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-Ipr/Ipr mice. These results demonstrate that the nature of an initial activation signal has a great influence on the fate of activated B cells after engagement of the antigen receptor. RP105, as well as CD40, may be important in this life/death decision.
We now demonstrate that B cell proliferation induced by RP105 ligation is dramatically affected by cross-linking of surface IgM (slgM)\(^1\), 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 slgM. 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, IgG1) (12) and rat anti-mouse CD40 mAb (LB429, IgG2a) (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. Shin'ya 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 (\(\mu\)-chain specific) were purchased from Zymed Laboratories, Inc. (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 slgM 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 \(\mu\)g/ml mouse anti-rat \(\kappa\) mAb (MAR.18.5; 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-\(\kappa\) 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.

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

| Ab added                  | Without anti-RP105 | With anti-RP105 |
|---------------------------|--------------------|-----------------|
| 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')\(_2\) anti-IgM     | 3,470 ± 163        | 33,624 ± 200    |
| Anti-Fas                  | 647 ± 49           | 62,847 ± 1,083  |

^{1} Abbreviations used in this paper: FcγRIIB, Fcγ receptor IIB, slgM, surface IgM.
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')
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')
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.

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')
Ab. Such an increase was not clearly observed with the intact anti-Ig-M. 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')
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 slgM 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')
Ab, 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-
tent responsiveness (Fig. 3A). 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 slgM-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 slgM, 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 slgM 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 slgM 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-
Table 3. Cell Cycle Arrest of RP105 Blasts but Not CD40 Blasts by Anti-IgM and Augmentation by Fc Receptor Coligation

| Cells          | Ab                  | G0/G1 | S       | G2/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  |
|               | 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  |

Most all RP105-positive B cells become blastic when cultured with anti-RP105 Ab (7, 8). As few as $5 \times 10^5$ B cells still showed a significant response to RP105 ligation (~30% of [H]Tdr uptake by $2 \times 10^5$ 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 slgM 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.
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 (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
Table 4.  Fas Antigen Is Not Required for B Cell Death by Antigen Receptor Ligation

| Exp. | Ab                  | 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 slgM-induced apoptosis.

The FcyRIIB has been shown to abort signaling through the B cell antigen receptor when it is engaged together with slgM (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 FcyRIIB 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-FcyRIIB 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 FcyRIIB but from coligation with slgM. A tyrosine phosphatase, PTP1C/HCP/SH-PTP1, has been recently implicated in negative regulation of antigen receptor signaling by FcyRIIB (36, 37). Tyrosine phosphorylation of FcyRIIB recruits PTP1C/HCP/SH-PTP1 to the antigen receptor complex, and allows for dephosphorylation of appropriate substrates. Similarly, PTP1C/HCP/SH-PTP1 or other signaling molecules may be recruited and modulate a slgM-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, FcyRIIB 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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