally to the time spent in culture. This clearly was not the case. Furthermore, flow cytometric analysis of high density cultures with T17-38 showed that the addition of cognate peptide caused an increase in the level of B cell activation over 12 h. These observations raise the possibility of an amplification loop created by initial cognate T-B interactions.

At present, we cannot safely generalize this interpretation to all Ags because our data are derived primarily from studies of one κ epitope. Although our experiments with normal B cells produced a compatible result with respect to a second (VH)′ epitope, these assays lacked high sensitivity because so few B cells expressed the corresponding VH gene. Moreover, there is good evidence that different Ags are processed differentially in subcellular compartments associated with MHC presentation (2, 61, 62). Thus, although there is still no clear precedent, it is conceivable that other BCR-associated epitopes may be presented by truly resting B cells. However, our argument can be extended when viewed quantitatively with respect to monovalent Ags. Uptake of self-Ags by resting B cells has been offered as a potential avenue of T cell tolerance, but unstimulated B cells almost invariably express low affinity Abs that must be improved by somatic mutagenesis and selection. Thus, it is unlikely that a monovalent Ag will ever come close to saturating the receptor on a resting B cell and approach the epitope density achieved in this study with a κ transgene. Despite “saturating” conditions, we found no convincing evidence of class II MHC-restricted peptide display by resting B cells.

The ability of resting B cells to induce T cell tolerance has been postulated for some time. Resting B cells loaded with exogenous peptide induced abortive activation in T lymphocytes in vitro (35, 63). Adoptive transfer of resting B cells from male mice induced unresponsiveness in naive but not primed CTLs from female mice (64). Adoptive transfer of resting B cells expressing a membrane-only form of a human μ transgene induced T cell tolerance to human μ, and treatment of mice with rabbit Fab anti-mouse IgG8 induced T cell tolerance to rabbit Fab (25–27). Studies with B cells expressing an engineered immunogenic epitope within the VH region of an IgG transgene also induced tolerance upon adoptive transfer (65). Finally, studies with transgenic TCR mice demonstrated that circulating IgG does not efficiently induce tolerance in T cells to an Ig V region epitope and thus suggested that B cells might be responsible for T cell tolerance to Ab V region determinants (66). It is clear from these studies that activating B cells via the BCR leads to T cell activation, not tolerance. However, the propensity of B cells to become at least partially activated by a simple isolation and culture procedure suggests that some degree of activation might have occurred after isolation in these preceding studies. Alternatively, it is conceivable that B cells attain distinct states of activation in vivo, some of which are immunogenic and others of which are tolerogenic. Interestingly, in the system described by Zambidis et al. (65), tolerance was induced by LPS-activated B cells, suggesting that IgG-expressing B cells may become tolerogenic through various modes of activation. This idea is also supported by recent studies suggesting that some degree of costimulation may be required during induction of T cell tolerance (43–45, 67–69). In all of the preceding studies, it was assumed that the activation state of the B cell remained constant throughout the experimental procedure and that no other cell type played a role in vivo.

Literature concluding that resting B cells can induce tolerance is predicated on the idea that the BCR traffics into MHC-containing compartments even in the absence of receptor cross-linking (2, 14, 15, 70). These and other studies have suggested that cross-linking simply accelerates the rate of receptor turnover without altering the trafficking pathway (2, 4). Still other data have suggested that BCR signaling is essential to induce efficient Ag presentation, irrespective of Ag internalization (1, 3, 5, 71, 72). Our hypothesis is consistent with these latter studies in that presentation is not induced without signaling. However, to our knowledge, neither of these phenomena has ever been studied in normal ex vivo B lymphocytes. The data presented here indirectly suggest that the BCR traffics differently in resting and activated B cells or fails to be processed for presentation in resting B cells.
We do not know how many types of signals might alter intracellular pathways of BCR processing and presentation. Clearly, BCR cross-linking induces robust class II MHC presentation of BCR-associated Ags. However, in our hands, the ability to present BCR-derived peptides is not dependent on BCR signaling. Both LPS and cell culture induced presentation of BCR-derived peptides. Therefore, it seems plausible that other signals mediated by cytokine, complement, and Toll-like receptors, for example, also alter subcellular trafficking and processing of BCR-associated Ags. Different activation states induced by these signals may also have distinct functional consequences for MHC-restricted T cells. Analogous hypotheses have recently been reported for dendritic APC (73–75).

It is clear that anergic B cells are able to present BCR-derived epitopes in class II MHC. In the transgenic B cells we examined, anergy is apparently induced upon BCR aggregation by a multivalent self-Ag because we can preclude the anergic state by blocking the transgenic BCR with a monovalent ligand (hapten) (53). This result underscores the importance of B cell activation in processing and presentation of BCR-associated epitopes and is consistent with work suggesting that anergic hen egg lysozyme-specific B cells can process and present Ag targeted to the B cell receptor (76–78). These results are intriguing in light of evidence suggesting that anergic B cells have a limited ability to signal through their BCR (53, 79–83). Nevertheless, the MHC display of a BCR-derived peptide by anergic B cells suggests that they may play an active role in inducing T cell tolerance to self-Ags. This is consistent with expression of costimulators on anergic B cells and recent in vivo and in vitro studies suggesting that T cell activation may be a necessary step leading to T cell tolerance (1, 43, 45, 83). Nevertheless, the MHC display of a BCR-derived peptide by anergic B cells suggests that they may play an active role in inducing T cell tolerance to self-Ags. This is consistent with expression of costimulators on anergic B cells and recent in vivo and in vitro studies suggesting that T cell activation may be a necessary step leading to T cell tolerance (1, 43, 45, 83).

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References


