Activation Mediated by RP105 but Not CD40 Makes Normal B Cells Susceptible to Anti-IgM–induced Apoptosis: A Role for Fc Receptor Coligation

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Summary

Signals through the B cell antigen receptor lead to a variety of cellular events such as activation, anergy, and apoptosis. B cells select these outcomes to establish and maintain self-tolerance, and to mount adequate antibody responses. However, it is not fully understood how one and the same signal causes such different consequences. In the present study, we have studied the effect of activation signals on the outcome of responses to antigen receptor ligation. Two distinct growth-promoting signals were used to activate B cells. Ligation of either RP105, a newly discovered B cell surface molecule, or the CD40 molecule, drove B cells to proliferate. Resultant blastic cells were then exposed to anti-immunoglobulin M (IgM). Blast cells that had been stimulated with anti-RP105 ceased growing and underwent apoptosis after cross-linking of surface IgM. Coligation of the Fcγ receptor IIB with surface IgM augmented, rather than aborted, this response. In contrast to RP105-activated B cells, blast cells that had been activated by CD40 ligation were unaltered by anti-IgM. On the other hand, CD40-activated B cells became extremely susceptible to Fas-mediated apoptosis, whereas RP105-activated B cells were much less sensitive. Anti-IgM–induced apoptosis in RP105 blasts was independent of Fas, because it was demonstrable with Fas-deficient MRL-*lpr/lpr* mice. These results demonstrate that the nature of an initial activation signal has a great influence on the fate of activated B cells after (re)engagement of the antigen receptor. RP105, as well as CD40, may be important in this life/death decision.

Signals through the B cell antigen receptor do not always lead to activation. They also result in anergy or apoptosis under certain circumstances (for a review see reference 1). Anergy and apoptosis have been implicated in establishing self-tolerance in immature B cells, whereas activation occurs in mature B cells. However, mature B cells may also have to undergo apoptosis in response to antigens during or at the terminating stage of immune responses. During affinity maturation in the germinal center, some of the newly emerged specificities may correspond to self-antigens. Also, excessive numbers of activated B cells have to be deleted to prevent an excessive response or to terminate an immune response, as is the case with T cells (for a reviews see references 2 and 3). It has been recently demonstrated that a large amount of soluble antigen, identical to that used for immunization, delivers an apoptotic signal to antigen-specific germinal center B cells when administered at the peak of the response (4–6). These models demonstrate that antigen-induced cell death occurs in mature B cells. However, it is not yet understood how these two opposite consequences, activation and apoptosis, are regulated by the same antigenic signal.

We have recently identified a cell surface molecule referred to as RP105 that is expressed on mature B cells but not on pre-B or T cells (7). The RP105 protein is monomeric, 105 kD in size, and a member of the leucine-rich repeat protein family (8). Tandem repeats of a leucine-rich motif in the extracellular portion are thought to be involved in processes such as cell adhesion or receptor–ligand interactions. An antibody against RP105 protects B cells from apoptosis induced by irradiation or dexamethasone, and drives them to proliferate (7). Therefore, the RP105 molecule transmits an activation signal that protects B cells from some types of apoptosis.

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We now demonstrate that B cell proliferation induced by RP105 ligation is dramatically affected by cross-linking of surface IgM (sIgM)[1], and is different in this respect from CD40-dependent B cell activation. CD40-activated B cells were induced to die via the Fas antigen as previously reported (9–11), but not via antigen receptor ligation. In contrast, growth of RP105-activated B cells was arrested; the cells died by apoptosis in a Fas antigen–independent manner upon exposure to anti-IgM. RP105-activated B cells also showed responsiveness to Fas ligation, but to a lesser degree than CD40-activated B cells. Thus, these two growth-promoting signals lead to different outcomes after engagement of sIgM. We also describe a positive regulatory effect of the Fcγ receptor IIB (FcγRIIB) on anti-IgM–induced growth arrest and cell death of activated B cells.

Materials and Methods

Mice. BALB/c, MRL/MpJ-+/+(MRL-+/+), and MRL/MpJ-*lpr/lpr* (MRL-*lpr/lpr*) mice were purchased from Japan SLC Co. (Hamamatsu, Japan) and used at 6–8 wk of age. All experiments were carried out according to the guidelines at Saga Medical School for the Care and Treatment of Laboratory Animals.

Abs. Rat anti–mouse mAbs against RP105 (RP/14 or RP/16) and IgM (AM/3) were established in our laboratory, and described previously (7). Hamster anti-Fas mAb (Jo2, IgG) (12) and rat anti–mouse CD40 mAb (LB429, IgG2a/κ) (13) were also previously reported. We also used another anti-CD40 mAb (HM40-3; hamster IgM) (14), that gave similar results to LB429. Rat anti–mouse CD45R/B220 antibody, RA3-6B2, was obtained from Dr. Shinya Murakami (Osaka University Medical School, Osaka, Japan). Rat anti–mouse FcγRIIB, 2.4G2 was purchased from PharMingen (San Diego, CA). Intact and $F(ab')_2$ fragments of affinity-purified rabbit anti–mouse IgM (μ-chain specific) were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). These anti-IgM Abs were able to activate freshly prepared B cells. It is known that intact rabbit anti-IgM coligates FcγRIIB and hardly activates B cells (see Table 1). Blocking the FcγRIIB with an antibody allowed the response to proceed (data not shown). As expected, $F(ab')_2$ fragments of rabbit anti-IgM activated splenic B cells in the absence of anti-FcγRIIB (see Table 1). These two anti-IgM Abs allowed us to see an effect of engagement of sIgM with or without coligation of the FcγRIIB. For subdiploid DNA analysis, we also used goat anti-IgM Abs (see Fig. 1 *A*). Intact and $F(ab')_2$ fragments were purchased from Zymed Laboratories, Inc. and Organon Teknika Co. (Durham, NC), respectively.

Enrichment of B Cells from the Spleen. B cells were enriched by a panning technique. Plastic dishes were coated with 10 μg/ml mouse anti–rat κ mAb (MAR18.5; obtained from American Type Culture Collection, Rockville, MD) in HBSS at room temperature for 2 h. After washing with HBSS, mixtures of culture supernatant containing rat mAb against mouse CD4 (GK1.5; 15) and mouse CD8 (LICR.LAU.RL172/4; 16) were added to the anti–rat κ-coated dishes and incubated for 2 h at room temperature. After washing out unbound mAbs, spleen cells were added to the dishes and incubated for 1 h at 4°C. The unbound cells were collected and used as splenic B cells.

[1] *Abbreviations used in this paper:* FcγRIIB, Fcγ receptor IIB, sIgM, surface IgM.

Cell Proliferation Assay. Splenic B cells (5×10^6 cells/well) were inoculated into a 6-well plate (Costar Corp., Cambridge, MA) and cultured with anti-RP105 (5 μg/ml) or anti-CD40 mAb (10 μg/ml) for 48 h. These two antibodies were used at optimal concentrations for inducing B cell proliferation. Cells were washed twice and cultured again in a 96-well plate (Becton Dickinson Labware, Lincoln Park, NJ) at 2×10^5 cells/well with indicated antibodies for 48 h. The cultures were pulsed with 1 μCi of [^{3}H]TdR (ICN Radiochemicals, Irvine, CA) for the final 4 h. They were then harvested onto glass fiber filters and the incorporated radioactivity was determined on a Beta plate flat-bed liquid scintillation counter (Pharmacia-Wallac, Gaithersburg, MD). The results are presented as means ± SD of triplicate wells.

Measurement of DNA Content. Splenic B cells were cultured with RP105 or CD40 mAb for 48 h as described above. Cells were harvested and viable cells were obtained by removing dead cells using density gradient centrifugation with Lympholyte-M (Cedarlane Laboratories Ltd., Ontario, Canada). Cells were then cultured again with the indicated antibodies for 24 or 48 h. Cells were harvested and cell cycle analysis was carried out with the CycleTEST™ Plus DNA reagent kit (Becton Dickinson Immunocytometry Systems, San Jose, CA). For determining percentages of cell with subdiploid DNA, harvested cells were fixed in 70% ethanol, and stained with PBS containing 50 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) and 10 μg/ml RNase (Nippon Gene, Toyama, Japan). Cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

Immunofluorescence Analysis. Cells in suspension (5×10^5 cells/200 μl) were incubated for 20 min on ice with anti-Fas. After two washes, FITC-labeled protein A (Zymed Laboratories, Inc.) was used as the second reagent. Propidium iodide was included during this incubation period and used as a gating parameter to exclude dead cells. Labeled cells were then analyzed on a FACScan® (Becton Dickinson & Co.).

Wright Giemsa Staining. Harvested cells were cytocentrifuged for 4 min at 400 rpm on slides (Shandon, Sewickley, PA). After

Table 1. *Inhibition of RP105-dependent B Cell Growth by Simultaneous Addition of Anti-IgM Abs*

Ab added	[^{3}H]TdR uptake	
	Without anti-RP105	With anti-RP105
	cpm	
None	281 ± 31	68,956 ± 528
Control Ab	636 ± 86	62,137 ± 3,360
Monoclonal anti-IgM	282 ± 12	2,785 ± 338
Intact anti-IgM	206 ± 34	5,130 ± 115
F(ab′) anti-IgM	3,470 ± 163	33,624 ± 200
Anti-Fas	647 ± 49	62,847 ± 1,083

Splenic B cells were cultured with the indicated antibodies for 2 d. Anti-CD45R/B220 was used as a control Ab. Rabbit antibodies were used as intact and $F(ab')_2$ antibodies. All the antibodies were used at a concentration of 10 μg/ml to achieve maximal effect (see Fig. 1). The same amount of antibodies was used in the following experiments except Fig. 1. [^{3}H]TdR was added during the last 4 h of the culture. The results are represented as mean ± SD for triplicate cultures.

being air-dried, slides were stained with Wright's solution (Muto Pure Chemicals, Ltd., Tokyo, Japan) for 4 min, washed in water, and stained again with 10% Giemsa's solution (Muto Pure Chemicals, Ltd.) diluted in PBS for 15 min.

Results

RP105-activated B Cells Are Sensitive to Anti-IgM–induced Growth Inhibition. Engagement of RP105 alone induces potent growth of mature B cells (7). Interestingly, simultaneous cross-linking of surface IgM drastically suppressed this RP105-dependent proliferation (Table 1). It was important to use intact anti-IgM Ab, and $F(ab')_2$ fragments were not as effective. Also, the inhibitory effect was less marked when B cells were first exposed to anti-IgM followed 1 d later by treatment with anti-RP105 Ab (data not shown). In contrast, blast cells generated by ligation of RP105 for 2 d were extremely sensitive to the antiproliferative effects of antigen receptor cross-linking (Table 2). Thus, the first signal markedly affected a responsiveness to a subsequent signal. The balance of our study was focused on determining the influence of activation signals on antigen receptor signaling. Therefore, we studied the fate of B cells that had been activated by ligation of either RP105 or CD40 and subsequently exposed to anti-IgM.

Both of these activation signals effectively induced B cell blasts and the activated cells were similar with respect to the expression of a number of cell surface markers (CD45R/B220, IgM, IgD, I-A, I-E, CD23, CD24, CD40, and RP105; data not shown). The resulting blasts were collected, washed, and cultured again with an antibody to either IgM or Fas. Incorporation of $[^3H]TdR$ was then assessed 2 d later. As was reported (9–11), B cells that were responding to the anti-CD40 Ab (CD40 blasts) were extremely sensitive to anti-Fas (Table 2). Splenic B cells that had been activated with anti-RP105 (RP105 blasts) were much less so. The inhibitory effect was obvious at a concentration of 0.01 μg/ml and during 1–5 d after the addition of the antibody (Figs. 1 and 2). In striking contrast, RP105 blasts were sensitive to anti-IgM (Table 2). The inhibition was dose dependent and most apparent between 1 and 3 d after the beginning of the culture (Figs. 1 and 2). The inhibition of proliferation was virtually complete with intact Ab but less so with $F(ab')_2$ Ab. The growth of CD40 blasts was not inhibited at all by either form of anti-IgM. Thus, the antigen receptor on activated B cells transmitted negative signals when the initial activation signal was delivered through RP105 but not CD40.

Table 2. *Sensitivity of B Cell Blasts to Receptor Ligation Depends on the Means of Activation*

Ab	$[^3H]TdR$ uptake	
	RP105 blasts	CD40 blasts
	cpm	
None	45,993 ± 4,056	43,651 ± 5,826
Control Ab	48,588 ± 1,463	50,971 ± 3,794
Intact anti-IgM	2,758 ± 581	56,845 ± 4,434
$F(ab')_2$ anti-IgM	28,512 ± 7,801	57,703 ± 2,695
Anti-Fas	15,673 ± 1,439	1,433 ± 136

Splenic B cells were cultured with either anti-RP105 (RP105 blasts) or anti-CD40 mAb (CD40 blasts). After 2 d of culture, cells were harvested, washed, and cultured again with the indicated antibodies for 2 d. The numbers of recovered cells were 1.6×10^6 (RP105 blasts: 89% recovery) and 6.5×10^5 (CD40 blasts: 36.1% recovery) out of 1.8×10^6 input cells. Anti-CD45R/B220 was used as a control Ab. Rabbit antibodies were used as intact and F(ab')2 antibodies. $[^3H]TdR$ was added during the last 4 h of the culture. The results are presented as means ± SD for triplicate cultures.

Engagement of sIgM Leads to Cell Cycle Arrest of RP105 Blasts and Coligation of the Fc Receptor Augments It. The inhibitory effect of anti-IgM on RP blasts was further studied. The reduction of $[^3H]TdR$ uptake could result from either growth arrest or apoptosis. Cell cycle status was assessed at 24 h or 48 h after ligation of the antigen receptor on RP105 or CD40 blasts. At 24 h, neither form of anti-IgM arrested cycling of CD40 blasts and similar results were obtained at 48 h (Table 3, and data not shown). In fact, the number of the S phase increased in many experiments with anti-IgM $F(ab')_2$ Ab. Such an increase was not clearly observed with the intact anti-IgM. Coligation of the FcγRIIB is likely to abort a signal through sIgM. In sharp contrast to CD40 blasts, cell cycling of RP105 blasts was arrested with either form of anti-IgMs. This time, intact Ab was more effective than $F(ab')_2$ Ab. As expected, cell cycle arrest by intact anti-IgM became less pronounced in the presence of anti-FcγRIIB (data not shown). Anti-Fas had no significant effect on either CD40 or RP105 blasts. We conclude from these results that engagement of sIgM arrests cycling of RP105 blasts but not CD40 blasts, and concurrent ligation of the FcγRIIB augments the cell cycle arrest.

B Cells Activated by Anti-RP105 Ab Undergo Apoptosis in Response to Anti-IgM. We observed few viable and many dead cells in RP105 blasts cultured with anti-IgM Ab for 24 h as compared with those cultured with control Ab or without Ab. This difference was more marked when the culture proceeded for another 24 h (data not shown). We further investigated the fate of RP105 blasts after sIgM ligation, and found evidence for apoptosis (Fig. 3). Percentages of cells with subdiploid DNA increased dramatically when RP105 blasts were exposed to monoclonal or intact anti-IgM. Additional evidence for apoptosis was obtained by an increase in orthogonal light scatter by flow cytometry, DNA fragmentation assessed from gel electrophoresis, and apoptotic figure with nuclear condensation revealed by Giemsa staining (Fig. 4). Inclusion of anti-FcγRIIB partially reduced apoptosis induction by intact fragments (data not shown). There was a smaller but consistent increase in apoptotic cells after addition of anti-IgM $F(ab')_2$ as compared with the intact Ab (Fig. 3). These results contrast with those obtained from CD40 blasts that were completely resistant to both forms of anti-IgM Abs. With regard to Fas-mediated apoptosis, although CD40 blasts were much more sensitive, RP105 blasts showed less but consis-

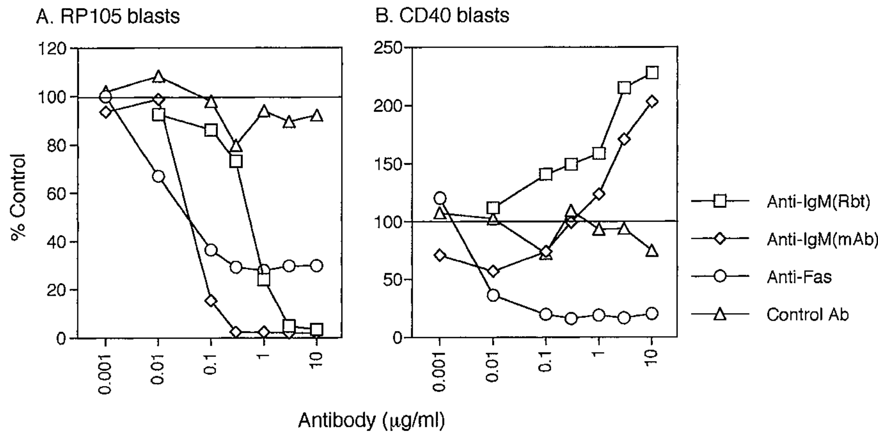

Figure 1. Inhibition of RP105 and/or CD40 blasts with an antibody to surface IgM or Fas (dose dependence). RP105 and CD40 blasts were prepared as in Table 2, and cultured with indicated antibodies at varying concentrations. Anti-CD44 mAb was used as a control mAb (△). Two anti-IgM antibodies were used, a monoclonal anti-IgM (AM/3; ◇) and the intact fragment of rabbit anti-IgM (□). Jo2 was used as an anti-Fas Ab (○). [^{3}H]TdR was added during the last 4 h of the culture. Each plot represents the mean of triplicate cultures and is shown as a percentage of the control response that did not include an antibody. Rabbit anti-IgM F(ab')$_2$ reached the maximal inhibition at 3–5 μg/ml, as did the intact antibody (data not shown).

tent responsiveness (Fig. 3 *A*). We conclude that RP105 but not CD40 blasts undergo apoptosis when surface antigen receptors are engaged, and coligation of the FcγRIIB enhances induction of apoptosis.

Fas Is Not Required for Receptor-mediated Apoptosis of RP105-stimulated B Cell Blasts. Previous studies of T cell blasts demonstrated an essential role of the Fas antigen in TCR-mediated apoptosis (17–19). We found that RP105 blasts differ from CD40 blasts in the amount of the Fas antigen expression (Fig. 5). RP105 blasts were similar to freshly isolated B cells in this respect. However, this was sufficient for some Fas-mediated cell death (Fig. 3). Therefore we employed Fas-deficient MRL-*lpr/lpr* mice (20) to ask whether sIgM–triggered cell death required Fas. In response to engagement of RP105 or CD40, B cells from MRL-*lpr/lpr* mice showed comparable proliferation to wild-type mice (data not shown). The Fas antigen was not detected on either blast cells or normal spleen cells of homozygous defective mice (data not shown). RP105 and CD40 blasts from MRL-*lpr/lpr* mice were then exposed to anti-IgM or anti-Fas, and DNA content of blast cells was examined (Table 4). As expected, monoclonal anti-Fas had no effect on either blasts. Nevertheless, RP105 blasts underwent apoptosis in response to anti-IgM Abs. From these results, we conclude that an anti-IgM–induced B cell apoptosis is not Fas mediated.

Discussion

One of the most important questions about B cell receptor signaling is how the same stimulus leads to quite different consequences that include activation, growth arrest, and cell death. It has been demonstrated that, whereas immature B cells easily undergo apoptosis after engagement of sIgM, mature B cells do not (21). We confirmed the latter finding by examining the DNA content of splenic mature B cells that had been cultured for 24 h with anti-IgM Abs (Yamashita, Y., K. Miyake, and M. Kimoto, unpublished observations). Mature resting B cells seem to have no alternative but activation in response to sIgM ligation. After activation by CD40 engagement, B cells remained resistant to anti-IgM–induced apoptosis (Fig. 3), and cell cycling was accelerated by anti-IgM F(ab')$_2$ (Table 3). In sharp contrast, RP105-mediated activation opened a pathway to anti-IgM–induced growth arrest and apoptosis (Table 3 and Fig. 3). Thus, mature B cells still retain a choice to arrest their growth and die in response to sIgM engagement, and activation signals have a great influence on this type of life/death decision.

Because normal heterogeneous B cells were used in the present study, it is possible that RP105 ligation selectively activated a very minor population of splenic B cells that were susceptible to anti-IgM–induced death before activation. However, most mature B cells express RP105, and al-

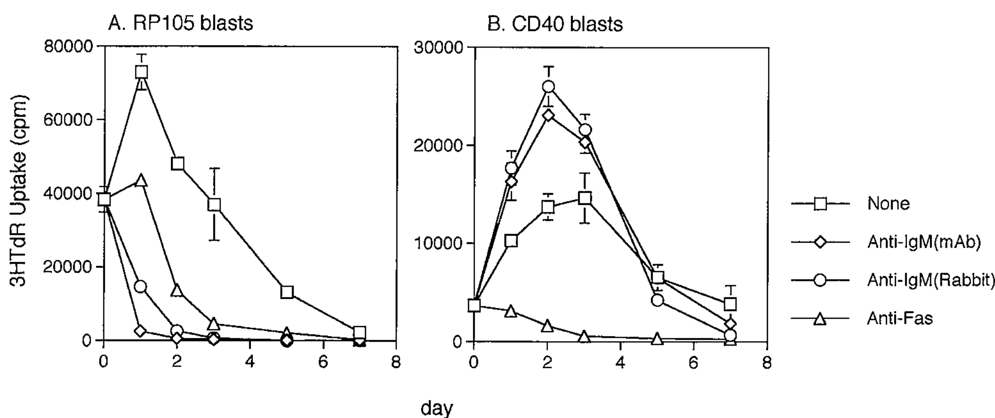

Figure 2. Inhibition of RP105 and/or CD40 blasts with an antibody to sIgM or Fas (time course). RP105 and CD40 blasts were prepared as in Table 2, and cultured with indicated antibodies for indicated periods. [^{3}H]TdR was added during the last 4 h of the culture. Antibodies were similar to Fig. 1, and used at 10 μg/ml. The results were presented as means ± SD for triplicate cultures. The results with rabbit anti-IgM F(ab')$_2$ were similar to those with the intact antibody with respect to time course (data not shown).

Table 3. *Cell Cycle Arrest of RP105 Blasts but Not CD40 Blasts by Anti-IgM and Augmentation by Fc Receptor Coligation*

Cells	Ab	Cells in Cell Cycle Phase		
		G_0/G_1	S	G_2/M
			%	
RP105 blasts	None	73.9	19.2	6.9
	Control Ab	74.1	17.9	8.1
	Intact anti-IgM	94.3	3.0	2.7
	F(ab')$_2$ anti-IgM	84.6	9.7	5.7
	Anti-Fas	69.7	23.7	6.6
CD40 blasts	None	89.6	6.3	4.1
	Control Ab	86.6	6.1	7.4
	Intact anti-IgM	89.0	7.9	3.2
	F(ab')$_2$ anti-IgM	74.9	22.4	2.7
	Anti-Fas	92.0	5.5	2.5

RP105 or CD40 blast cells were incubated with the indicated antibodies for 24 h. Anti-CD44 Ab was used as a control Ab. Rabbit antibodies were used as intact and F(ab')$_2$ antibodies. DNA was stained with propidium iodide and analyzed as described in Materials and Methods. Percentages of each cell cycle phase are shown. One representative result is presented from three independent experiments.

most all RP105-positive B cells become blastic when cultured with anti-RP105 Ab (7, 8). As few as 5 × 10^3 B cells still showed a significant response to RP105 ligation (~30% of [^{3}H]TdR uptake by 2 × 10^5 B cells: Yamashita, Y., K. Miyake, and M. Kimoto, unpublished observation). The number of recovered RP105 blasts after 2-d culture was even larger than that of CD40 blasts (see the legend to Table 2). Moreover, we compared RP105- with CD40-activated B cells in terms of cell surface markers such as CD23, CD24, IgD, and IgM, and found no differences. Thus, RP105 ligation results in activation/proliferation of a majority of, not a minority of B cells. It is more likely that RP105-dependent activation has an effect on the relationship between antigen receptor signaling and final cellular outcomes. Engagement of the B cell antigen receptor initiates a cascade of biochemical events including protein tyrosine kinase activation, phosphatidylinositol hydrolysis, and calcium mobilization (for review see references 22 and 23). Bifurcations are anticipated in these signaling events to lead to different cellular responses. For example, Lyn tyrosine kinase is differentially utilized in anti-IgM–induced cell cycle arrest and death. It is defined as a critical component for the former (24), but dispensable for the latter (25). In this regard, it is interesting to study early signaling events after sIgM ligation in resting, CD40- and RP105-activated B cells.

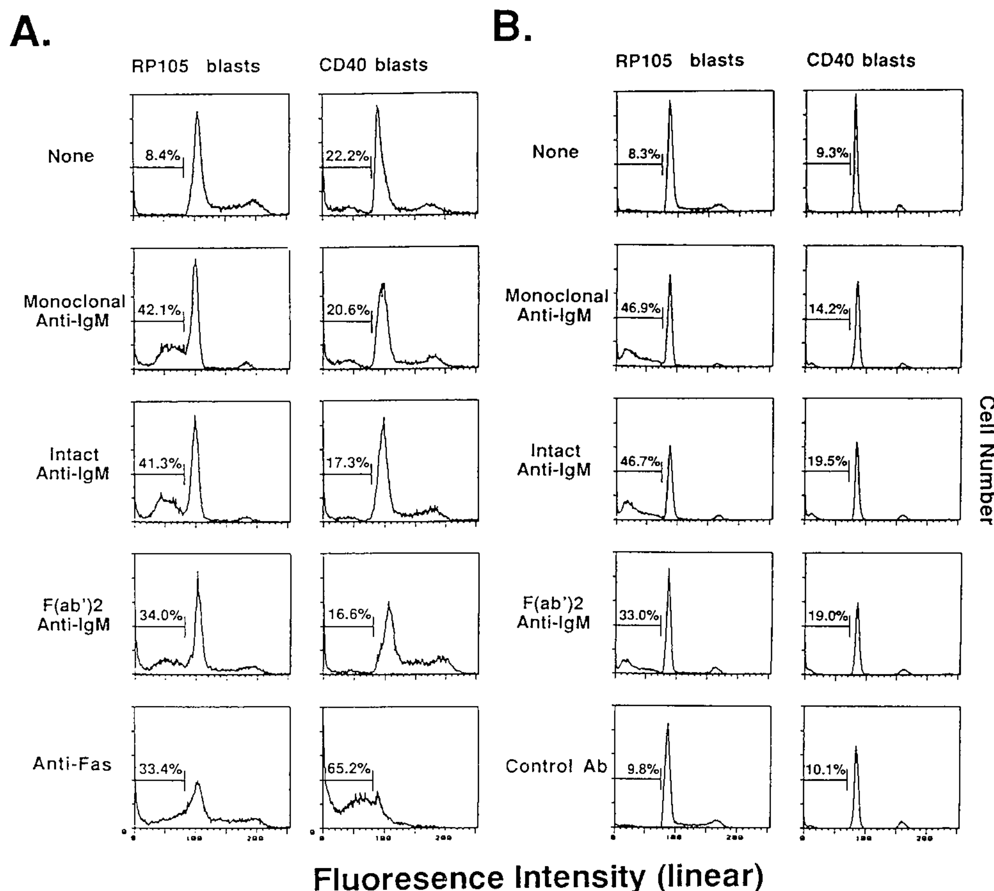

Figure 3. Anti-IgM induces apoptosis in RP105 but not in CD40 blasts. RP105 and CD40 blasts were prepared as in Table 1, and cultured with the indicated antibodies for 24 h. Goat (*A*) or rabbit (*B*) antibodies were used as intact and F(ab')$_2$ anti-IgM. Cells were then fixed and DNA was stained with propidium iodide. Percentages of subdiploid DNA are shown in each panel.

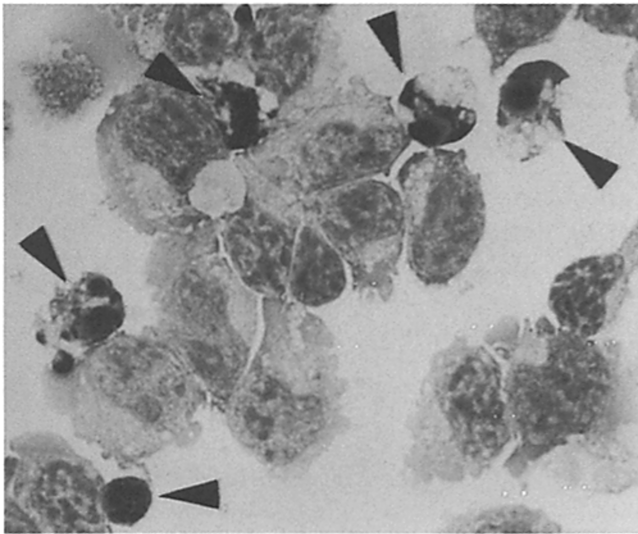

Figure 4. RP105 blast cells show morphological features of apoptosis in response to anti-IgM. Wright Giemsa staining of RP105 blast cells that had been exposed to anti-IgM mAb for 12 h. (▶) Apoptotic cells.

The present study demonstrates that anti-IgM–induced apoptosis of activated B cells does not utilize the Fas molecule. RP105 as well as CD40 blasts are able to receive a death signal via the Fas antigen (Fig. 3). However, antigen receptor signaling probably does not result in Fas ligand expression. Our finding is consistent with a recent study by Han et al. (6) which showed that C57BL/6.*lpr* mice remain fully susceptible to antigen-driven B cell apoptosis in germinal centers. Moreover, Rathmell et al. (26) demonstrated that elimination of B cells recognizing membrane-bound autoantigen occurs normally despite the absence of the Fas antigen. Thus, Fas-mediated apoptosis is dispensable for antigen-induced B cell death and some other category of molecules may be involved. T cells use an autocrine TNF/TNF receptor system as well as the Fas-mediated apoptosis for executing antigen-induced cell death (27, 28). It is possible that antigen receptor signaling leads to activation of a comparable system in certain B cells.

There are a number of functional similarities between CD40 and the more recently described RP105 antigen, although the two proteins are structurally unrelated. Whereas CD40 is a member of the TNF/nerve growth factor receptor family (for a review see reference 29), RP105 is related to proteins with tandem repeats of a leucine-rich motif (8). Ligation of either molecule with mAb leads to protection against irradiation-induced apoptosis, massive proliferation, but little or no Ig secretion (7, 29, and 30). Also, B cells from immunodeficient *xid* mice fail to respond to either antibody (7, 31). The majority of normal spleen cells that expresses RP105 also displays CD40 and blast cells prepared by stimulation with either antibody express both antigens (Yamashita, Y., K. Miyake, and M. Kimoto, unpublished observations). Further study is needed to determine if the same subsets of B cells are responsive to stimulation via either molecule. However, we now demonstrate that populations of actively dividing B lymphocytes are distinct, depending on which of these molecules provided the activation stimulus. The engagement of the antigen receptor induces acceleration of cell cycle in those arising from ligation of CD40, whereas growth arrest and cell death in B cell blasts results from RP105 ligation.

The importance of CD40 ligation in immune responses is demonstrated by CD40-deficient mice (32). These mice show impaired Ig class switching and germinal center formation. Proliferating cells in that site are thought to be selected for survival/proliferation if they display high affinity antigen receptors, and simultaneously receive signals via other surface molecules such as CD40 (33). Less is known about RP105, but it is possible that it could also contribute to cell survival/proliferation in germinal centers. The results reported here indicate that cells rescued only on the basis of this molecule may be destined to die when their antigen receptors are utilized. Recent studies (4–6) demonstrated that some B cells in germinal centers are poised to die in response to antigen. Germinal centers are sites for antigen-induced deletion of B cells as well as for survival/proliferation. It is difficult to imagine how CD40 alone governs these quite different processes. Indeed, a blocking antibody specific for the CD40 ligand did not abrogate antigen-driven B cell death in germinal centers (6). Galibert et al. (34) showed that dual triggering of CD40 and sIg induces B cells to express a germinal center phenotype, including high susceptibility to spontaneous apoptosis that becomes apparent after 6-d stimulation. In our culture system, it was difficult to study blast cells after 6-d culture, be-

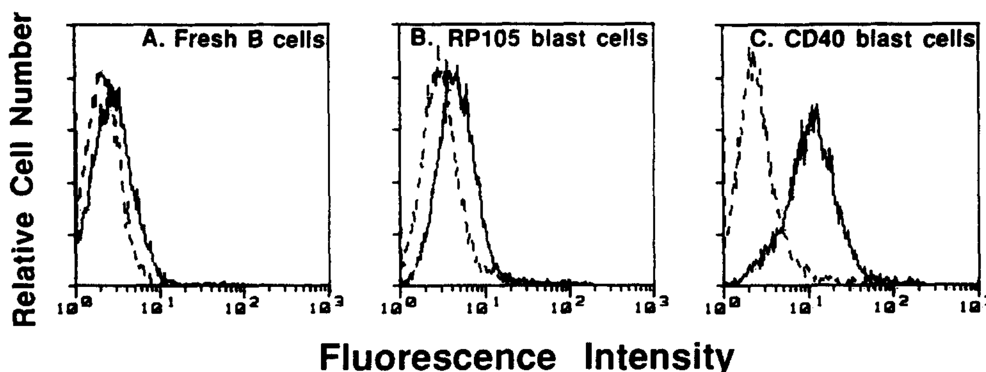

Figure 5. The expression of Fas antigen increases on CD40 but not on RP105 blasts. Purified B cells from spleen, RP105 blasts, and CD40 blasts were stained with Jo2 (anti-Fas mAb) followed by protein A-FITC.

Table 4. *Fas Antigen Is Not Required for B Cell Death by Antigen Receptor Ligation*

Exp.	Ab	Apoptotic cells	
		RP105 blasts	CD40 blasts
		%	
1	None	16.3	23.9
	Monoclonal anti-IgM	43.1	23.6
	Anti-Fas	15.8	27.8
2	None	23.8	19.5
	Control Ab	31.4	28.7
	Monoclonal anti-IgM	48.0	22.9
	Intact anti-IgM	43.9	23.8
	Anti-Fas	22.4	24.8

RP105 or CD40 blast cells from MRL-*lpr/lpr* mice were incubated with the indicated antibodies for 24 h. Anti-CD45/B220 was used as a control Ab. Rabbit antibodies were used as intact anti-IgM Ab. The content of DNA was determined by staining with propidium iodide and analyzed on a FACScan®. Percentages of cells with subdiploid DNA are shown.

cause only a small number of cells remained to grow (Fig. 2). Galibert et al. (34) used a fibroblast that expresses the human Fc receptor and CD40 ligand. It is worth trying to stimulate B cells with anti-RP105 immobilized on a fibroblast for a longer period, and to see their susceptibilities to sIgM-induced apoptosis.

The FcγRIIB has been shown to abort signaling through the B cell antigen receptor when it is engaged together with sIgM (35). Indeed, $F(ab')_2$ fragments, but not intact anti-IgM, were capable of activating splenic B cells (Table 1) or influencing the cell cycle of CD40 blasts (Table 3). Therefore, it was expected that coligation of the FcγRIIB might prevent anti-IgM–induced growth arrest and apoptosis of RP105 blasts. However, this was not the case, and intact anti-IgM was required for optimal inhibition. Anti-FcγRIIB itself did not induce cell cycle arrest or cell death, and did not augment the effects of anti-IgM (Yamashita, Y., K. Miyake, and M. Kimoto, unpublished observations). Thus, responses did not result from independent signaling through the FcγRIIB but from coligation with sIgM. A tyrosine phosphatase, PTP1C/HCP/SH-PTP1, has been recently implicated in negative regulation of antigen receptor signaling by FcγRIIB (36, 37). Tyrosine phosphorylation of FcγRIIB recruits PTP1C/HCP/SH-PTP1 to the antigen receptor complex, and allows for dephosphorylaton of appropriate substrates. Similarly, PTP1C/HCP/SH-PTP1 or other signaling molecules may be recruited and modulate a sIgM-mediated signal that results in growth arrest and apoptosis.

T cells use antigen-induced apoptosis for deletion of excessive T cells that have been previously activated (For reviews see references 2 and 3). Similar cell death must occur in antibody responses, and the anti-IgM–induced cell death described here may represent such a mechanism. In this regard, FcγRIIB coligation has been proposed as a mechanism for immune complex–dependent feedback inhibition of antibody responses (38). However, previous investigators have studied only resting, and not actively proliferating B cells. We have now demonstrated a circumstance where immune complexes can effectively induce growth arrest and deletion of activated B cells.

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