Cross-linking of Membrane Immunoglobulin D, in the Absence of T Cell Help, Kills Mature B Cells In Vivo

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Summary

In vivo experiments were performed to determine whether the cross-linking of membrane immunoglobulin (mIg) D on mature B cells, in the absence of T cell help, leads to B cell death. Mice were injected with either a monoclonal antibody (mAb) that cross-links mIgD effectively or a mAb that binds to mIgD avidly but cross-links it to a limited extent, and effects on B cell number and B cell Ia, mIgM, and mIgD expression were observed. In most experiments, mice were pretreated with anti-interleukin 7 mAb to prevent the generation of new bone marrow B cells, and with anti-CD4 mAb to prevent the generation of T cell help. In some experiments, mice also received anti-FcγRII mAb to prevent cross-linking of mIgD with FcγRII, and cobra venom factor to prevent possible mIg–complement receptor interactions and complement-mediated killing of B cells. The results of these studies demonstrate that (*a*) even limited cross-linking of mIgD on mature B cells can lead to B cell death; (*b*) increased cross-linking of mIgD leads to increased B cell death; (*c*) the loss of B cells is first detected 2 d after anti-IgD mAb injection and increases during the subsequent 3 d; (*d*) sustained modulation of mIgD may be necessary to cause B cell death; (*e*) $mIgM^{dull}$ but not $mIgM^{bright}$ B cells are lost in mice injected with anti-IgD mAbs; and (*f*) T cell help prevents or minimizes B cell death.

The two-signal theory of B lymphocyte activation predicted that an interaction between antigen and B cell membrane immunoglobulin (mIg)[1] would trigger a B cell– activating event that would lead, in the presence of additional signals, to clonal expansion and antibody production, but, in the absence of additional signals, to death (1). Since this theory was proposed, the cross-linking of B cell mIg has been shown to costimulate B cell proliferation and differentiation in the presence of such stimuli as T cell–produced cytokines (2, 3) and T cell membrane costimulatory molecules (4, 5), while the cross-linking of mIg on newly generated B cells has been shown to lead to B cell unresponsiveness and death (6–8). The ultimate effects of mIg cross-linking on mature B cells, in the absence of additional stimuli, have, however, been less well defined. Cross-linking of mIg, in the absence of T cell help, stimulates enhanced B cell expression of receptors involved in proliferation and cellular interactions (9–11) and, under some conditions, can stimulate DNA synthesis, although clonal expansion and antibody secretion are not induced (12–16). It is not known, however, whether these activated B cells eventually return to a resting state, survive but become anergic, or die. This issue has been difficult to resolve in vitro, where unstimulated B cells have a short life span and start to undergo apoptosis within 24 h (17). Study of the effects of mIg cross-linking on B cell life span has also been difficult, in part because of a long-standing controversy about whether resting B cells live for a long or short time in vivo (18–22). Recent experiments that have either labeled dividing B cells and B cell precursors in vivo (21) or used antibodies to IL-7 to prevent the in vivo generation of new B cells (22) have provided compelling evidence that most mature B cells have a life span that is measurable in weeks, rather than days. This conclusion has made it reasonable to question whether the cross-linking of B cell mIg, in the absence of additional signals, shortens that life span. To investigate this, we have injected mice with anti-IgD antibodies, including two rat IgG2a anti-IgD mAbs that bind to IgD with similar avidity but differ considerably in their abilities to cross-link mIgD and activate B cells in a T cell–independent fashion (23). In most of these experiments, the generation of new B cells was blocked by pretreating mice with anti-IL-7 mAb (22), and the generation of T cell help was blocked by pre-

[1] *Abbreviations used in this paper:* GαMδ, affinity-purified goat anti-mouse IgD antibody; HEL, hen egg lysozyme; HNA, HBSS supplemented with 10% newborn bovine serum and 0.2% sodium azide; mIg, membrane immunoglobulin.

treating mice with anti-CD4 mAb (24). In addition, in some experiments, potential complement-mediated killing of B cells and potential inhibitory interactions between mIg and FcγRII were blocked by treating mice with cobra venom factor (25) and anti-FcγRII mAb (26), respectively. The results of these experiments demonstrate that anti-IgD antibody treatment under these conditions causes B cells to die over a 2- to 7-d period, defines differences in the susceptibility of different B cell populations to the lethal effects of anti-IgD mAbs, and shows that T cell help can prevent cell death.

Materials and Methods

Mice. Female BALB/c mice were purchased from the Small Animals Division of the National Cancer Institute (Frederick, MD) and were used at age 8–14 wk.

Immunological Reagents. The following antibodies were prepared as previously described: m25, a mouse IgG1 mAb that neutralizes both human and mouse IL-7 (22), a gift of Dr. Kenneth Grabstein (Immunex Research Corp., Seattle, WA); GK1.5, a rat IgG2b mAb that kills $CD4^+$ T cells and blocks helper T cell function (24); 11-26, a rat IgG2a mAb that binds mouse IgD avidly but cross-links it poorly (23); HBδ6, also known as LO-MD-6, a rat IgG2a mAb that effectively cross-links mouse IgD (23, 27); FF1-4D5, a mouse IgG2a of the *b* allotype that binds to an epitope of mouse IgD of the *a* allotype that is not blocked by HBδ6 (23); DS-1, a mouse IgG1 of the *b* allotype that binds to mouse IgM of the *a* allotype (28); MKD6, a mouse IgG2a alloantibody specific for $I-A^d$ (29); 6B2, a rat IgG2a specific for mouse B220 (CD45R), the B cell form of CD45 (30); 24G2, a rat IgG2b that binds to mouse FcγRII and blocks its ability to bind IgG (26); J1.2, a rat IgG2b mAb specific for the hapten NP (3-nitro-4-hydroxyphenylacetyl) (a gift of Dr. John Abrams, DNAX Research Institute, Palo Alto, CA), and GαMδ, an affinity purified goat antibody to mouse IgD (31). Some of these antibodies were labeled with FITC (32) or biotin-*N*-hydroxysuccinimide (33). Antibodies were also labeled with the fluorochrome Cy5 (Research Organics, Inc., Cleveland, OH) according to the directions provided by the manufacturer. Lyophilized cobra venom factor was purchased from Diamedix Corp. (Miami, FL) and reconstituted according to the manufacturer's instructions. Reconstituted cobra venom factor was aliquoted and stored at −70°C until used.

Immunofluorescence Staining. Single-cell suspensions of spleen, peripheral lymph node, or bone marrow were depleted of erythrocytes, suspended at 10–20 × 10^6 cells/ml in HBSS with 10% newborn bovine serum and 0.2% sodium azide (HNA). 100 μl of cells was stained for 30 min on ice with 1 μg each of a FITC-labeled antibody, a biotin-labeled antibody, and, in some experiments, a Cy5-labeled antibody. Cells were washed three times with HNA and then exposed to streptavidin-R-phycoerythrin (GIBCO BRL, Gaithersburg, MD) for 30 min on ice. All staining was done in the presence of 10 μg/ml of unlabeled anti-FcγRII mAb (24G2) to block the binding of IgG staining reagents to FcγRII. After washing twice more with HNA, cells were washed once with HBSS/0.2% sodium azide, then fixed in PBS/2% paraformaldehyde. Cells were analyzed with a FACScan® (Becton Dickinson & Co., Mountain View, CA) and FACScan® software. Light scatter gates were set to exclude most cells that had died before fixation, as well as nonlymphoid cells, except that light scatter gates for analysis of bone marrow cells were set to include all living nucleated cells. Spleen cells that had been stained with a single fluorochrome-labeled antibody were used to determine the settings used to compensate for overlap between FITC and PE emission spectra. Data were analyzed to determine percentages of specifically stained cells and the mean and/or median fluorescence intensities of specifically stained cells. Spleen cells from mice injected with HBδ6 1 d before killing were used to define the fluorescence channel that separates IgM^{dull} from IgM^{bright} spleen cells, since this treatment increases the phenotypic difference between these two populations (see below). In one experiment, cells stained with FITC-, R-PE-, and Cy5-labeled reagents were analyzed by flow cytometry with an Epics V (Coulter Corp., Hialeah, FL), and R-PE fluorescence histograms of $Cy5^+FITC^{dull}$ and $Cy5^+FITC^{bright}$ cells were prepared.

Cell Counts. Cells were counted with a counter (Coulter Corp.) that was set to exclude dead cells. Total spleen cell number was multiplied by the percentages of IgM^{bright} or IgM^{dull} spleen cells to calculate numbers of IgM^{bright} or IgM^{dull} spleen cells.

Treatment Protocol. Unless otherwise noted, mice were treated with immunological reagents according to the following schedule: 3 mg of m25 anti-IL-7 mAb was injected intraperitoneally 3 d/wk, starting 2 wk before injection of anti-IgD antibody and continuing for the duration of the experiment; 1 mg of GK1.5 anti-CD4 mAb was injected intravenously once a week, starting 2 wk before injection of anti-IgD antibody and continuing for the duration of the experiment; 2 U of cobra venom factor was injected intravenously twice daily for 2 d and 1 d before the injection of anti-IgD antibody, then once every other day, starting the day after injection of anti-IgD antibody and continuing for the duration of the experiment; and 1 mg of 24G2 anti-FcγRII mAb was injected intravenously along with or shortly before the injection of anti-IgD antibody. In some experiments, mice were injected intravenously with 1 mg of IgD (TEPC-1017 or TEPC-1033 [31]) 5 and 6 d after injection of anti-IgD antibody to neutralize remaining anti-IgD antibody (34), and then killed 1 d after the second dose of IgD.

Results

Treatment with Anti-IL-7 mAb Blocks the Development of New B Lymphocytes. Our procedure for evaluating the in vivo effects of mIgD cross-linking on B cell survival depended, in many experiments, on blocking the production of new B cells that might replace B cells that die. For this reason, studies were initially performed to confirm the effectiveness of the technique used to prevent B cell production in these experiments. BALB/c mice were injected intraperitoneally with 3 mg of m25 anti-IL-7 mAb three times a week for 2 wk. Mice were then killed, their spleen and bone marrow cells stained for mIgM and B220, and dual parameter flow microfluorimetry was used to determine percentages of bone marrow and spleen cells that expressed B220 and/or mIgM. Anti-IL-7 mAb treatment decreased the percentage of bone marrow cells that had the pre-B cell phenotype ($B220^+IgM^-$) by ~85% and the number of immature B cells ($B220^{dull}IgM^+$) (22, 35) to a barely detectable level (Fig. 1). The percentage of bone marrow B lymphocytes that had a more mature phenotype ($B220^{bright}IgM^+$) was much less affected by anti-IL-7 mAb treatment (Fig. 1), and the number of splenic B cells was not significantly affected (data not shown). These observations confirmed the demonstration by Grabstein et al. (22) that depletion of IL-7 prevents B cell generation and that mature B cells have a relatively long in vivo life span, and validated the use of anti-IL-7 mAb–treated mice as a closed system in which B cell loss would not be compensated for by the generation of new, bone marrow–derived, B cells.

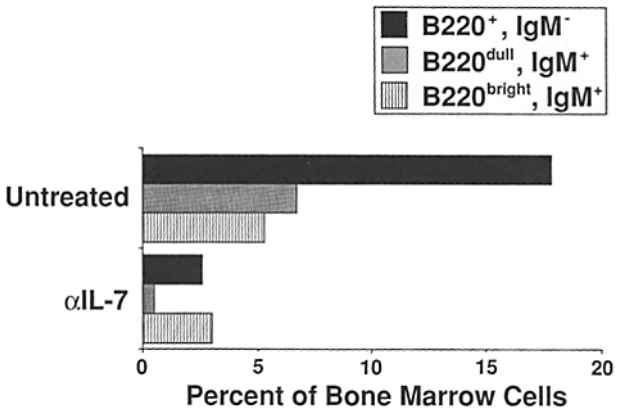

Figure 1. Treatment of mice with anti-IL-7 mAb prevents the generation of new bone marrow B cells. BALB/c mice (three/group) were left untreated or were injected intraperitoneally with 3 mg of neutralizing anti-IL-7 mAb (m25) three times a week for 2 wk. Mice were killed 2 wk after the initial injection, and bone marrow cell suspensions were prepared. Pools of bone marrow cells from the three mice in each group were stained with FITC-anti-B220 and biotin-anti-IgM mAbs, followed by streptavidin-R-PE. Stained cells were analyzed with a FACScan® with light scatter gates set to include all living nucleated cells, and percentages of pre-B cells ($B220^+IgM^-$), immature B cells ($B220^{dull}IgM^+$), and mature B cells ($B220^{bright}IgM^+$) were determined.

In Vivo Treatment with Anti-IgD mAbs Causes Increased Ia Expression by Both IgM^{dull} and IgM^{bright} Spleen Cells and Decreased mIgM Expression by IgM^{dull} Spleen Cells. To determine the initial effects on splenic B cell populations of injecting mice with anti-IgD mAbs, spleen cells were obtained from anti-IL-7 mAb-treated mice that had been injected 1 d before killing with either 11-26 (a rat IgG2a mAb that binds IgD avidly but ineffectively cross-links IgD) or HBδ6 (a rat IgG2a mAb that cross-links mIgD effectively) (23). Mice were also treated with anti-CD4 mAb (to prevent T cell help), and, in some experiments, with anti-FcγRII mab (to block interactions between antibody-bound mIgD and the B cell Fcγ receptor that might negatively signal B cells [36, 37]) and cobra venom factor (to prevent the possibility of complement-mediated killing of B cells and interactions between mIgD and B cell complement receptors) (38, 39). Spleen cells from treated and control mice were counted, then stained for mIgM and either B220 or I^d, and analyzed by flow microfluorimetry. Treatment with either anti-IgD mAb had no effect on IgM expression by IgM^{bright} B cells but considerably decreased mIgM expression by IgM^{dull} B cells (Fig. 2, *left*; note the considerable shift to the left of the modal population of IgM^{dull} cells from mice that received anti-IgD mAb). In three separate experiments, Ia expression on IgM^{dull} spleen cells was considerably upregulated by injection of HBδ6 but only slightly upregulated in most experiments by injection of 11-26 (Fig. 3). In contrast, both anti-IgD mAbs considerably and equally increased Ia expression by the IgM^{bright} splenic B cells. Total numbers of splenic $mIgM^{bright}$ or $mIgM^{dull}$ spleen cells were not consistently affected 1 d after injection of anti-IgD mAb (Fig. 3 and see Fig. 6), regardless of whether mice were pretreated with anti-IL-7 mAb and whether they received anti-FcγRII mAb and cobra venom factor.

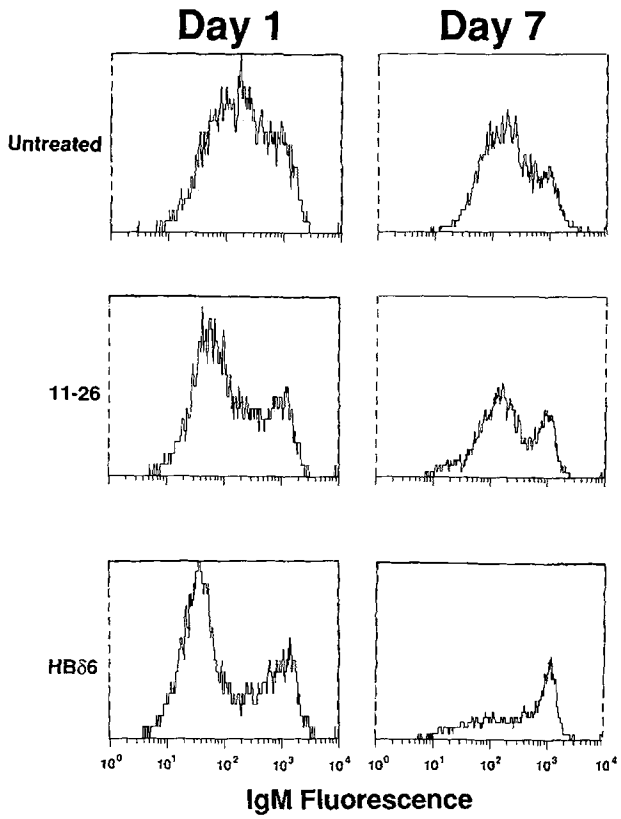

Figure 2. Effects of anti-IgD mAbs on splenic B cell number and IgM expression 1 and 7 d after injection. BALB/c mice (three/group) were treated with anti-CD4 mAb, anti-FcγRII mAb, and cobra venom factor (*left*) or with the same reagents plus anti-IL-7 mAb (*right*) according to the schedule described in Materials and Methods. Mice received no additional treatment (*top*, *"Untreated"*) or a single intravenous injection of 11-26 (*middle*) or HBδ6 (*bottom*). Mice were killed 1 d after anti-IgD mAb injection (*left*) or were injected intravenously, with 1 mg of an IgD mAb 5 and 6 d after anti-IgD mAb injection and then killed 7 d after anti-IgD mAb injection. Spleen cell suspensions were prepared and stained with FITC-anti-B220 plus biotin-anti-IgM mAbs, and then analyzed with a FACScan® for IgM-associated fluorescence on B220-expressing cells. Representative fluorescence histograms generated by the analysis of 10^4 spleen cells from individual mice are shown. Full scale on the ordinate is 16 cells/channel for panels on the left and 32 cells/channel for panels on the right.

Anti-IgD mAb Treatment Causes the Selective Loss of IgM^{dull} Spleen Cells over a 5- to 7-d Period. To determine if the in vivo interaction of splenic B cells with poor or effective cross-linkers of IgD for >1 d would cause a loss of B cells from the spleen, mice that were injected with anti-IL-7, anti-CD4, and anti-FcγRII mAbs, with or without cobra venom factor, received no further treatment or were injected with 11-26 or HBδ6. 5 and 6 d after anti-IgD antibody injection, mice were injected with IgD (TEPC-1017 or TEPC-1033) to neutralize remaining anti-IgD antibody. Mice were killed 1 d after the second dose of IgD, and numbers of IgM^{dull} and IgM^{bright} spleen cells were determined. The number of IgM^{dull} spleen cells decreased by the seventh day after anti-IgD mAb injection by a factor of three to five in HBδ6-injected

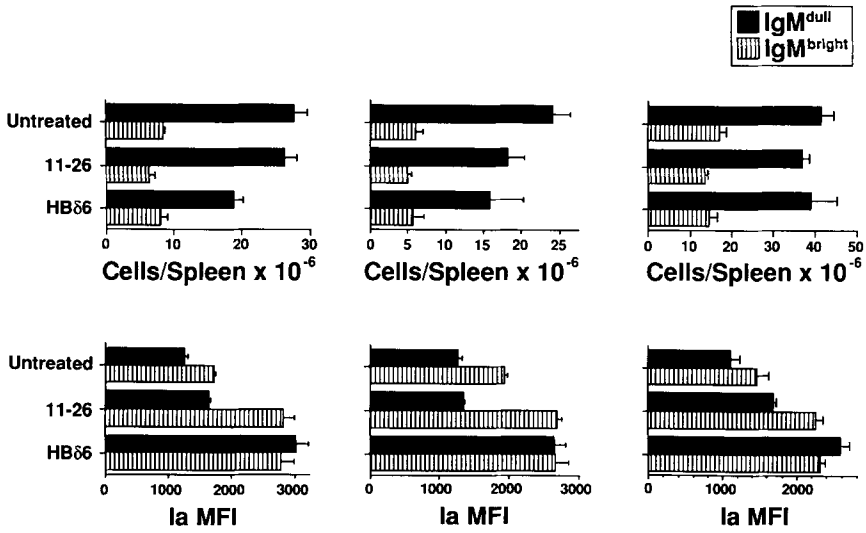

Figure 3. Effects of anti-IgD mAbs on IgM^{bright} and IgM^{dull} spleen cell number and Ia expression 1 d after injection. In three separate experiments, BALB/c mice (three/group) were pretreated with anti-CD4 mAb and anti-IL-7 mAb (*left* and *middle*) or with anti-CD4 mAb, anti-FcγRII mAb, and cobra venom factor (*right*), according to the schedule described in Materials and Methods. Mice received no further treatment or were injected intravenously with 100 μg of 11-26 or HBδ6, then killed 1 d later. Spleen cell suspensions prepared from individual mice were counted and stained with FITC-anti-B220 and biotin-anti-IgM mAbs, followed by streptavidin-R-PE, or with FITC-anti-I^d and biotin-anti-IgM mAbs followed by streptavidin-R-PE. Cells were analyzed with a FACScan® for percentage of $B220^+IgM^{bright}$ and IgM^{dull} cells, and for Ia median fluorescence intensity (*MFI*) of IgM^{bright} and IgM^{dull} Ia^+ cells. Means and standard errors are shown.

mice, compared with mice that did not receive anti-IgD mAb (Fig. 2, *right*, and Fig. 4). The number of IgM^{dull} spleen cells also consistently decreased in 11-26-injected mice, but the decrease was less than twofold. Similar results were obtained with mice that received anti-FcγRII mAb but not cobra venom factor, or neither anti-FcγRII mAb nor cobra venom factor (compare upper, middle, and lower panels in Fig. 4). In contrast to their effects on IgM^{dull} B cells, neither anti-IgD mAb caused a reproducible decrease in the splenic IgM^{bright} B cell population (Figs. 2 and 4).

To determine if the persistence of IgM^{bright} spleen cells in anti-IgD mAb-treated mice might reflect decreased mIgD expression by $mIgM^{bright}$ spleen cells compared with $mIgM^{dull}$ spleen cells, spleen cells from mice treated with anti-IL-7, anti-CD4, and anti-FcγRII mAbs, plus cobra venom factor, were stained with FITC-anti-IgM mAb, Cy5-anti-B220 mAb, and biotin-anti-IgD mAb (FF1-4D5), followed by streptavidin-R-PE. Stained cells were fixed and analyzed for IgD-associated fluorescence on $B220^+IgM^{dull}$ and $B220^+IgM^{bright}$ cells. While IgM^{dull} spleen cells were almost uniformly $mIgD^{bright}$, IgM^{bright} spleen cells contained a majority IgD^{bright} population and a minority IgD^{dull} population (Fig. 5). Inasmuch as the entire population of IgM^{bright} spleen cells is maintained in anti-IgD mAb-treated mice, this result indicates that resistance to the B cell-depleting effect of anti-IgD mAb treatment is not simply a result of decreased mIgD expression.

To determine the kinetics of B cell loss in response to injection of anti-IgD mAb, mice were treated with anti-IL-7, anti-CD4, and anti-FcγRII mAbs, as well as cobra venom factor, and killed before or 1–5 d after injection of HBδ6. Numbers of IgM^{dull} and IgM^{bright} spleen cells were determined by cell counting and flow microfluorimetric analysis after staining for mIgM and B220. No significant loss of IgM^{bright} spleen cells was detected during the course of the experiment, and no loss of IgM^{dull} spleen cells was detected during the first 24 h after HBδ6 injection (Fig. 6). The number of IgM^{dull} spleen cells, however, decreased significantly by 2 d after HBδ6 injection and continued to decrease, at a relatively constant rate, during the next 3 d.

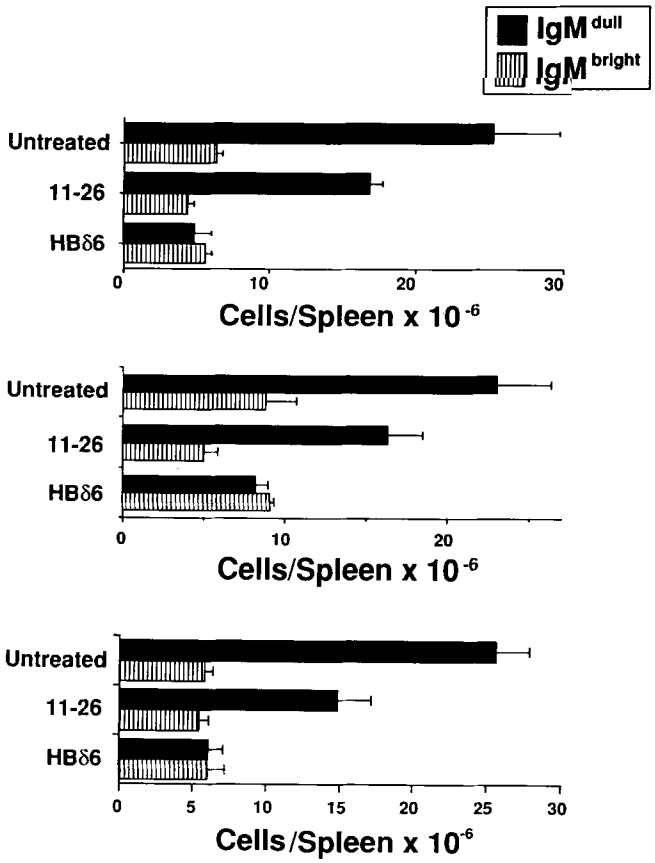

Figure 4. Selective depletion of IgM^{dull} B cells in mice treated with anti-IgD mAbs. In three separate experiments, BALB/c mice (three/group) were pretreated with anti-CD4 and anti-IL-7 mAbs (*top*) anti-CD4, anti-IL-7, and anti-FcγRII mAbs (*middle*), or the same mAbs plus cobra venom factor (*bottom*). Mice received no further treatment or were injected intravenously with 100 μg of 11-26 or HBδ6, and, 5 and 6 d later, 1 mg of IgD. Mice were killed 7 d after anti-IgD mAb injection. Spleen cells suspensions were prepared from individual mice, counted, and stained with FITC-anti-B220 and biotin-anti-IgM mAbs, followed by streptavidin-R-PE. Stained cells were analyzed with a FACScan® to determine percentages of $B220^+IgM^{bright}$ and IgM^{dull} cells. Means and standard errors are shown.

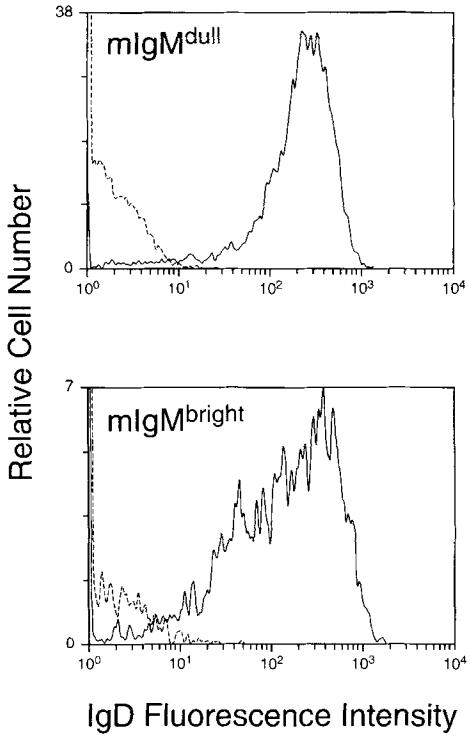

Figure 5. mIgD expression by IgM^{dull} and IgM^{bright} B cells. A BALB/c mouse was treated with anti-IL-7, anti-CD4, and anti-FcγRII mAbs plus cobra venom factor according to the schedule described in Materials and Methods. After 3 wk of treatment, spleen cells were stained with FITC-anti-IgM, biotin-anti-IgD, and Cy5-anti-B220 mAbs followed by streptavidin-R-PE (*solid line*) or with the same reagents except for biotin-anti-IgD (*dashed line*) and analyzed by flow microfluorimetry with a Coulter Epics V fluorescence-activated cell sorter. IgD (R-PE) fluorescence profiles of $B220^+mIgM^{dull}$ and $B220^+mIgM^{bright}$ cells are shown. Similar results were seen with spleen cells from two additional similarly treated mice.

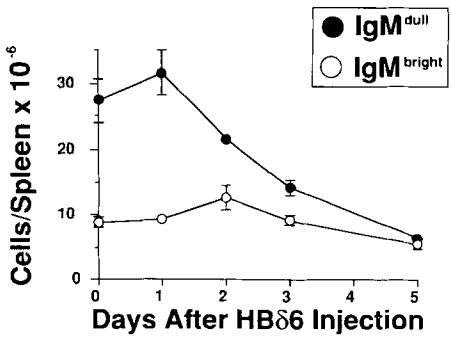

Figure 6. In vivo treatment with anti-IgD antibody causes a slow decline in the number of IgM^{dull} spleen cells. BALB/c mice (three/group) were pretreated with anti-CD4, anti-IL-7, and anti-FcγRII mAbs and cobra venom factor. Mice received no further treatment or were injected intravenously with 100 μg of HBδ6. All mice were killed at the same time, 1–5 d after HBδ6 injection. Spleen cell suspensions were prepared, counted, and stained with FITC-anti-B220 and biotin-anti-IgM mAbs followed by streptavidin-R-PE. Stained cells were analyzed by FACScan® for the percentages of $B220^+$ IgM^{bright} and IgM^{dull} cells. Means and standard errors are shown.

Anti-IgD mAb Treatment Selectively Causes the Loss of $mIgM^{dull}$ Peripheral Lymph Node Cells. The selective loss of IgM^{dull} cells from the spleen might represent selective destruction of these cells, selective migration of these cells to another organ, or loss of both IgM^{dull} and IgM^{bright} spleen cells that is accompanied by acquisition of increased mIgM by some IgM^{dull} cells. To distinguish among these possibilities, we examined the effects of anti-IgD mAb injection on IgM^{dull} and IgM^{bright} peripheral lymph node cells. The peripheral lymph node B cell population differs from that of the spleen in that very few IgM^{bright} cells are normally present (33). Thus, if treatment with anti-IgD mAb caused some initially IgM^{dull} spleen cells to become IgM^{bright}, then the percentage of IgM^{bright} lymph node cells would be expected to increase in anti-IgD mAb–treated mice. Instead, 5 d after mice that had been pretreated with anti-IL-7, anti-CD4, and anti-FcγRII mAbs, as well as cobra venom factor, were injected with HBδ6, we observed a four- to fivefold decrease in the percentage of IgM^{dull} lymph node cells, with little change in the percentage of IgM^{bright} lymph node cells (Fig. 7). As was observed with spleen, the effects of injection of 11-26 were qualitatively similar, but considerably less marked than those induced by HBδ6. The observation that anti-IgD mAbs have the same effects on peripheral lymph node B cells as on splenic B cells suggests that the selective loss of IgM^{dull} spleen cells in anti-IgD mAb–injected mice is not a result of expression of increased mIgM by some IgM^{dull} cells and rules out the possibility that anti-IgD mAb causes the migration of IgM^{dull} spleen cells to peripheral lymph nodes.

Low Doses of Anti-IgD mAb Do Not Cause the Loss of Splenic B Cells. Experiments were performed to determine the quantity of anti-IgD mAb that is required to decrease the number of IgM^{dull} spleen cells and to try to correlate modulation of IgD from B cell surface and B cell activation with the B cell depletion. Mice were treated with anti-CD4 and anti-FcγRII mAbs plus cobra venom factor, then left without further treat-

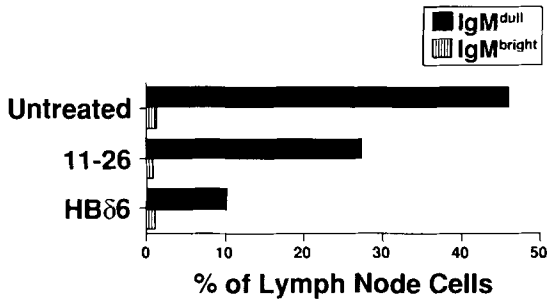

Figure 7. Selective depletion of lymph node IgM^{dull} B cell in node treated with anti-IgD mAbs. Percentages of IgM^{dull} and IgM^{bright} cells in pooled axillary, superclavicular, and popliteal lymph nodes from groups of three mice that had been pretreated with anti-CD4, anti-IL-7, and anti-FcγRII mAbs and cobra venom factor, and then left without further treatment or injected intravenously with 100 μg of 11-26 or HBδ6, were determined 7 d after anti-IgD mAb injection in the same experiment from which spleen cell data are shown in the right panels of Fig. 2 and the bottom panel of Fig. 4. No attempt was made to calculate absolute numbers of lymph node cells of each phenotype because of considerable variation in lymph node size.

ment or injected with 10, 33, or 100 μg of HBδ6. These mice were killed 1 d after anti-IgD mAb injection. A second set of mice was treated with the same reagents, as well as with anti-IL-7 mAb, and killed 5 d after HBδ6 injection. Injection of 33 or 100 μg of HBδ6 caused a three-to fourfold decrease in the number of splenic IgM^{dull} cells 5 d after HBδ6 injection, while injection of 10 μg of HBδ6 had only a minor effect on the number of these cells (Fig. 8, *right*). Although the injection of 10, 33, or 100 μg of HBδ6 substantially decreased B cell mIgD expression and increased B cell Ia expression 1 d later (Fig. 8, *left*), these changes only persisted 5 d after injection in mice that received 33 or 100 μg of HBδ6. The reappearance of normal quantities of mIgD and the decrease to baseline Ia levels on B cells 5 d after mice had received 10 μg of HBδ6 suggests that this dose of anti-IgD mAb was neutralized or catabolized by this time. The failure of this quantity of anti-IgD mAb to cause a significant loss of splenic IgM^{dull} B cells 5 d after injection, even though it is more effective than 100 μg of 11-26 at upregulating Ia expression by these cells 1 d after injection, is compatible with the view that signaling through mIgD needs to be maintained beyond 24 h to cause B cell loss.

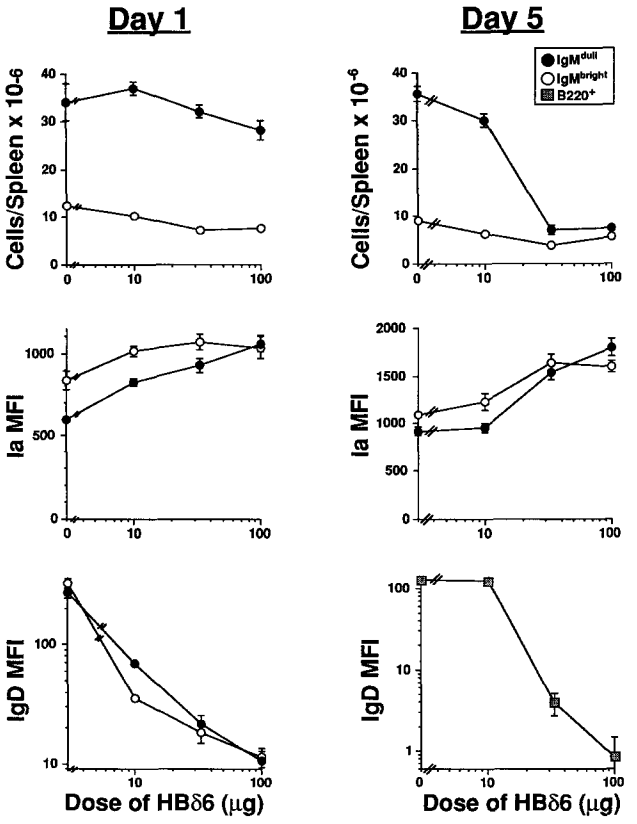

Figure 8. A high dose of anti-IgD mAb is required to deplete IgM^{dull} spleen cells. BALB/c mice (three/group) were pretreated with anti-CD4 and anti-FcγRII mAbs plus cobra venom factor (*left*) or these reagents plus anti-IL-7 mAb (*right*), after which they received no further treatment or were injected intravenously with 10, 33, or 100 μg of HBδ6. Mice were killed 1 d (*left*) or 5 d (*right*) after HBδ6 injection. Spleen cell suspensions were prepared, counted, and stained with FITC-anti-B220 and biotin-anti-IgM mAbs (*top*), FITC-anti-Ia^d and biotin-anti-IgM mAbs (*middle*), FITC-anti-IgD (FF1-4D5) and biotin-anti-IgM mAbs (*bottom left*) or FITC-anti-B220 and biotin-anti-IgD mAbs (*bottom right*), followed in all cases by streptavidin-R-PE. Stained cells were analyzed with a FACScan® for percentages of $B220^+IgM^{dull}$ and IgM^{bright} cells, the median fluorescence intensity of Ia^d staining of $B220^+IgM^{dull}$ and IgM^{bright} cells, and the median fluorescence intensity (*MFI*) of IgD staining of either $B220^+IgM^{dull}$ and IgM^{bright} cells (*bottom left*) or all $B220^+$ cells (*bottom right*). Means and standard errors are shown.

T Cell Help Decreases the Anti-IgD Antibody-induced Loss of IgM^{dull} B Cells and Induces the Appearance of an $Ia^+B220^-IgM^-$ Cell Population. To investigate whether the generation of T cell help during the course of an immune response could prevent anti-IgD antibody-induced loss of splenic B cells, mice were treated with anti-IL-7 and anti-FcγRII mAbs plus cobra venom factor and either anti-CD4 mAb or an isotype-matched control mAb (J1.2). Mice were then injected with GαMδ, which is a stronger inducer of T cell help than HBδ6, and killed 7 d later. Immunofluorescence staining and flow microfluorimetry were used to determine mIgM expression by $B220^+$ and Ia^+ spleen cells from these mice, and from mice that received no GαMδ. GαMδ treatment caused an ~40% decrease in spleen cell number in anti-CD4

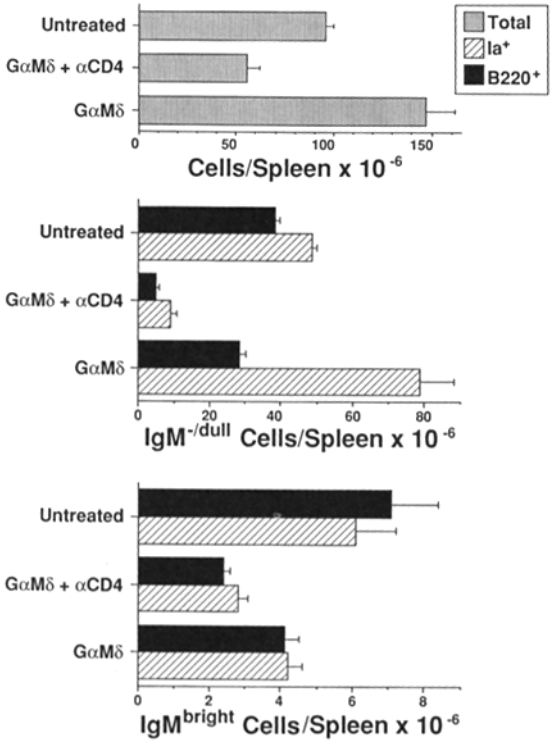

Figure 9. T cell help prevents the loss of splenic B cells in anti-IgD antibody-injected mice. BALB/c mice (three/group) were pretreated with anti-IL-7 mAb, anti-FcγRII mAb, cobra venom factor, and either anti-CD4 mAb or an isotype-matched control mAb, then injected intravenously with 800 μg of GαMδ. Mice were killed 7 d after GαMδ injection, and spleen cell suspensions were prepared, counted, and stained with FITC-anti-B220 and biotin-anti-IgM mAbs or FITC-anti-I-A^d and biotin-anti-IgM mAbs, followed in all cases by streptavidin-R-PE. A FACScan® was used to determine percentages of $B220^+$ and Ia^{d+} IgM^{dull} and IgM^{bright} spleen cells. Means and standard errors are shown.

mAb-treated mice and an ~50% increase in spleen cell number in T cell-sufficient mice (Fig. 9, *top*). In anti-CD4 mAb-treated mice, GαMδ caused a greater than fivefold decrease in the number of IgM^{dull} spleen cells and a two- to threefold decrease in the number of IgM^{bright} spleen cells (Figs. 9 and 10). In contrast, in T cell-sufficient mice, GαMδ caused a less marked decrease in the number of $B220^+$ IgM^{dull} and $B220^+IgM^{bright}$ spleen cells (Fig. 9, *middle*, and Fig. 10, *lower left*) and induced the appearance of a large population of Ia^+ spleen cells that lack B220 and express little or no mIgM (Fig. 9, *middle*, and Fig. 10, compare lower left and right panels). This latter population probably corresponds to the large population of $Ia^+B220^-IgM^-IgG$-secreting spleen cells that are present at this time in GαMδ-injected T cell-sufficient mice (26, 40).

Discussion

Our experiments demonstrate that anti-IgD mAbs cause a substantial decrease in the number of IgM^{dull} spleen and lymph node B cells when injected into mice in which the generation of new B cells and T cell help are blocked by anti-IL-7 and anti-CD4 mAbs, respectively. It is likely that B cell loss in these mice results from cell death, rather than migration of B cells from the spleen and lymph nodes to other organs or a change in B cell phenotype, because (*a*) anti-IgD antibody also causes loss of mature B cells from the bone marrow; (*b*) histologic studies of lung and liver from anti-IgD antibody-treated mice fail to show increased lymphoid infiltrates (data not shown); (*c*) parallel decreases are observed in numbers of Ia^+ and $B220^+$ cells; and (*d*) no increase in the number of $mIgG^+$ cells is seen (data not shown). The consequences of the interaction of mIgD and anti-IgD mAb in our model should resemble those that follow an interaction between antigen and the mIg of an antigen-specific B cell. Features of IgG antibodies that differentiate them from most antigens, such as complement fixation and interaction with Fc receptors, cannot account for the induction of B cell death in our system, inasmuch as (*a*) rat IgG2a mAbs, which were used for most of our experiments, have little ability to directly kill targeted cells in vivo (41), and, at least in rats, do not bind to FcγRI (42); (*b*) anti-IgD mAb-induced B cell death is not blocked by cobra venom factor and anti-FcγRII receptor mAb; (*c*) the loss of B cells occurs slowly, over a period of 2–7 d, unlike complement-mediated cell lysis or clearance of opsonized B cells, which would be expected to occur more rapidly; (*d*) IgM^{dull} B cells are much more susceptible than IgM^{bright} B cells to the cytocidal effects of anti-IgD mAbs, although both cell populations are bound to a similar extent by these antibodies (Figs. 5 and 8); and (*e*) the loss of B cells, unlike complement-mediated lysis or clearance of opsonized cells, is prevented by the presence of T cell help (Figs. 9 and 10). These same considerations suggest that death is likely to occur by apoptosis. Increased numbers of apoptotic B cells have not been observed in anti-IgD mAb-injected mice (Ashman, R., personal communication), however, presumably because the slow progression of cell death in this system, coupled with rapid in vivo removal of apoptotic cells, prevents the accumulation of detectable numbers of apoptotic cells. The failure to detect apoptotic lymphocytes in vivo when the rate of cell death is slow has also been noted by other investigators (43, 44).

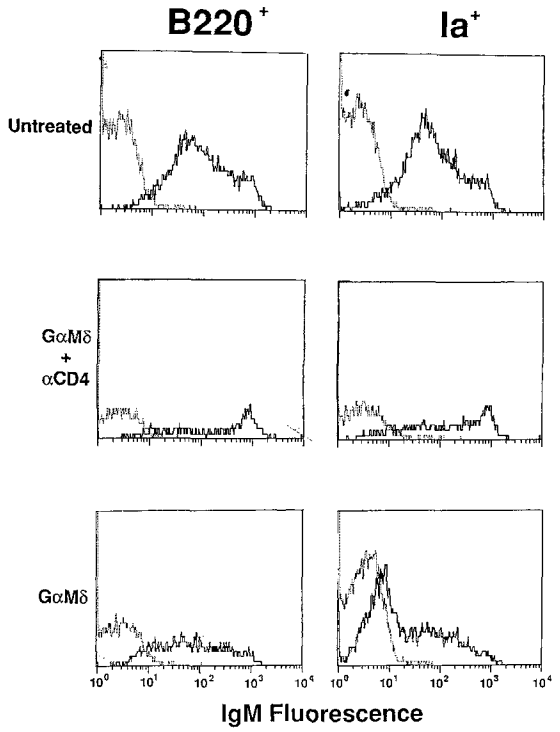

Figure 10. T cell help prevents the loss of splenic B cells in anti-IgD antibody-injected mice. Representative fluorescence histograms of stained spleen cells from the same experiment depicted in Fig. 8 are shown. Solid lines are histograms of $B220^+$ or Ia^{d+} spleen cells stained with biotin-anti-IgM mAb followed by streptavidin-R-PE; dotted lines are histograms of the same cells stained with streptavidin-R-PE in the absence of biotin-anti-IgM mAb.

The slow loss of B cells in this system resembles that described recently in double-transgenic mice, in which B cells express mIgM and mIgD specific for hen egg lysozyme (HEL) and HEL is present in serum (45). B cells in these double-transgenic mice are anergic (45) and have an in vivo half-life of ~5 d, while the half-life of HEL-specific B cells in transgenic mice that do not have serum HEL is ~4 wk (46). The double-transgenic study differs from ours in that B cell loss was measured indirectly, by determining percentages of B cells that have synthesized DNA during a defined period of time, rather than by directly recording decreases in B cell numbers. This methodological difference may be important, because the half-life of anergic, HEL-specific B cells was considerably longer when measured in the same study by a cell transfer technique (46), although it was still shorter than that of competent HEL-specific B cells. In addition to differences in the methodology used to determine B cell life span, our studies differ from investigations with double-transgenic mice in that antigen-specific B cells in the double-transgenic mice become exposed to antigen as soon as they have acquired mIg, while

the 2-wk pretreatment with anti-IL-7 mAb before injection of anti-IgD mAb in our system allows newly generated B cells to mature before mIgD is cross-linked. Thus, while the results in these two systems appear to be consistent, our observations demonstrate that the decrease in B cell life span that results from signaling through mIg does not require that this signaling start when B cells are immature, even though immature B cells have been shown to be more easily killed or tolerized than mature B cells by exposure to antigen or anti-Ig antibody (6–8). Results that are consistent with ours have been obtained in a variant of the double-transgenic system in which HEL-specific B cells that were initially exposed to a subtolerogenic concentration of HEL during their development still became anergic if exposed to a higher HEL concentration after they had matured, and in an experiment in which the transfer of HEL-specific B cells to an HEL-expressing mouse caused the donor B cells to become anergic (47). Although it was not determined whether the anergic B cells in these double-transgenic studies had a decreased in vivo life span, a likely interpretation of all of the double transgenic studies, when combined with ours, is that Ig cross-linking, in the absence of additional signals, inactivates and eventually deletes most autoreactive B cells, regardless of whether autoreactivity results from expression of germline or somatically mutated Ig genes.

In addition to methodological differences and differences in time of exposure to ligand for mIg, our system and the double-transgenic system differ in that HEL interacts with both mIgM and mIgD in the double-transgenic mice, while only mIgD is cross-linked in anti-IgD antibody–injected mice. Signaling through mIgM has been reported to be more effective than signaling through mIgD at inducing the growth arrest of B cell tumor lines that resemble immature B cells (48, 49). Furthermore, the removal of mIgD from mature B cells has been reported to make them easier to tolerize than mIgD-expressing B cells by exposure to antigen (50–52), and B cells from mice that lack a functional δ chain gene are more easily tolerized by in vitro antigen exposure than are B cells from conventional mice (53). In contrast, experiments with transgenic mice that expressed either IgM or IgD anti-HEL on their B cells as well as serum HEL or a cell membrane–bound form of HEL demonstrated that either mIg isotype can transduce signals that lead to B cell anergy or clonal abortion, respectively (54). In addition, B cells from conventional mice are stimulated in vitro to rapidly apoptose by extensive cross-linking of either mIgM or mIgD with biotinylated anti-IgM or anti-IgD mAbs and avidin (55). Our studies now establish that the in vivo interaction of a soluble ligand with mIgD on fully mature B cells can decrease the B cell's life span to that characteristic of anergic B cells.

Our studies, combined with previous in vivo and in vitro experiments, suggest that the difference between B cell anergy and clonal abortion is quantitative rather than qualitative. Relatively limited mIg cross-linking, as is induced by a mAb such as 11-26 or an antigen such as HEL, decreases B cell life span to a relatively limited extent, which cannot be demonstrated in vitro and is not easily observed in vivo unless the generation of new B cells is blocked (46). More extensive mIg cross-linking, as is induced by a mAb such as HBδ6, reduces B cell life span to a greater extent. Still more extensive mIg cross-linking, as would be induced by cells that express multiple plasma membrane representations of the epitope that is recognized by B cell mIg, can abort the development of epitope-specific B cells (7, 56) and cause B cells specific for that epitope to apoptose within a 24-h period (57). For practical purposes, this would mean that B cells specific for self-antigens, for which T cell help is not available, would have their half-life shortened in proportion to the extent to which their mIg is cross-linked by self-antigens. Increased antigen valency and concentration, and higher affinity of the mIg on a B cell clone for that antigen, would increase the extent of mIg cross-linking and the limitation of B cell life span. As a result, autoreactive B cells that could cause the greatest threat to health by avidly binding antigens that are abundantly present on cell membranes would be eliminated most rapidly, while lower avidity binding, binding of less abundant antigen, and binding of nonpolymeric antigen would be associated with a less profound decrease in B cell life span that might allow less threatening autoreactive B cells to still be stimulated by a foreign antigen. Because elimination of all B cells that are even slightly autoreactive might eliminate cells that are required for optimal antibody responses to foreign pathogens, a continuous inverse relationship between extent of autoreactivity and B cell life span may represent the optimal compromise between preventing autoimmune disease and allowing maximal protective antibody responses to foreign pathogens.

The results of our experiments also provide evidence that not all populations of mature B cells are equally susceptible to negative signaling through mIg. The mature IgM^{bright} B cell population, which includes marginal zone B cells (58, 59), is retained to a much greater extent in mice injected with anti-IgD mAbs than is the more predominant $mIgM^{dull}$ B cell population, which predominantly consists of mantle layer B cells (58, 59). The resistance of IgM^{bright} B cells to the cytocidal effects of anti-IgD mAb does not reflect an absence of mIgD from these cells or an inability of anti-IgD antibody to signal these cells. Many mature $mIgM^{bright}$ B cells express considerable quantities of mIgD (Fig. 8), and the mAb 11-26, which cross-links mIgD poorly, is actually more effective at inducing increased Ia expression by IgM^{bright} than IgM^{dull} spleen cells. These observations suggest that, although the cross-linking of mIg induces stimulatory signals for both IgM^{bright} and IgM^{dull} B cells, the signals that are generated may not be identical in these two B cell subsets, and/or IgM^{bright} B cells may be more resistant to the induction of cell death by the signals that are generated. Inasmuch as splenic polysaccharide-reactive B cells are predominantly in the splenic marginal zone B cell population (60, 61), and polysaccharide antigens generally express multiple representations of a given epitope and are unable to induce antigen-specific T cell help (62, 63), the ability of IgM^{bright} B cells to resist the tolerogenic effects of mIg cross-linking in the absence of T cell help may contribute to the generation of antipolysaccharide

antibody responses. The resistance of IgM^{bright} B cells to killing by cross-linking of their mIg, however, appears to be relative rather than absolute: the injection of mice with GαMδ, which should have a greater ability than anti-IgD mAbs to cross-link mIgD, causes the loss of a considerable percentage of IgM^{bright} B cells when the generation of T cell help has been blocked (Figs. 8 and 9).

The induction of T cell help in mice injected with anti-IgD antibody both maintains the survival of most $mIgM^{dull}$ B cells and stimulates the generation of a large number of B cells that exhibit a phenotype ($Ia^+B220^-mIgM^-$) that is typical of newly generated IgG-secreting cells (16, 40). Manipulation of this model should allow in vivo identification of the important signals by which T cell help prevents mIg cross-linking–induced B cell death and investigation of the extent to which mIg cross-linking–induced anergy is reversible.

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