Tumor Dormancy and Cell Signaling. II. Antibody as an Agonist in Inducing Dormancy of a B Cell Lymphoma in SCID Mice

By E. Racila,* R. H. Scheuermann,‡ L. J. Picker,‡ E. Yefenof,§ T. Tucker,* W. Chang,* R. Marches,* N. E. Street,* E. S. Vitetta,* and J. W. Uhr*

*From the *Department of Microbiology and Cancer Immunobiology Center,‡ Laboratory of Molecular Pathology and Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas 75235; and the §The Lautenberg Center for General and Tumor Immunology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel*

Summary

Tumor dormancy can be induced in a murine B cell lymphoma (BCL_1) by immunizing BALB/c mice with the tumor immunoglobulin (Ig) before tumor cell challenge. In this report, we have investigated the immunological and cellular mechanisms underlying the induction of dormancy. BCL_1 tumor cells were injected into SCID mice passively immunized with antibody against different epitopes on IgM or IgD with or without idiotype (Id)-immune T lymphocytes. Results indicate that antibody to IgM is sufficient to induce a state of dormancy. Antibodies against other cell surface molecules including IgD and CD44 (Pgp1) had no effect on tumor growth. Id-immune T cells by themselves also had no effect on tumor growth in SCID mice. However, simultaneous transfer of anti-Id and Id-immune T cells enhanced both the induction and duration of the dormant state. In vitro studies indicated that antibody to IgM induced apoptosis within several hours and cell cycle arrest by 24 h. Hyper cross-linking increased apoptosis. The FcγRII receptor played little or no role in the negative signaling. Antibodies that did not negatively signal in vitro did not induce dormancy in vivo. The results suggest that anti-IgM plays a decisive role in inducing tumor dormancy to BCL_1 by acting as an agonist of IgM-mediated signal transduction pathways.

Cancer dormancy is a well-recognized clinical phenomenon in which tumor cells are present but the population does not increase for long periods of time. However, tumor cells can regrow many years or even decades later. Cancer dormancy therefore represents an important clinical problem. At a basic level, it is also intriguing that malignant cells (characterized by uncontrolled growth) can survive for decades in vivo without apparent expansion of the cell population.

In an effort to gain more insight into the mechanisms underlying tumor dormancy, we have used a well-characterized B cell lymphoma, BCL_1[1] (1, 2), in mice immunized with the BCL_1 idiotype-positive (Id^+) IgM (3). We have shown that the major outcome of such immunization and challenge is a state of dormancy in which dormant lymphoma cells (DLC) persist in stable numbers for the 200 d of observation and are maintained in the majority of animals throughout life as evidenced by cell transfer experiments (4, 5). Isolation of DLC by multiparameter flow cytometry indicates that they are different from the growing BCL_1 cells with regard to size, morphology, and cell cycle status. We therefore suggested that signals induced by cross-linking of mIg with antibodies to BCL_1 Id are responsible for the induction and maintenance of the dormant tumor state.

In the present studies, we have explored the roles of anti-IgM, anti-IgD, and Id-specific T cells in inducing tumor dormancy by passively immunizing SCID mice. We have also analyzed the mechanisms underlying the antibody-induced dormant state by use of an in vitro–adapted cell line derived from the BCL_1 tumor (designated BCL_1 3B3) (6). We present evidence that anti-μ antibodies act as agonists rather than via conventional effector mechanisms to induce and maintain tumor dormancy. They do this by inducing both apoptosis and cell cycle arrest (CCA). Anti-IgM but not anti-IgD induces these antitumor effects and the FcγRII plays little

[1] *Abbreviations used in this paper:* BCL_1, B cell lymphoma; CCA, cell cycle arrest; DLC, dormant lymphoma cell; GARIg, goat anti-rabbit IgG; Id^+, idiotype positive; MA BCL_1 Id, polyclonal mouse anti-BCL_1 Id; MARK, mouse anti–rat κ; MFI, mean fluorescence intensity; RtAMλ, rat anti–mouse λ; RtAMδ, rat anti–mouse δ; $RAGM_1$, rabbit anti-asialo GM_1; 7-AAD, 7-amino actinomycin D.

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or no role. Id-specific T cells cannot by themselves induce dormancy, but they synergize with anti-IgM to induce a more long-lived dormant state.

Materials and Methods

BCL_1 3B3 Tumor. The BCL_1 tumor and its 3B3 subline were maintained in vivo by i.v. and i.p. passage in BALB/c or SCID mice. 5 wk after inoculation of 5–10 × 10^5 spleen cells from a tumor-bearing animal, mice were killed and their splenocytes were used as a source of tumor cells. Alternatively, BCL_1 3B3 cells were maintained in vitro and 3 × 10^4 cells were injected directly into each SCID mouse. Tumor cells in the spleens of dormant mice were determined by flow cytometry and by adoptive transfer of graded numbers of splenocytes from dormant mice into naive BALB/c mice. Splenomegaly was determined by palpation as previously reported (4).

BCL_1 Id-KLH Conjugation and Immunization. Conjugation of the Id^+ BCL_1 IgM to KLH and the immunization of BALB/c mice have been described previously (4).

Antibodies. Hybridoma cells secreting rat IgG2a anti-BCL_1 Id (RtA Id) (6A5) were a gift from Dr. Freda Stevenson (Southampton, UK). The Id^+ IgMλ was purified from the ascites of C5D5 hybridoma which was generated by fusing BCL_1 cells with SP2/0 myeloma cells (7). Rat anti-mouse λ (RtAMλ) (B1.1) was generated in our laboratory and previously described (8). Rat anti-mouse δ (RtAMδ) (JA12.5) was produced by Abbott Biotech, Inc. Normal rat IgG (NRt IgG) and normal mouse IgG (MIgG) were prepared from pooled normal sera by chromatography on Sephadex A-50. Rat anti-mouse CD44 (IM7) (RtAM CD44), RtAMδ (JA 12.5), and rat anti-mouse IgM (RtAMμ) (Bet-2) were purified from the supernatants of hybridomas obtained from the American Type Culture Collection (Rockville, MD). Polyclonal mouse anti-BCL_1 Id (MA BCL_1 Id) ascites was produced by intraperitoneal injection of pristane into Id-immune mice followed 1–2 wk later by intraperitoneal injection of SP2/0 myeloma cells. Polyclonal rabbit anti-BCL_1 Id (RA BCL_1 Id), rabbit anti-mouse μ (RAμ), rabbit anti-ovalbumin (RAOVA), goat anti-mouse δ (GAδ), goat anti-mouse μ (GAμ), goat anti-ovalbumin (GAOVA), and goat anti-rabbit IgG (GARIg) were produced and affinity purified as previously described (2). Aggregation of RAOVA was performed by heating it at 60°C for 10 min until the solution became slightly turbid.

Immunofluorescent Staining and Cytofluorometry of Cells. For multicolor flow cytometry on a FACScan® (Becton Dickinson Immunocytometry Systems USA, San Jose, CA), the fluorochromes FITC, PE, Red 670, and Red 613, were used. The following procedure was employed to stain the cells for cytofluorometric analysis. 10^6 cells were incubated with either RtAMλ, RtA Id, or their biotinylated conjugates. The binding of these antibodies was detected using a secondary FITC-conjugated mouse anti-rat κ (MARK) or a fluorochrome bound to streptavidin (SA), respectively. Cells were examined directly on the FACS® or residual MARK binding sites were blocked with NRt IgG and the cells were further incubated with antibodies coupled to PE or biotin before FACS® analysis. The final incubation included Red 670-SA or Red 613-SA (GIBCO BRL, Gaithersburg, MD).

To determine the cycle status of the BCL_1 3B3, cells were fixed in 0.5% paraformaldehyde and then incubated with the DNA-binding fluorochrome Hoechst 33342 (Sigma Chemical Co., St. Louis, MO) (220 μl of 15 μM Hoechst in 5% Tween 20) overnight. The DNA content of single cells was determined in a specially prepared FACStar Plus® (Becton Dickinson) (320 and 488-nm excitation) by measuring the blue light emission of DNA-bound Hoechst. Cell doublets and large aggregates were excluded from the analysis using appropriate gating on pulse process signals from the Hoechst emission (signal width vs. area). Apoptotic cells were identified as above except that cells were stained with 400 μM 7-amino actinomycin D (7-ADD) (Molecular Probes, Inc., Eugene, OR) in PBS at 4°C for 30 min before fixation (9). For tumor cells from spleens of BCL_1-bearing mice, splenocytes were stained for surface markers using RtAMλ and FITC-MARK with or without additional PE-conjugated antibodies, stained with 7-AAD and Hoechst, and analyzed as described above.

Flow cytometry data were analyzed by "Paint-a-Gate" software as described (10). This analysis enables a multi-dimensional identification of cells reactive with the antibodies as well as the determination of their relative sizes (small vs. large) based on the position of the cells in the correlative display of forward vs. orthogonal light scattering.

[^{3}H]Thymidine Incorporation. 3 × 10^4 cells were incubated in RPMI 1640 5% FCS (GIBCO) at 37°C for 8 h with antibody in 0.2 ml of medium before the addition of 1 μCi [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL). Cells were harvested after a 16-h pulse. All values are presented as the mean of triplicate samples.

Purification of T Cells. B and $Fc\gamma RII^+$ cells were depleted by two methods: (*a*) GARIg magnetic beads (Advanced Magnetics Inc., Cambridge, MA); or (*b*) a mouse T cell enrichment column (R & D Systems, Inc., Minneapolis, MN). Briefly, spleens from normal BALB/c or Id-immune BALB/c mice were removed, teased into a single cell suspension, and the RBCs were lysed by incubating the resuspended pellet in lysis buffer (0.1 M ammonium chloride, 0.7 mM KH_2PO_4) for 5 min at 20°C. The cells were washed in HBSS and resuspended at 2.5 × 10^7 cells/ml HBSS. 10 μg of rabbit anti-mouse Ig (RAMIg)/ml was added and the cells were incubated on ice for 40 min. 0.7 ml of GARIg magnetic beads was washed in HBSS and added to the spleen cells in a 25-cm flask. A magnet was attached to the bottom of the flask and the cells were incubated for 10 min at room temperature. The flask was gently inverted, decanted, an additional 0.7 ml beads was added, and the procedure was repeated. The depleted cells were then pelleted, pooled, and resuspended at 10^8 per ml. Alternatively, spleen cells with lysed RBCs were adjusted to 1.5–2 × 10^8 cells/ml and B cells and $Fc\gamma RII^+$ cells were removed by use of mouse T cell enrichment columns. Cells from each spleen were purified on a single column. Aliquots of cells were sampled before and after both purifications and the numbers of B and T cells were determined by FACS® analysis. The two methods gave similar results.

Agarose Gel Electrophoresis of DNA. 10^7 cells were treated with proteinase K (1 mg/ml) in 50 mM Tris-HCl, pH 8.0/100 mM NaCl/100 mM EDTA/1% SDS, and the DNA was isolated by phenol/$CHCl_3$, 1:1 (vol/vol) extraction and ethanol precipitation. DNA isolated from 3 × 10^5 cell equivalents was treated with RNase A (1 mg/ml) and resolved in 2% agarose gels with 1× TAE buffer (40 mM Tris acetate, 2 mM EDTA) (11).

Elimination of NK Cells from SCID Mice. Rabbit anti-asialo GM_1 ($RAGM_1$) (Wako Pure Chemical Industries, Inc., Osaka, Japan) was used to eliminate NK cells from SCID mice. 25 μl of this antibody was calibrated by Wako and found to diminish NK-mediated killing of YAC-1 at an E/T ratio of 50:1 by 97% when injected into mice. 25 μl of this antibody was injected into SCID mice and the NK activity was measured by lung clearance of ^{125}I-UdR-labeled YAC-1 cells as previously described (12). A sixfold reduction in NK function was demonstrated in the SCID mice treated with $RAGM_1$.

Results

Antibody-induced Dormancy in SCID BCL_1 Mice. The capacity of antibody to induce dormancy in the absence of BCL_1-specific cytotoxic T cells was studied by passive immunization of SCID mice. A series of polyclonal and monoclonal antibodies specific for various epitopes on Ig were injected into the mice before injection of BCL_1 or BCL_1 3B3 cells. Dormancy was defined as the absence of splenomegaly at 60 d since in virtually all control mice, splenomegaly was detected by 35–45 d after challenge with BCL_1 or BCL_1 3B3 cells. As shown in Table 1, 53–79% of BCL_1-inoculated mice developed dormancy depending upon the particular anti-Ig antibody used. The polyclonal antibodies were more effective as judged by the duration of dormancy. Essentially similar results were obtained using BCL_1 3B3 cells (Table 2) except that the tumor cells grew slightly faster (controls had splenomegaly by 25–35 d) and the incidence of dormancy was lower and duration shorter than those observed when the parent BCL_1 tumor cells were used (data not shown).

When the spleens from dormant SCID/BCL_1 mice injected with BCL_1 3B3 were stained with RtAMλ and examined by flow cytometry, DLC were identified by staining with anti-λ (Fig. 1). These cells represented 0.1–1% of the spleen cells and were few in number ($\sim 10^5$ DLC) because of the small size of the SCID mouse spleens and because only 3×10^4 BCL_1 3B3 cells were inoculated. In the 66 mice injected with either BCL_1 cells or BCL_1 3B3 cells and classified as dormant based on the lack of splenomegaly at 60 d, all the mice harbored tumor cells as evidenced by delayed tumor growth, FACS® analysis, or by cell transfer. Hence, these mice had not been cured of their tumor by treatment with anti-Ig. Because of the small number of tumor cells present, we were unable to evaluate their cell cycle status.

We considered the possibility that the role of antibody was to divert the tumor cells from the spleen to another organ and/or to deplete them by Fc-mediated effector mechanisms, e.g., opsonization or ADCC. To test these possibilities, RtAM CD44 and RtAMδ, were administered to SCID mice challenged with BCL_1 3B3 cells. These antibodies bind to BCL_1 3B3 cells without affecting their growth in vitro. Mice treated with these antibodies showed no antitumor immunity. GAμ, which has little or no affinity for the FcγRII (13), was highly effective in inducing dormancy. In addition, when mice were first injected with BCL_1 cells and 3–7 d later with anti-μ (to allow the tumor cells to home to the spleen before treatment with antibody) antibody was completely effective 3 d and moderately effective 1 wk after BCL_1 challenge, despite the 3–7 d of tumor growth before treatment (anti-μ injected at -1, $+3$, and $+7$ d later showed dormancy in six of nine, four of five, and three of five mice, respectively). These results make it unlikely that anti-Ig diverts the tumor cells to another anatomical site.

SCID mice lack T and B cells but have normal or above normal levels of NK cells (14). To test the possibility that NK cells contributed to the induction of dormancy, SCID mice were treated with 25 μl of $RAGM_1$ antibody 3 d before injection of BCL_1 cells and 4 and 11 d thereafter. A single injection of 25 μl of $RAGM_1$ reduced NK function in SCID mice more than sixfold. MA BCL_1 Id was injected on day 1 and weekly thereafter for 7 wk. The results demonstrated that $RAGM_1$ had no significant effect on the ability of MA BCL_1 Id to induce dormancy. Anti-asialo GM_1 injected with anti-μ induced dormancy in four of five mice (average of 84 ± 15 d before splenomegaly appeared); injection of anti-μ alone induced dormancy in five of five mice (average 91 ± 13 d before splenomegaly appeared). This suggests that antibody-mediated effector mechanisms do not contribute to the induction of dormancy in a major way, and this result is consistent with our working hypothesis that the antitumor effects of MA BCL_1 Id are caused by signal transduction.

The Effect of Id-immune T Cells on Dormancy in SCID Mice. In prior experiments using SCID mice injected with antibody and challenged with BCL_1 3B3 or BCL_1 cells, the duration of dormancy was usually less than that previously reported for BALB/c Id-immune mice (3, 5, 15). A possible explanation is that Id-specific T cells contribute to the induction and maintenance of dormancy. Therefore, Id-immune T cells were injected into SCID mice with or without anti-Id to determine if T cells had any role in the induction of dormancy. To facilitate the detection of an additive effect by Id-immune T cells, SCID mice were treated with suboptimal doses of MA BCL_1 Id, which alone induced dormancy in

Table 1. *Dormancy in Antibody-treated Mice Injected with BCL_1 Tumor Cells*

Antibody	Percent dormant (No. of mice injected with tumor)	Average day of onset of splenomegaly ± SD
	%	
–	0 (8)	36 ± 5.9
MIgG	0 (12)	32 ± 7.2
GAOVA	0 (3)	28 ± 0
RtAM CD44	0 (10)	35 ± 7.0
RtAMδ	0 (3)	35 ± 1.6
MA BCL_1 Id	77 (35)	85 ± 21
GAμ	53 (19)	75 ± 5.1
RtAMλ	60 (5)	60 ± 11
RtAMμ	67 (9)	76 ± 22
RA BCL_1 Id	79 (14)	153 ± 60
RAμ	62 (13)	99 ± 16

SCID mice were injected intravenously with 50 μg of antibody on day 0 and weekly thereafter for 7 wk. On day 1, mice were injected with 3×10^4 BCL_1 tumor cells intraperitoneally and palpated twice weekly for splenomegaly (4). Animals without splenomegaly on day 60 were considered dormant.

Table 2. *Dormancy in Mice Injected with the BCL_1 3B3 Cell Line Tumor*

Exp. No.	Control	Antibody injected into SCID mice*			BALB/c Mice‡	
		RA BCL_1 Id	RAμ	GAμ	Control	Id immune
1	0/4	4/4	–	–	0/5	2/10
2	0/2	3/3	5/5	0/5	1/5	3/5
3	0/2	0/3	0/5	2/4	0/5	4/5
Total	0/8	7/10	5/10	2/9	1/15	9/20

* Animals received 50 μg each of antibodies intravenously on day 0 and weekly thereafter for an additional 7 wk. 3 × 10^4 BCL_1 3B3 were injected intraperitoneally on day 1. Animals were then palpated twice weekly for splenomegaly. Control animals received BCL_1 3B3 but not antibodies. Animals without splenomegaly at 60 d were considered to have dormant tumor. Number of dormant mice/total number of mice is presented.
‡ BALB/c mice were immunized with BCL_1 Id, injected intraperitoneally with 10^6 BCL_1 3B3 cells and followed for splenomegaly by biweekly palpations. Naive BALB/c mice injected with BCL_1 3B3 cells served as controls.

only 50% of the mice (vs. 77% observed with optimal doses of this antibody). As shown in Table 3, MA BCL_1 Id alone induced dormancy in 5 of 10 mice which lasted an average of 72 d. Id-immune T cells did not by themselves induce dormancy. However, when administered with anti-Id antibody, all 10 mice developed dormancy with an average duration of 164 d. The differential onset of splenomegaly in mice treated with MA BCL_1 Id and Id-immune T cells compared to antibody alone was statistically significant. By contrast, there was no statistical difference in dormancy between mice treated with antibody alone and antibody together with normal T cells. These results indicate that although T cells from Id-immune animals cannot by themselves induce dormancy under the above conditions (10^7 cells/SCID animal), they can enhance the incidence and duration of dormancy achieved with anti-Id.

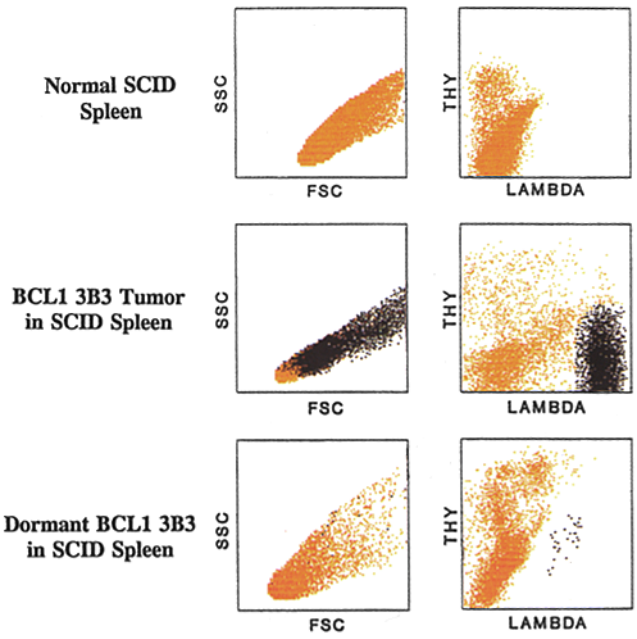


Figure 1. Flow cytometric analysis of dormant lymphoma cells (BCL_1 3B3) in spleens of SCID mice. 3 × 10^4 BCL_1 3B3 cells were injected into SCID mice. 7 d after injection of tumor cells, animals received weekly injections of 50 μg RA BCL_1 Id for 8 wk. 66 d after the administration of BCL_1 cells, a dormant mouse and a naive mouse (no Ab) with growing tumor were killed along with a naive SCID mouse without tumor. The splenocytes were stained for FACS®. The animal harboring growing tumor cells (*middle*) had 67% λ^+ cells (BCL_1 3B3 tumor shown in *black*), the dormant animal had 0.5% λ^+ cells (*bottom*), and no λ^+ cells were detected in splenocytes from a control mouse (*top*). Forward scatter (*FSC*) and side scatter (*SSC*) indicate the size of the cells. Staining with Thy-1 shows that the λ^+ population is distinct from NK cells in the spleen.

Effect of Polyclonal Antibodies on [^{3}H]Thymidine Incorporation in BCL_1 3B3 Cells In Vitro. To analyze the mechanisms underlying the induction of dormancy, in vitro, studies using BCL_1 3B3 were undertaken. Fig. 2 demonstrates that polyclonal antibodies proven to induce dormancy of BCL_1 3B3 in SCID mice (see Table 2) also significantly reduced [^{3}H]thymidine incorporation in BCL_1 3B3 cells in vitro. RtAM CD44 and RtAMδ antibodies that were ineffective in vivo had no effect on thymidine incorporation in vitro (to be discussed below).

The reduction in [^{3}H]thymidine incorporation induced by RA BCL_1 Id is related to receptor occupancy (Fig. 3). Receptor occupancy was measured by determining the mean fluorescence intensity (MFI). As can be seen, MFI reached a plateau at 1 μg/10^6 cells at which point [^{3}H]thymidine incorporation diminished incrementally up to the highest concentration used (8 μg/10^6 cells). The continued decrease in [^{3}H]thymidine uptake after saturation of surface IgM (mIgM) could be explained by continued cross-linking of newly expressed mIgM molecules when an excess of anti-Id was present. It is unlikely that dissociation of antibody from mIgM could account for this finding since hyperimmune polyvalent antibodies (which have a high affinity and low rate of dissociation) were used. These results indicate that antibodies that induce dormancy in vivo, have antiproliferative effects in vitro and that these effects occur when receptor occupancy is complete and continued for many hours.

Cell Cycle Arrest and Apoptosis Are Induced by Cross-linking IgM. The above experiments demonstrate that anti-μ or anti-

Table 3. *Effect of Purified T Cells on Dormancy in SCID Mice*

MA BCL_1 Id 50 μg/wk for 5 wk*	Source of T cells (10^7)‡	Number of dormant mice / Total No. of mice	Average day to splenomegaly ± SD§
–‖	–	0/8	45 ± 3.9
–	Normal spleen	0/10	49 ± 7.4
–	Id-immune spleen	0/10	52 ± 8.7
+	–	5/10	72 ± 13
+	Normal spleen	6/10	88 ± 30
+	Id-immune spleen	10/10¶	164 ± 96

* This regimen of MA BCL_1 Id gives suboptimal dormancy of BCL_1 cells. Eight injections each of 50 μg (one injection per week) gives 77% dormancy with an average day to loss of dormancy of 85 ± 21 d when a total of 35 animals were followed.
‡ B cells and Fc receptor positive cells were depleted either by magnetic beads (Advanced Magnetics Inc.) or by a T cell column. In the two experiments represented in this table, contamination of B cells and Fc receptor positive cells were <2% (magnetic beads) and <0.1% (T cell column).
§ The time to onset of splenomegaly in mice treated with MA BCL_1 Id plus Id-immune T cells is statistically different by the *t* test from mice receiving only MA BCL_1 Id ($p < 0.02$). There is no statisical difference between mice treated with MA BCL_1 Id or MA BCL_1 Id plus normal T cells.
‖ – = None.
¶ One dormant animal died at day 175 and two animals were killed at day 350. Both animals had small spleens and these splenocytes passed tumor into naive BALB/c mice indicating the presence of DLC.

Id antibodies induce antiproliferative activity in BCL_1 3B3 cells. To determine the cellular mechanisms underlying this inhibition, treated cells were analyzed by flow cytometry for cell permeability (death) vs. DNA content (cell cycle). As shown in Fig. 4, 45% of the BCL_1 3B3 cells treated with RAμ were dead as compared to 3.3% of the RAOVA-treated cells. Cell death was a result of apoptosis, since gel analysis of DNA from treated cells revealed the typical nucleosomal ladder (data not shown).

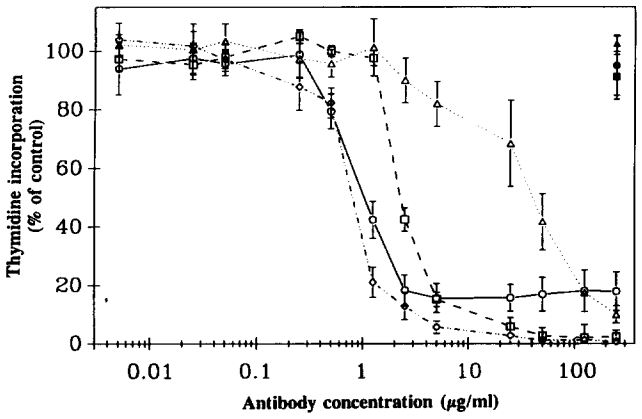


Figure 2. The effect of anti-μ on DNA synthesis in BCL_1 3B3 cells. 3×10^4 BCL_1 3B3 cells were plated in triplicate into a 96-well plate with GAμ (O), RAμ (□), RA BCL_1 Id (◇), MA BCL_1 Id (Δ), or with each of their control Ig antibodies (*closed symbols*, respectively). After 8 h of incubation, 1 μCi [^{3}H]thymidine was added to each well for the next 16 h. The cells were then harvested and thymidine incorporation measured. Results represent the average of three experiments. Thymidine incorporation is presented as the percent of incorporation in cells treated with medium alone. All values are the mean of triplicate samples. Error bars represent ± 1 SD.

The RAμ-treated cells also showed a marked decrease in the proportion of viable cells in S, G_2, and M phases of the cell cycle as compared to RAOVA-treated cells (9.3% vs. 24.3%, respectively) (Fig. 4). To prove that the decrease in the proportion of cycling cells was due to CCA, cells were treated with vinblastine which inhibits mitosis by interacting with the microtubular protein, tubulin (16). If cells are arrested in G_0/G_1, then the observed increase in the G_0/G_1 fraction will be maintained after vinblastine treatment, whereas if the cells are cycling more slowly as a result of anti-Id treatment, then the G_0/G_1 fraction will decrease as the cells progress to the block in mitosis. In these experiments, cells

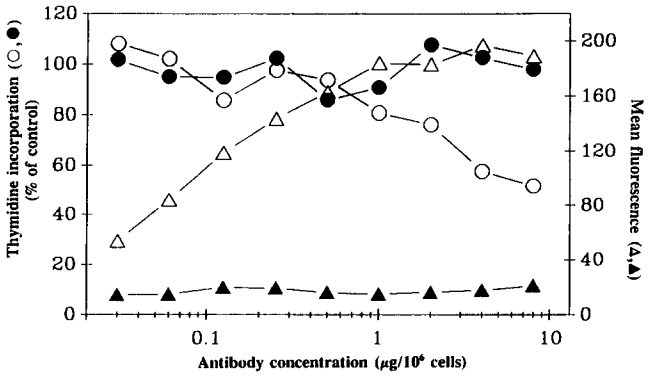


Figure 3. The relationship of mIg occupancy to the inhibition of growth. 10^6 cells were incubated for 15 min at 4°C with RAOVA (*closed symbols*) or RA BCL_1 Id (*open symbols*). This was performed in a volume of only 20–30 μl because the large number of cells and titrations performed required a large quantity of antibody. The cells were washed twice in BSS-Eagle, stained with FITC-GARIg, and the MFI (*triangles*) was determined. Duplicate samples were plated in triplicate and DNA synthesis measured by [^{3}H]thymidine uptake (*circles*).

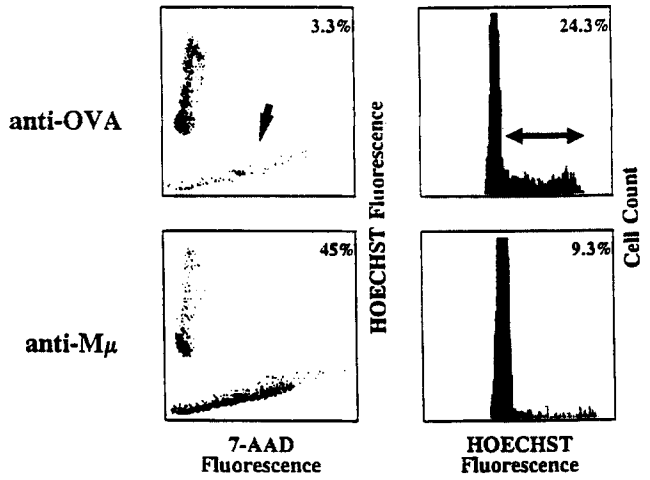


Figure 4. Flow cytometric analysis of membrane integrity and DNA content. 10^6 BCL_1 3B3 cells were treated with either RAOVA (*top*) or RAμ (*bottom*) at a concentration of 35 μg/ml and analyzed by flow cytometry for membrane integrity using 7-AAD and for DNA content using Hoechst 33342. Viable cells exclude 7-AAD and bind intermediate to high amounts of Hoechst dye, including cells in the G_0/G_1 and $S/G_2/M$ phases of the cell cycle. Apoptotic cells (*down arrow*) have relatively low Hoechst fluorescence primarily due to induced DNA fragmentation, and most show a loss of membrane integrity (7-AAD^+). The percentage of cells that is apoptotic is indicated in the upper right hand corner of 7-AAD vs. Hoechst fluorescence plots (*left*). Hoechst fluorescence gating only on viable cells is indicated in histograms (*right*), and the percentage of cells in $S/G_2/M$ phases (*horizontal arrow*) of the cell cycle is indicated.

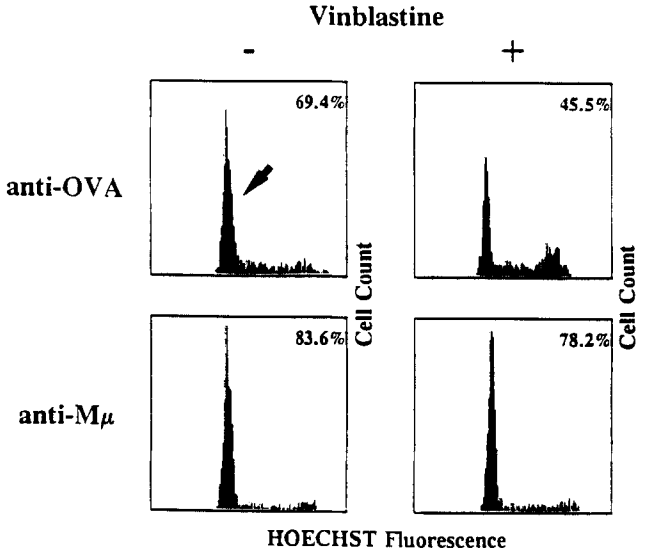


Figure 5. Effect of vinblastine on the cell cycle status of BCL_1 3B3 cells treated with anti-Id. Samples were treated with RAOVA (*top*) or RA BCL_1 Id (*bottom*) for 48 h. Duplicate cultures (*right*) were incubated with vinblastine (5 ng/ml) for 24 h before analysis. The cells were then stained with Hoechst 33342 and 7-AAD and analyzed by flow cytometry. The percentage of cells in the combined G_0 and G_1 phases (*arrow*) of the cell cycle is indicated in the upper right hand corner of each panel.

were incubated with RA BCL_1 Id for 24 h before 5 ng/ml of vinblastine (Sigma Chemical Co.) was added. The cells were then analyzed by flow cytometry 24 h later. As shown in Fig. 5, the elevated proportion of cells in G_0/G_1 after treatment with anti-Id and vinblastine was not affected. In contrast, exposure of RAOVA-treated cells to vinblastine resulted in a marked decrease in the percentage of G_0/G_1 cells. These results indicate that treatment with anti-Id itself arrests cells in G_0/G_1.

Kinetics of Cell Death and CCA in anti-BCL_1 Id–treated BCL_1 3B3 Cells. Signal transduction by anti-Ig in lymphoma or immature B cells results in CCA or apoptosis or both (17–23). We investigated the kinetics of induction of CCA and apoptosis by flow cytometry. Fig. 6 represents a compilation of four experiments in which cell death and CCA were induced with RA BCL_1 Id. Loss of membrane integrity and DNA degradation in apoptotic cells was detectable as early as 2 h after treatment and their percentage continued to increase throughout the time course of 24 h. In contrast, CCA was not detectable until 8 h after antibody treatment and increased in the residual viable cells at 24 h. These results indicate that a portion (or all) the cell death observed can occur independently of CCA. These findings are consistent with the existence of at least two partially independent signal transduction pathways in BCL_1 3B3 cells after mIg cross-linking, that is, one leading directly to apoptosis/necrosis and one leading to CCA (11).

The Effect of "Hyperaggregation" of Surface IgM. The cross-linking of IgM is required for signaling in BCL_1 3B3 cells. The question therefore arose as to whether increased cross-linking of occupied receptors can increase negative signaling, and whether such a putative increase in signaling would have equivalent effects on CCA and apoptosis. To test this, BCL_1 3B3 cells were treated for 15 min at 4°C with 1 μg RA BCL_1 Id per 10^6 cells (a concentration that causes 90–95% receptor occupancy but inhibits [^{3}H]thymidine incorporation by only 5–10% because the time of exposure was very short before excess antibody was removed). An aliquot of the cells was then treated with 10 μg/ml of GARIg to induce "hyper cross-linking". As shown in Fig. 7, superaggregation of mIgM more than doubled the percentage of dead cells observed after treatment with RA BCL_1 Id alone (25.1 vs. 11.8%, respectively). Thus, increased cross-linking of a fixed number of antibody-bound receptors results in increased cell death. These results indicate that the extent of cross-linking determines the number of cells that die. Similar findings have been reported by Parry et al. (24).

The Role of the Fc Receptor in the Antibody-mediated Negative Signaling of BCL_1 3B3 Cells. The potential role of the Fc receptor in the antibody-induced negative signaling of BCL_1 3B3 cells was investigated. In mature B cells when the FcγIIR is coligated with IgM or IgG, it induces a codominant negative signal that can override the positive signal induced by mIg (25, 26). In the first series of experiments, we demonstrated that $F(ab')_2$ fragments of goat or rabbit antibody were highly effective at inducing CCA and apoptosis in vitro. Goat $F(ab')_2$ was as effective as goat IgG (Fig. 8 *A*), however, $F(ab')_2$ fragments of rabbit IgG were slightly less effective than the rabbit IgG antibody in vitro (data not shown).

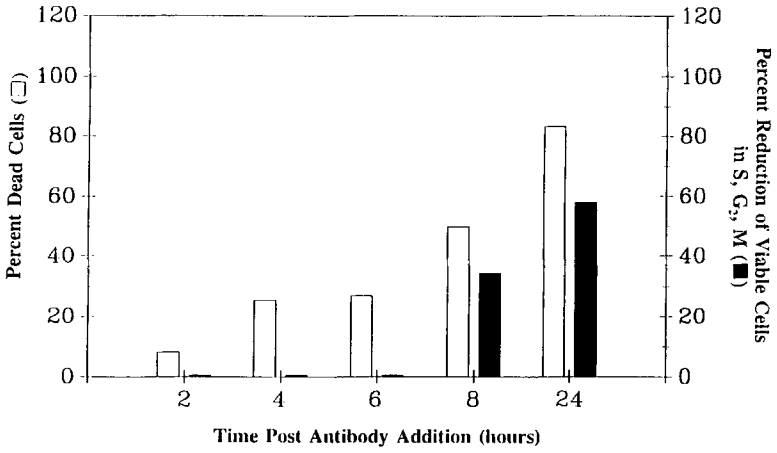


Figure 6. Kinetics of RA BCL_1 Id–induced cell death and CCA. 10^6 BCL_1 3B3 cells were plated in 1.0 ml in 24-well plates with 35 μg/ml of rabbit anti-BCL_1 Id, rabbit anti-OVA, or no antibody. There was no significant difference between the RAOVA-treated and the nontreated BCL_1 3B3 cells. Wells were harvested at 2, 4, 6, 8, and 24 h (the figure is a compilation of four experiments – not all time points were done in each experiment). Cells were analyzed by FACS® for percentage of cells in S_1, G_2, and M phases of the cell cycle and percentage of dead cells. The percentage of dead cells in untreated controls at each time point was subtracted from the percentage of dead cells in anti-BCL_1 Id–treated cells. The difference between the percentage of cycling cells in controls and cycling cells in anti-BCL_1 Id–treated samples was compared to the percentage of control cycling cells and plotted as percent reduction of cycling cells. Two additional experiments utilizing GAμ gave similar results except that significant CCA did not occur until 24 h after antibody treatment and the maximum percentage of dead cells did not exceed 25%.

This could be due to the ~17% decrease of the binding affinity of the rabbit $F(ab')_2$ compared to the intact antibody. Therefore, further experiments were carried out to block the coligation of the FcγRII with crosslinked Ig. As shown in Fig. 8 *B*, blocking the Fc receptor by pretreating the cells with nonaggregated or heat-aggregated RAOVA before the addition of RAμ had no effect on the capacity of RAμ to inhibit thymidine incorporation. Taken together, these data indicate that the FcγRII does not contribute significantly to the inhibitory signal induced by cross-linking mIgM.

Effect of Anti-δ on the Induction of Dormancy. There are contradictory reports indicating whether cross-linking IgD can induce an inhibitory signal (24, 27, 28). One study suggested

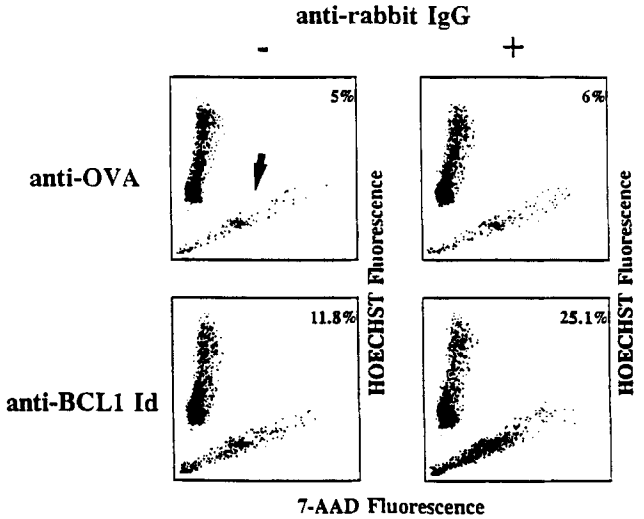
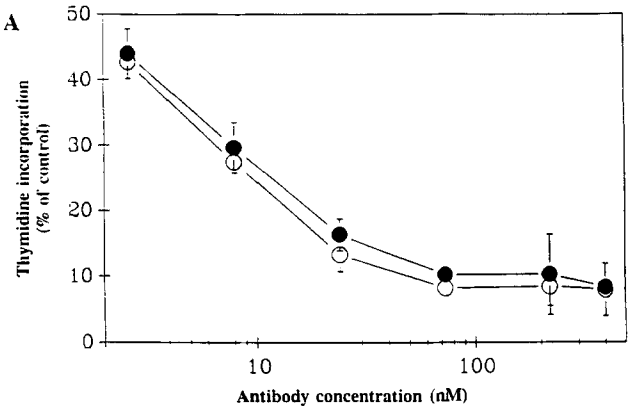


Figure 7. Enhanced cross-linking of mIg increases apoptosis in BCL_1 3B3 cells. Duplicate samples of 10^6 cells were incubated in the presence of 1 μg of either RAOVA (*top*) or RA BCL_1 Id (*bottom*) at 0°C for 15 min. The cells were washed twice in BSS-Eagle and plated with or without GARIg (10 μg/ml) for 24 h and then analyzed by flow cytometry as described in Materials and Methods. The percentage of apoptotic cells (*arrow*) is indicated for each sample.



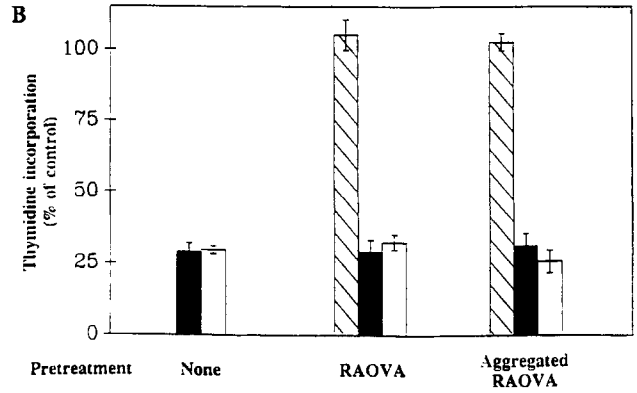


Figure 8. The role of Fc receptors in anti-Ig–induced negative signaling. (*A*) Different concentrations of goat anti-μ (*open circles*) or its $F(ab')_2$ (*closed circles*) fragments were incubated with 3×10^4 BCL_1 3B3 cells for 24 h. [^{3}H]Thymidine incorporation was measured and is shown as a percentage of that incorporated in untreated cells. (*B*) 3×10^4 BCL_1 3B3 cells were plated in 200 μl per well and 10 μg RAOVA, either native or heat aggregated, were added. After 15 min of incubation at 37°C, 1 μg of rabbit anti-μ (*closed boxes*), goat anti-μ (*open boxes*), or no anti-μ antibodies (*cross-hatched boxes*) were added to the appropriate wells for the next 24 h. [^{3}H]Thymidine incorporation was measured and is shown as a percentage of untreated cells.

that although anti-δ by itself cannot induce a negative signal in WEHI231 cells, it can increase the negative signaling induced by anti-μ antibodies (28). We, therefore, performed several experiments to determine the role of IgD signaling in BCL_1 3B3 cells. Polyclonal (goat) or monoclonal (rat) anti-δ antibodies at various concentrations (2–500 μg) had no effect on 3×10^4 BCL_1 3B3 cells in vitro. Moreover, there was no increase in inhibition observed when a constant suboptimal dose of RAμ (1.75 μg/ml) was compared to the same dose of anti-μ administered simultaneously with increasing doses of polyclonal or monoclonal anti-δ antibodies. Thus, there was no evidence that negative signaling could occur through IgD. However, although all the 3B3 cells stained with monoclonal rat anti-δ, the density of mIgD was only 10% that of mIgM as determined by MFI using FITC-labeled MARK. Hence, we cannot exclude the possibility that the negative result would be due to the low density of mIgD.

Anti-μ Treatment of BCL_1 3B3 Cells Growing in SCID Mice. To determine what roles cell death and CCA play in inducing dormancy, SCID mice were injected with 10^6 BCL_1 3B3 cells and palpated weekly for splenomegaly. When the spleen enlarged to a size consistent with the presence of 5×10^8 tumor cells, mice were injected with a single dose of 500 μg of RAμ or GAμ or their respective control antibodies. After 24 and 48 h, animals were killed, the spleens were removed, and splenocytes were analyzed by flow cytometry for cell death and CCA. Cells were also lysed and analyzed by agarose gel electrophoresis for DNA fragmentation. When GAμ antibodies (Fig. 9 *A*) were injected, 4.1% of the cells were dead at 24 h and 30.8% were dead at 48 h. Cell death after treatment with RAμ peaked at 24 h with 62.5% dead cells vs. 3.6% after RAOVA treatment (Fig. 9 *B*). By 48 h, RAμ-treated cells had only 7.9% more dead cells than the controls (14.4 vs. 6.5%). The sharp decline in the proportion of dead cells is probably related to rapid cell lysis and clearance of dead cells by the host. Agarose gel analysis for DNA laddering was performed on the aliquots of cells used for flow cytometric analysis (Fig. 9 *C*). The results demonstrate the presence of nucleosome multimer-sized fragments only in the cells removed from mice treated with GAμ after 48 h and RAμ after 24 h. Thus, a portion, if not all of the GAμ- or RAμ-induced cell death of BCL_1 3B3 cells results from signal-induced apoptosis. These experiments suggest that signal-induced cell death is an important mechanism contributing to induction of dormancy. In the mice in which apoptosis was induced by anti-μ, there appears to be CCA as well (Fig. 9, *A* and *B*). However, there is considerable variation in the proportion of cycling cells in control mice so that a much larger number of control and anti-μ–treated mice will have to be studied before a conclusion can be reached.

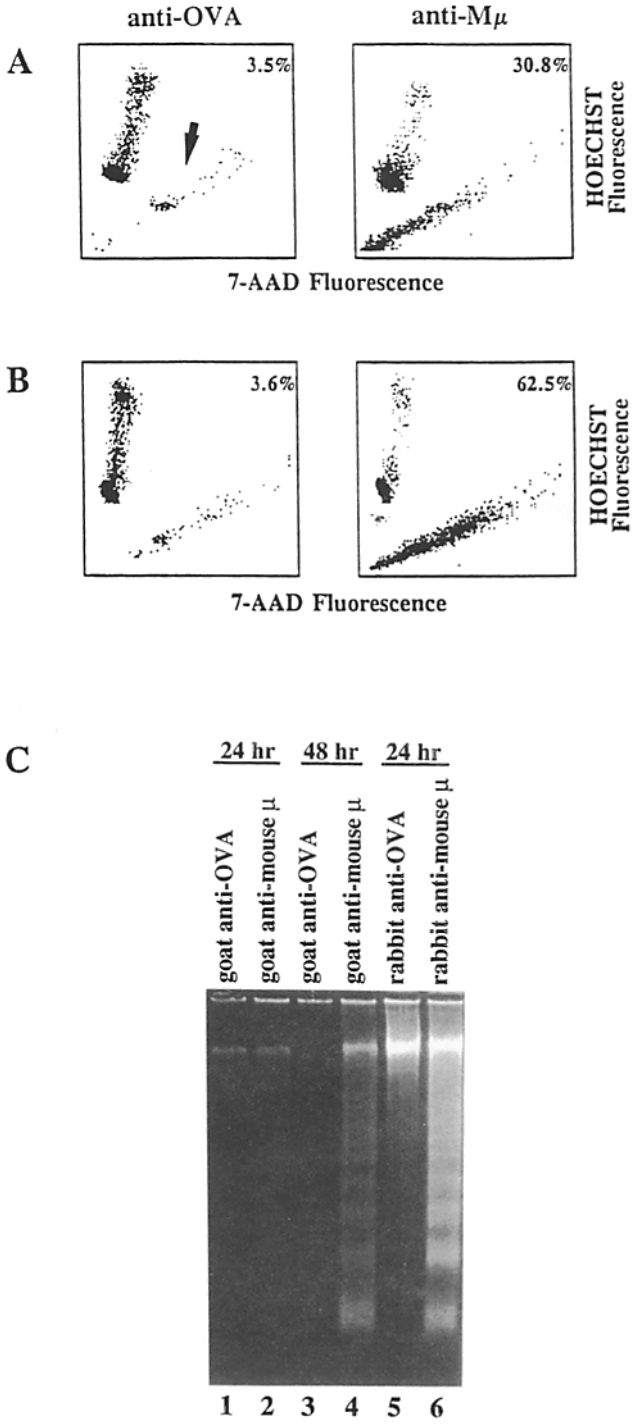


Figure 9. Flow cytometric analysis of BCL_1 3B3 cells recovered from SCID spleens after anti-μ treatment. SCID mice were injected with 10^6 tumor cells and palpated weekly. When the spleen index was 2–3 (representing ~5×10^8 tumor cells [4]), animals were grouped by spleen size and injected with 0.5 mg of either goat (*A*) or rabbit (*B*) antibodies. Due to the large volume injected (~0.5 ml), the dose was given both intravenously and intraperitoneally. Animals were killed at 24 h (*B*) or 48 h (*A*) and the splenocytes were gated on λ^+, Thy-1^- cells and analyzed for membrane integrity and DNA content. The percentage of apoptotic cells (*arrow*) is indicated for each plot. (*C*) Aliquots of the splenocytes from each animal were lysed, the DNA extracted and analyzed by gel electrophoresis as described in Materials and Methods.

Discussion

The major findings to emerge from this study are: (*a*) Antibodies directed against a variety of epitopes on mIgM induce tumor dormancy in a proportion of SCID mice chal-

lenged with the murine lymphoma, BCL_1. (*b*) Id-immune T cells by themselves are unable to induce dormancy, but they enhance the frequency and duration of dormancy when administered with anti-IgM. (*c*) The capacity of antibody to induce dormancy in vivo depends primarily on its capacity to signal rather than on conventional immune effector mechanisms. (*d*) The major effects of antibodies in vitro are to induce apoptosis and CCA. Apoptosis was also demonstrated in tumor cells removed from treated mice. (*e*) Superaggregation of IgM increased the proportion of cells undergoing apoptosis. (*f*) Occupation or coligation of the Fc receptor is not necessary for negative signaling either in vitro or in vivo.

Taken together, the in vivo and in vitro data suggest that signal transduction rather than classical immunological effector functions are the major contributors to tumor dormancy. This conclusion is based on the following observations: (*a*) There is a strict correlation between the capacity of antibodies to induce negative signaling in vitro and their ability to induce dormancy or, indeed, to have any affect on tumor growth in vivo. Thus, RtAMδ and RtAM CD44 that bind to BCL_1 3B3 cells were ineffective, whereas RAμ, RA BCL_1 Id, and RtAMλ were effective. (*b*) An interaction between the antibody and the FcγIIR is not required for negative signaling. (*c*) Virtual elimination of NK cell activity by treatment with $RAGM_1$ had no effect on the induction of dormancy. (*d*) In vivo experiments in mice with actively growing BCL_1 tumors indicated that injection of RAμ can cause massive apoptosis in vivo.

These results are consistent with a growing body of evidence indicating that antibodies against surface molecules associated with the Ig signaling complex have powerful antitumor effects and that these are due to their signaling properties. Thus, lymphoma regression induced by monoclonal anti-Id antibodies correlates with their ability to induce an increase in tyrosine phosphorylation in vitro (29). Both anti-CD19 (30) and anti-CD20 (31) inhibit the growth of human lymphoma cells in SCID mice. Of particular importance, the $F(ab')_2$ fragment of anti-CD19 is as effective as intact antibody in preventing tumor growth, proving that its antitumor activity is not due to Fc-mediated effector mechanisms (30). The ability of anti-CD19 to induce CCA in B lymphoma lines in vitro is consistent with this conclusion (11, 30). Anti-CD21, anti-CD23, and anti-CD24 can downregulate the growth of EBV-positive B cell lymphomas in SCID mice (32) and in humans (33), although the mechanisms responsible have not been explored. Anti-CD81 has antiproliferative activity in B cell lymphomas (34). Recent studies have shown that anti-CD40 can inhibit the growth of B cell lymphomas in vitro (35, 36) and display antitumor activity in SCID/lymphoma mice; the mechanisms have not been elucidated. We have obtained similar results using anti-CD5, -CD20, and -CD21 in vitro (Vitetta, E. S., and J. W. Uhr, unpublished data). It is not surprising that the therapeutic potential of the signaling function of mAbs has not been previously appreciated. Thus, mAbs selected for therapeutic use were selected on the basis of their specificity for tumor cells and their effector function (37–40). They were not screened in vitro for negative signaling which may be the major contributor to their antitumor effects.

In contrast to antibodies, Id-specific T cells by themselves were unable to induce dormancy under the conditions of our experiments. However, when Id-specific T cells were administered with anti-BCL_1 Id antibody, they synergized in the induction and maintenance of dormancy. This could be due to the secretion of cytokines or cytotoxic T cell activity. Cytokines released from T cells could increase the CCA or apoptosis mediated by anti-Ig. If CTLs are involved, then the intracellular pathway used may increase the proportion of tumor cells undergoing apoptosis since cytotoxic T cells can also induce this effect on target cells (41).

There is accumulating information on the molecular events underlying the capacity of anti-μ to induce CCA and apoptosis by cross-linking surface IgM in B lymphoma cells and in immature B lymphocytes. The present concept of B cell signaling (reviewed in 42, 43) is that cross-linking of IgM initiates the phosphorylation of the Ig α and β chains, that one or more of the *src* family kinases, Lyn, Fyn, Lck, and Blk, then bind to docking sites on the phosphorylated Igα and β chains, that this interaction activates the kinases which, in turn, leads to phosphorylation of a set of cytoplasmic proteins, possibly including another tyrosine kinase, Syk. PLCγ is activated resulting in generation of inositol triphosphate and diacylglycerol. Intracellular Ca^{++} levels are elevated and, in turn, protein kinase C is activated. A series of second messengers that are serine/threonine kinases are phosphorylated by pathways that have not been clarified. Eventually there is activation of transcription regulators such as *c-fos*, *jun B*, *erg*, and other early proteins. The result is activation and differentiation of mature B cells.

Although there are many similarities in the physiological changes associated with activation of mature B cells and negative signaling in B lymphoma cells, there are differences between the biochemical events in these two cell types. Thus, coligation of the FcγRII with membrane Ig in a normal B cell or a B cell tumor that cannot be negatively signaled results in a dominant negative effect, that is, the activation by cross-linking IgM can be overridden by coligation of FcγRII (25). This effect of FcγRII has been shown to be dependent on exogenous calcium ions (26). In contrast, in the BCL_1 3B3 cell line, neither coligation of FcγRII with IgM nor the absence of extracellular Ca^{2+} ion (data not shown) significantly affects negative signaling. Another feature that distinguishes signaling in the two cell types is the role of IgD. Cross-linking IgD on normal B lymphocytes results in activation (44). Cross-linking IgD by itself or together with IgM on BCL_1 3B3 cells does not affect signaling. Finally, as indicated above, positive signaling results in replication and differentiation, whereas negative signaling causes CCA and apoptosis. Hence, although portions of the signaling pathways in activated mature B cells and growth inhibited B lymphoma cells are probably identical, there must be differences in the pathways from the cell surface to the transcriptional response that remain to be characterized. These could depend on inherent differences between the two cell types.

There is also evidence that the signaling pathways involved in CCA and apoptosis may be partially independent. We have reported that antisense oligonucleotides targeted to the *lyn* gene prevent induction of CCA, but not apoptosis resulting from cross-linking surface IgM on murine or human lymphoma cells (11). This finding suggests that there is a bifurcation point depending on Lyn that separates the two pathways. In contrast, experiments with antisense *blk* suggests that this PTK is essential for induction of apoptosis (21).

What is the relationship between signaling lymphoma cells to undergo CCA and death and signaling their normal cellular counterparts with physiological ligands? The simplest explanation is that the stimuli and signaling pathways are identical. There is a large body of evidence to suggest that after contact with antigen, CCA and apoptosis can occur in immature B cells and in mature B cells if no accessory signals are concurrently received (18, 45). These cellular events have been implicated in the induction of anergy (45, 46) and clonal deletion of self-reactive cells (47) leading to B cell tolerance.

There are several arguments to suggest that the signaling described here may be abnormal. Thus, it is unlikely that physiological concentrations of self antigens (particularly those in low concentrations) are sufficient to occupy all the specific receptors on B cells. Many self antigens may be paucivalent or even univalent and might not be effective at extensive cross-linking even when presented on a cell surface. Also, the L and H chain variable genes of naive B cells have not yet undergone hypermutation. Hence, their sIg will have a relatively low binding affinity for self antigens. Thus, it is likely that lesser degrees of cross-linking are sufficient to negatively signal B cells. In contrast, the use of anti-Ig antibodies (particularly polyclonal ones) saturate and cause massive clustering of these receptors. There is abundant literature to suggest that hyper cross-linking can increase negative signaling and in the present studies, apoptosis (24). Thus, increasing haptenization of proteins can render them more tolerogenic (48, 49). Using fluorescence photobleaching to measure lateral diffusion of surface Ig receptors, it has been observed that there is a relationship between the increase in the fraction of mobile surface Ig receptor aggregates and the induction of B cell tolerance (50). It has recently been shown that immobilization of anti-μ or anti-δ antibodies on plastic can induce apoptosis in mature B cells (51). This is not the normal response of B cells to specific soluble antigens or, indeed, to soluble anti-IgD. We speculate, therefore, that antibody-mediated signaling of tumor cells may be different from physiological signaling, either in quantity (It is possible that during development, concentrations of physiological ligands are markedly increased in order to induce apoptosis in particular cell lineages and in that sense are physiological.) or quality. Hyperaggregation of large numbers of cell surface molecules could significantly alter the architecture of the plasma membrane or its underlying structures to such a degree that physiological signaling cascades are disrupted. This might result in apoptosis or CCA in normal or malignant cells. This does not exclude the possibility that tumor cells may be more susceptible than normal cells to such negative signaling.

Regardless of the mechanisms, these considerations have implications for clinical intervention. Thus, super cross-linking could be enhanced by the generation of multivalent antibodies using recombinant DNA technology. These could be multispecific against different epitopes on the same molecule or, indeed, on different molecules to achieve hyper cross-linking. If this approach is proved effective in inducing apoptosis, it might be useful in eliminating undesired cells, that is, autoimmune B or T cells as well as neoplastic ones.

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Address correspondence to Dr. J. W. Uhr, Department of Microbiology and Cancer Immunobiology Center, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75235.

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