Murine B Cell Proliferation and Protection from Apoptosis with an Antibody against a 105-kD Molecule: Unresponsiveness of X-linked Immunodeficient B Cells

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Summary

We established a novel monoclonal antibody, RP/14, that can protect B cells from apoptosis induced by irradiation or dexamethasone. A molecule recognized by RP/14 (the RP antigen) was expressed on B cells with $B220^{bright}$, IgM^{dull}, and IgD^{bright}. Immunoprecipitation experiments revealed that RP/14 recognized a monomeric protein with an approximate molecular mass of 105 kD. Stimulation of B cells with RP/14 for 48 h induced B cell proliferation and blastogenesis. In contrast to B cells of wild-type mice, X-linked immunodeficient (XID) B cells did not proliferate upon stimulation with RP/14, although the RP antigen was expressed to the same extent as that of wild-type B cells. These results suggest that the RP antigen-mediated signaling pathway is important for rescuing B cells from apoptosis and is deficient in XID B cells.

B cells have to get through several selection steps during differentiation from an immature to a mature stage B cell, or into an antibody-secreting cell (reviewed in 1 and 2). Only B cells that have experienced selections are allowed to join a mature B cell pool and to differentiate into antibody-secreting cells with appropriate stimuli. These selections would enable mice to maintain a stable B cell pool without autoreactive B cells particularly against cell membrane-anchored self-antigens (2–4) and to produce efficiently antibodies with adequate specificity and affinity (1, 5, 6). An execution of these selections would involve apoptosis, survival, or proliferation of B cells. Analysis of double transgenic mice, reported by Goodnow et al. (3 and reviewed in 4), revealed that apoptosis is critically involved in a negative selection that trims off B cells bearing aberrant specificity against self-antigens. Autoantigen itself or in concert with other signals would trigger apoptosis in these hazardous cells. A positive selection requires a condition where only cells that receive a signal are able to survive or grow. Otherwise, cells undergo apoptosis. In the germinal center, only cells bearing a surface immunoglobulin with sufficient affinity against an antigen are allowed to survive and grow (5). In this situation, stimulatory signals such as antigen itself or the CD40 ligand are shown to protect cells from apoptosis and induce survival or proliferation (5, 6). In either selection, apoptosis seems to play an essential role, and a decision whether cells undergo apoptosis or are allowed to survive or grow would be the most critical part of the selections. Accordingly a signal that is capable of inducing or blocking apoptosis can be implicated in the pivotal systems for selecting B cells. An identification of a molecule mediating such a signal profoundly contributes to the understanding of the systems underlying B cell selections during their maturation.

Lymphocytes are one of the most sensitive cells to immediate radiation-induced damage (7). In contrast to other cell types, quiescent lymphocytes are more sensitive than actively cycling cells, (7, 8), and the damaged cells are thought to undergo apoptosis. This process does not involve cell division and is referred to as interphase death. It is conceivable that some lymphocyte-specific signaling systems underlie interphase death in irradiation-induced damage, and that these lymphoid cell–specific signaling systems also work at a time when B cells undergo apoptosis during B cell selection. These assumptions prompted us to search for a mAb with radioprotective activity.

In the present study, we established mAb RP/14, which was able to protect B cells from irradiation-dependent apoptosis. RP/14 mAb was able to drive B cells in culture into cycling as well. The antigen recognized with RP/14 (the RP antigen) was expressed on mature B cells but not on either immature or pre-B cells. Moreover, B cells from X-linked immunodeficient mice (XID)[1] (9) did not respond to RP/14

[1] *Abbreviations used in this paper:* BTK, Bruton's tyrosine kinase; XID, X-linked immunodeficient.

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for proliferation in spite of comparable expression of the RP antigen with B cells of wild type. The RP antigen was monomeric and 105 kD in size. These results suggest that the RP antigen delivers signals into B cells for blocking apoptosis and proliferation. Moreover, the signal transduction through the RP antigen was shown to be defective in B cells with XID.

Materials and Methods

Mice and Rats. BALB/c and C57BL/6 mice were obtained from Japan SLC Co. (Hamamatsu, Japan), and used at 8–15 wk of age. CB17 *scid/scid* mice were from Japan Clea Laboratories Inc. (Tokyo, Japan). Wistar rats were purchased from Charles River Japan Inc. (Yokohama, Japan). All mice and rats were maintained in the Animal Facility of Saga Medical School. BALB/c.*xid* and C57BL/6.*xid* mice (10, 11) were kindly provided by Dr. Alfred Singer (National Institutes of Health, Bethesda, MD) through Dr. Toshiyuki Hamaoka (Osaka University Medical School) and maintained in the Animal Facility of the Institute of Medical Science (the University of Tokyo). All experiments were performed according to guidelines in Saga Medical School and in the Institute of Medical Science for the care and treatment of Animal Experimentation.

mAbs. Wistar rats were immunized with spleen cells from BALB/c mice. 3 d after the third immunization, spleen cells of immunized rats were fused with SP2/0 myeloma cells (obtained from the American Type Culture Collection, Rockville, MD). The strategy for screening of mAbs is described in Results section. The established series (RP) of antibodies were all IgG2a/κ. The antibody against B220, RA3-6B2 (12) was provided by Dr. Shinya Murakami (Osaka University Dental School, Osaka, Japan). The rat antibodies against mouse IgM (AM/3; IgG2a/κ) and IgD (CS/15: IgG2a/κ) were established in our laboratory. These antibodies were purified from ascitic fluids from CB17 *scid/scid* mice that had been transplanted with hybridomas.

Protection from Cell Death by Irradiation, or Dexamethasone. For experiments of irradiation-induced apoptosis, spleen cells were placed in a 96-well plate (2×10^5/well), and precultured with antibodies (10 μg/ml) for 2 h. Then, cells were irradiated (100 or 200 rad) and cultured for an additional 18 h. As for dexamethasone-induced apoptosis, varying concentrations of dexamethasone (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added to cultures simultaneously with antibodies, and cells were cultured for 20 h. In these experiments, viable cells were determined after culture by a trypan blue dye exclusion test. Percentages of viable cells were determined by the formula: percent viable cells = 100 × (viable cell number after culture/input cell number). The results were represented as mean ± SD for triplicate cultures.

For measuring DNA contents, cells were harvested and fixed with 70% ethanol. Fixed cells were stained with PBS containing 50 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) and 10 μg/ml RNase (Nippon Gene, Toyama, Japan). After washing twice with PBS supplemented with 2% FCS and 0.1% sodium azide, cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

Cell Proliferation Assay. Spleen cells or T cell–depleted spleen cells were inoculated into a 96-well plate at 2×10^5/well (10) and cultured with or without mAb (10 μg/ml) for 2–3 d. The cultures were pulsed with 1 μCi/well of [^{3}H]TdR (ICN Radiochemicals, Irvine, CA) for the last 6 h of the culture. They were then harvested onto glass fiber filters and the incorporated radioactivity was determined in a liquid scintillation counter. The results were presented as the average cpm ± SD from triplicate wells.

Immunofluorescence Analysis. Cells in suspension (5×10^5 cells/200 μl) were incubated for 20 min on ice with mAbs. After two washes, FITC-labeled mouse anti–rat κ (MAR18.5) mAb was added for an additional 20-min incubation. Propidium iodide was included during this incubation period and used as a gating parameter to exclude dead cells. For dual staining, cells were incubated with a biotinylated antibody, and after washing twice, PE-labeled avidin (Vector Laboratories Inc., Burlingame, CA) and a FITC-labeled antibody were added and incubated for 20 min on ice. In this case, propidium iodide was not included. Labeled cells were then analyzed using a FACScan® (Becton Dickinson & Co.).

Cell Surface Biotinylation and Immunoprecipitation. Cell surface biotinylation and immunoprecipitation were carried out as described previously (13). Briefly, cells (5×10^7/ml) were washed twice with HBSS, and suspended in saline containing 100 mM Hepes (pH 8.0). Sulfosuccinimidobiotin (Pierce Chemical Co., Rockford, IL) was added to cell suspension at a concentration of 0.5 mg/ml. After a 40-min incubation at room temperature with occasional shaking, cells were washed and lysed in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 50 mM iodoacetamide, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.1% sodium azide. After a 30-min incubation on ice and following centrifugation, the cell lysates were recovered and incubated with antibody-coupled Sepharose 4B for 2 h at 4°C. After washing with lysis buffer, bound proteins were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and visualized with avidin-peroxidase (Bio-Rad Laboratories, Richmond, CA) followed by an appropriate substrate.

Results

Radioprotective Activity of mAbs. We immunized rats with spleen cells from BALB/c mice, and fused with SP2/0 myeloma cells. In an attempt to search for a molecule that modulates apoptosis, mAbs were screened by an assay system for protective effects from apoptosis caused by low dose irradiation. Mouse spleen cells were cultured with supernatants from resultant hybridoma cells for 2–4 h at 37°C. Spleen cells were then irradiated at 100–200 rad, cultured for another 18 h, and viable cells were counted. The supernatants with which more cells remained viable were selected and subjected to a subsequent cloning. Among over 2,000 of resultant hybridoma clones, 5 clones (RP/12, RP/14, RP/15, RP/16, and RP/19) were selected and analyzed in this study because of their remarkable radioprotective activities. Since functional activities of these five hybridoma clones were similar to each other and the antigen recognized with these antibodies appeared identical as judged by flow cytometry and immunoprecipitation experiments, the results obtained with RP/14 are shown in the present study, except the experiments of immunoprecipitation in which the results obtained with RP/14 and RP/16 are shown. Fig. 1, one of representative results of a series of experiments repeated more than five times, showed the radioprotective activity of RP/14. Two to three times more spleen cells remained viable with RP/14 than those without antibody or with a control antibody. Then DNA contents of irradiated cells were examined with an aim to analyze apoptotic cells, because cells with subdiploid DNA corresponded to those undergoing apoptosis (14). For this experiment, irradiated spleen cells were harvested at earlier periods of time

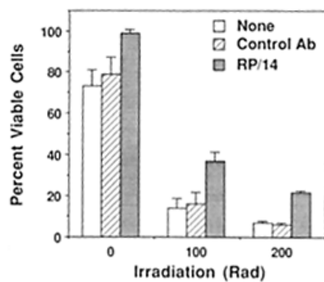

Figure 1. Radioprotective activity of RP/14 on spleen cells. Spleen cells from C57BL/6 mice were cultured for 2 h with or without an antibody before irradiation. Isotype-matched rat anti-mouse B220 mAb (RA3-6B2) was used as a control antibody. Subsequently cells were cultured for 18 h. Viable cells were counted by trypan blue dye exclusion, and percent viable cells are shown. The values are mean ± SD for triplicate cultures.

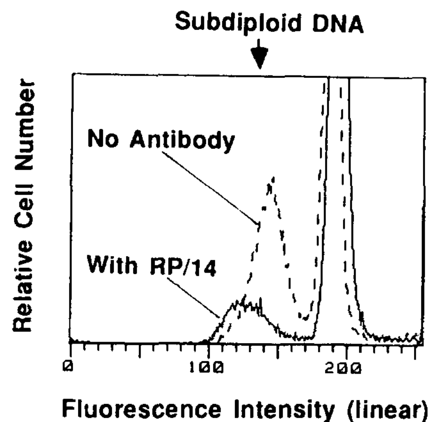

Figure 2. RP/14 reduced percentage of cells with subdiploid DNA that was undergoing apoptosis upon irradiation. DNA content of irradiated spleen cells were shown with propidium iodide. After irradiation (200 rad), cells were cultured for 6 h, fixed in 70% ethanol, stained with propidium iodide. Stained cells were analyzed on a FACScan®. 30,000 cells were analyzed.

(6 or 12 h) after the irradiation, because in apoptotic cells, nuclear changes including DNA degradation precedes cell membrane death (15), and cells undergoing DNA degradation still exclude trypan blue. As shown in Fig. 2, an inclusion of RP/14 reduced cells with subdiploid DNA in percentage. The radioprotective effect of RP/14 was more remarkable at 6 h after irradiation than that at 12 h after irradiation (Table 1). When cells were incubated on ice for 6 h after irradiation, as little as 0.6% of cells showed subdiploid DNA. Therefore DNA degradation is a metabolically active process, and RP/14 seemed to disturb this process. At higher dose irradiation of 2,000 rad, spleen cells were shown to die by necrosis rather than apoptosis (8). Under these conditions, RP/14 did not rescue spleen cells from irradiation-induced damage (data not shown). We also induced cell death by apoptosis using dexamethasone (16) and examined the effect of RP/14 on its protection. RP/14 was again effective in rescuing spleen cells from dexamethasone-induced cell death (Fig. 3). These results indicate that RP/14 can rescue spleen cells from apoptosis induced by irradiation or dexamethasone.

Tissue Distribution and Immunoprecipitation of the Target Antigen Recognized by RP/14. To characterize the antigen recognized by RP/14 mAb (the RP antigen), we examined the tissue distribution of the RP antigen by immunofluorescence analysis. Spleen, lymph nodes, and bone marrow, but not thymus contained positive cells for the RP antigen (data not shown). To analyze in more detail, dual staining was carried out with cells from spleen or bone marrow using various mAbs (Fig. 4). The expression of the RP antigen in spleen cells was restricted on B220-positive cells (Fig. 4 *A*). Among

Table 1. *RP/14 mAb Reduced Cells with Subdiploid DNA that Underwent Apoptosis after Irradiation*

Experiment	Spleen cell treatment: RP/14 mAb	Spleen cell treatment: Irradiation	Hour after Irradiation	Cells with subdiploid DNA	Relative increase
	10 μg/ml	*200 rad*		%	%
1	–	–	6	7.4	–
	–	+		28.3	20.9
	+	+		10.6	3.2
2	–	–	12	17.4	–
	–	+		66.4	49.0
	+	+		41.3	23.9

Spleen cells from BALB/c mice were cultured with RP/14 or control antibody (10 μg/ml) for 2 h before irradiation (200 rad). Then, cells were cultured for additional 6 h (experiment 1) or 12 h (experiment 2), fixed with 70% ethanol, and stained with propidium iodide. Percentage of cells with subdiploid DNA are shown. Relative increase (%) corresponds to the values from which percentages with cells cultured without irradiation was subtracted. The percentage of cells with subdiploid DNA was 0.6 when cells were incubated on ice for 6 h after irradiation (experiment 1). In both experiments, 3×10^4 cells were analyzed.

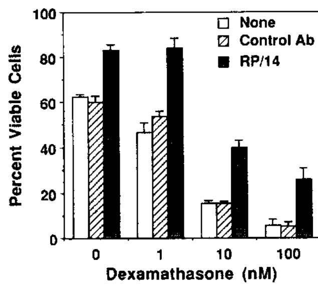

Figure 3. Rescue of spleen cells from dexamethasone-induced apoptosis by RP/14 mAb. Spleen cells from BALB/c mice were cultured for 20 h with or without dexamethasone at concentrations indicated in the figure. RP/14 or a control antibody (anti-B220: RA3-6B2) were present during culture at a concentration of 10 μg/ml. Viable cells were counted by trypan blue dye exclusion, and percent viable cells are shown. The values are mean ± SD for triplicate cultures.

them, cells expressing the RP antigen appeared to bear bright B220, dull IgM, and bright IgD (Fig. 4, *A*, *C*, and *E*). This phenotype represents rather mature and long-lived B cells (17–19).

In bone marrow, the RP antigen was expressed on B cells expressing bright B220 (Fig. 4 *B*), and dull IgM (Fig. 4 *D*), but not on pre-B cells, because the RP antigen was not expressed on either $B220^{dull}$ or IgM-negative cells. B cells in bone marrow with bright B220 and dull IgM are reported to be derived from long-lived recirculating B cells but not from newly generated B cells (17, 18). Therefore the expression pattern of the RP antigen in bone marrow would be consistent with that in spleen. Collectively, the RP antigen appears to be expressed on mature B cells but not either pre-B cells or newly generated B cells as judged by the two-color analysis with B220, IgM and IgD. Immunoprecipitation experiments were, then, performed to identify the antigen recognized with RP/14 or RP/16. A monomeric protein was immunoprecipitated, and its molecular mass was approximately 105 kD (Fig. 5). No differences in size were observed between reduced and nonreduced (data not shown) condition.

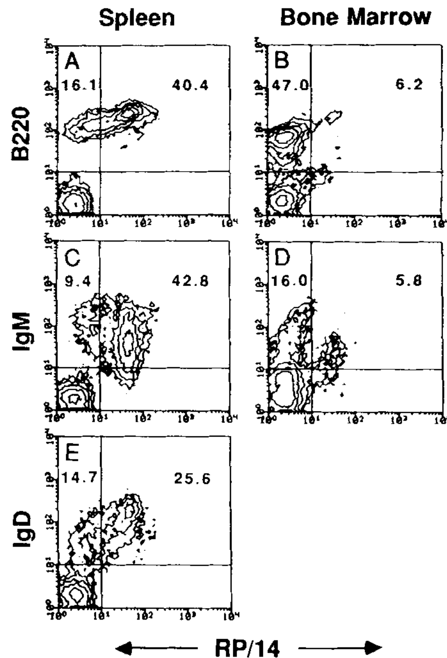

Figure 4. Dual staining of spleen and bone marrow cells with RP/14 mAb and B cell markers. Spleen cells or bone marrow cells were stained with FITC-labeled RP/14 mAb (*horizontal axis*) and biotinylated antibodies against B220, IgM, or IgD (*vertical axis*). Forward and side scatter were used as gating parameters to examine lymphoid cells in spleen and total nucleated cells in bone marrow. The percentages of cells are indicated in each of the upper quadrants. 30,000 cells were analyzed.

RP/14 Is Agonistic to IgM^+ IgD^+ B Cells. Spleen cells rescued from irradiation by RP/14 were harvested and cell surface markers were analyzed. Most of them expressed B220, IgM, and IgD as well as the RP antigen (data not shown). Therefore B cells expressing the RP antigen would be allowed to survive by RP/14. Most cells rescued by RP/14 from irradiation or dexamethasone appeared to be blastic. This blastic change occurred also to spleen cells cultured with RP/14 in the absence of irradiation or dexamethasone. Therefore RP/14 itself induces the blastic change on spleen cells. If RP/14 is agonistic, blastic cells should express the RP antigen. Spleen cells were cultured with or without RP/14 for 2 d, harvested, and then the RP antigen was stained (Fig. 6). Indeed, blasts generated by culturing with RP/14 expressed the RP antigen. These blasts retained other B cell markers including B220, IgM, and IgD (data not shown). Thus, it would be B cells expressing the RP antigen that showed blastic change and were rescued from irradiation- or dexamethasone-dependent apoptosis.

The uptake of [^{3}H]TdR was examined to determine whether or not blastic change of B cells achieved with RP/14 proceed to B cell proliferation. As shown in Table 2, spleen cells showed remarkable uptake of [^{3}H]TdR upon stimulation with RP/14. The response peaked around day 2–4, and

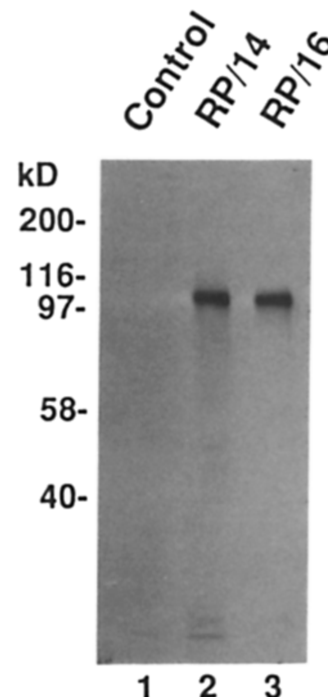

Figure 5. RP mAbs recognize a monomeric molecule with 105 kD molecular mass. B lymphoma cell, BCL_1, was surface-biotinylated, detergent-extracted, and precipitated with RP/14- or RP/16-bound beads. Bound proteins were subjected to SDS-PAGE (9% acrylamide) under a reduced condition, blotted onto a nitrocellulose membrane, and detected with avidin-peroxidase and appropriate substrates.

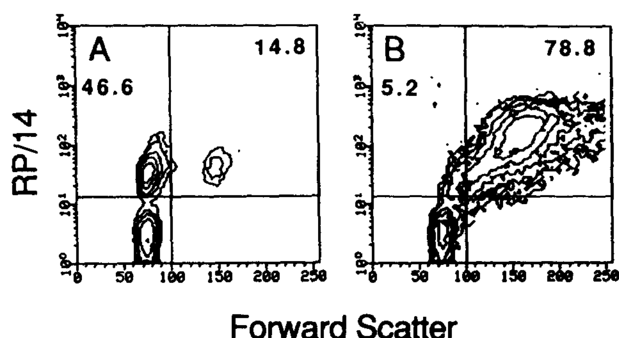

Figure 6. Responding blasts to the stimulation with RP mAb express the RP antigen. Spleen cells were cultured with (*B*) or without (*A*) RP/14 mAb (10 μg/ml) for 2 d. Then, cells were stained with RP/14 followed by FITC-labeled mouse anti-rat κ mAb. In *A*, some RP-positive blasts appeared in the upper right region. These cells seemed to have been already activated in vivo. The percentages of cells are indicated in each of the upper quadrants.

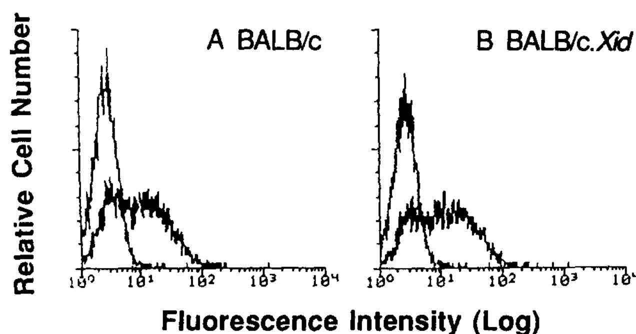

Figure 7. Comparable expression of the RP antigen on B 220-positive B cells with XID. Spleen cells from BALB/c or BALB/c.*xid* were stained with RP mAb and anti-B220 (RA3-6B2). B220-positive cells were gated and the expression of the RP antigen was shown.

Table 2. *Proliferation of Spleen Cells Induced by RP/14 mAb*

mAb added	[^{3}H]TdR uptake Experiment	Experiment
10 μg/ml	*cpm*	
None	587 ± 174	288 ± 34
Control Ab	964 ± 149	751 ± 141
RP/14	61,660 ± 3,374	27,626 ± 1,050

Spleen cells from BALB/c mice were inoculated into a 96-well microtiter plate (2 × 10^5 cells/well), and cultured with or without antibodies for 2 d. Cells were pulsed with 1 μCi of [^{3}H]TdR for the last 4 h of the culture, and cell-associated cpm were measured. The values were mean ± SD for triplicate cultures.

then gradually declined (data not shown). We obtained similar proliferative responses by using T cell–depleted spleen cells (Table 3). Moreover, spleen cells did increase in number when RP mAb was added to culture (data not shown). Thus, RP/14 appeared to drive B cells into proliferation. After stimulation with RP/14, supernatant from spleen cells were collected and the amount of IgM was determined by ELISA. No significant production of IgM was observed (data not shown).

B Cells with XID Do Not Respond to RP mAb in Spite of Comparable Expression of the RP Antigen to B Cells of Wild Type. The results that mature B cells expressed the RP antigen and were activated with RP/14 mAb prompted us to study B cells from XID mice (9) because the *xid* mutation results in drastic decrease in mature B cells bearing dull IgM, and bright IgD (20). At first, the expression of the RP antigen was studied. As shown in Fig. 7, the RP antigen was expressed on B220-positive B cells from BALB/c.*xid*. The amount of the RP antigen appeared to be comparable between BALB/c and BALB/c.*xid*. Then, T cell–depleted spleen cells were cultured with RP/14 mAb. XID B cells did not

Table 3. *Unresponsiveness of X-linked Immunodeficient B Cells to RP mAb*

Mouse	[^{3}H]TdR uptake stimulated with: (10 μg/ml) None	Control Ab	RP/14	LPS
			cpm	
BALB/c	103 ± 16	132 ± 35	37,702 ± 2,020	33,067 ± 3,167
BALB/c.*xid*	58 ± 15	108 ± 28	100 ± 11	3,364 ± 375
C57BL/6	89 ± 12	137 ± 22	46,678 ± 1,503	24,172 ± 2,008
C57BL/6.*xid*	64 ± 23	44 ± 24	64 ± 13	32,533 ± 246

T cell–depleted spleen cells from BALB/c or BALB/c.*xid* mice were inoculated into a 96-well microtiter plate and cultured with antibodies or LPS for 3 d. Anti-CD45 (RA3-6B2) mAb was used for an control antibody. Cells were pulsed with 1 μCi of [^{3}H]TdR for the last 6 h of the culture, and cell-associated cpm were measured. The values were mean ± SD for triplicate cultures.

respond to RP/14 mAb as judged by [^{3}H]TdR uptake (Table 3). As a control, B cells from BALB/c.*xid* and C57BL/6.*xid* showed good proliferative responses to LPS. Wild-type B cells from BALB/c or C57BL/6 responded to both RP/14 mAb and LPS. Similar results were obtained with B cells from CBA/N mice (data not shown). RP/14 did not protect spleen cells from CBA/N mice against apoptosis induced by irradiation. Neither uptake of [^{3}H]TdR nor blastic change was observed in CBA/N spleen cells when cultured with RP/14 mAb. These results strongly suggest that signal transductions through the RP antigen appear to be defective in XID mice.

Discussion

We established novel mAbs that are able to protect B cells from apoptosis induced by irradiation or dexamethasone. One of mAbs RP/14 can drive B cells into proliferation. The antigen recognized by RP/14 is approximately 105 kD in size and is expressed on mature B cells bearing IgM^{dull}, IgD^{bright}, and B220^{bright}, but not on either pre-B cells or immature B cells. Because responding B cells are positive for the RP antigen, RP/14 would deliver a signal into mature B cells through the RP antigen. To our knowledge, no molecule with similar molecular weight and expression profiles is reported, although NH_2-terminal amino acid sequencing and isolation of cDNA for the RP antigen is required to define that the RP antigen is novel.

In contrast to other cell types, lymphocytes in a quiescent state are more vulnerable to irradiation-induced apoptosis than those in an actively dividing state (7, 8). Activation makes B cells resistant to irradiation (8). RP/14 protected B cells from irradiation-induced apoptosis. At the same time RP/14 activated B cells as judged by a blastic change and drove them into proliferation. Moreover, both rescued cells and blastic cells expressed the RP antigen. Therefore B cell activation and protection from apoptosis by RP/14 would be explained by a signal generated by RP/14. In other words, radioprotective activity by RP/14 would stem from B cell activation by RP/14. A signal delivered with RP/14 through the RP antigen activates B cells, and activated B cells then would turn resistant against irradiation-induced apoptosis. Accordingly, protection from apoptosis, a blastic change, and [^{3}H[TdR uptake, all these readout systems would observe one phenomenon, B cell activation achieved by a signal with RP/14.

Signals to B cells delivered by RP/14 mAb appear to be similar to that delivered by the antibody against CD40 (21). Although a mAb against murine CD40 has not been reported yet, the antibody against human CD40 is able to induce B cell proliferation and immunoglobulin secretion particularly in concert with antigen or anti-IgM, and cytokines such as IL-4 or IL-10 (22, 23). Moreover, anti-CD40 has been shown to protect germinal center cells from apoptosis (6), and a B cell line, WEHI231, from anti-IgM–mediated apoptosis (24). The signal through CD40 into B cells results in both activation and protection from apoptosis, phenomena as were observed with the RP antigen. It is interesting to study the effect of RP/14 on anti-IgM–mediated apoptosis of WEHI231 cells (24).

The RP antigen can be, however, discriminated from CD40 by the fact that the RP antigen is expressed on B220^{bright} IgM^{dull} IgD^{bright} B cells, but not on pre-B cells or immature B cells. On the contrary, an acquisition of human CD40 precedes the expression of CD20, and shows a pan-B reactivity in peripheral lymphoid organs (22). CD40 is expressed on germinal center cells (6), and plays critical roles in germinal center formation (5, 6, 25–29). However, it is unlikely that germinal center cells possess the bright expression of the RP antigen, because the RP antigen is expressed on IgD-positive B cells (Fig. 4), which do not reside in germinal center (6). The restricted expression of the RP antigen in B cell ontogeny might suggest a very specified role of the RP antigen in B cell activation or maturation as compared with CD40.

It is well acknowledged that immature B cells in spleen can be discriminated from mature B cells on the basis of differential expression of B220, surface IgM, and surface IgD (17–19). B cells with B220^{dull}, IgM^{bright}, and IgD^{dull} are supposed to belong to an immature B cell population that would be recently generated in the bone marrow. Long-lived mature B cell subpopulation has B220^{bright}, IgM^{dull}, and IgD^{bright}. Cell labeling studies with 5-bromo-2′-deoxyuridine (BrdU) revealed that the immature B cell population has rapid turn over rate whereas the mature B cell population is stable (18). Consequently it is assumed that some maturation stages exist between immature B cells and mature long-lived B cells (19). Although more information is required concerning the precise expression pattern of the RP antigen in association with other markers such as the heat stable antigen (19), it could be a transition stage from immature to mature B cells where the RP antigen is acquired. In this regard, RP/14 would be useful as a novel marker for this transition stage. Moreover, because RP/14 is shown to deliver a signal into IgM-positive, IgD-positive B cells, the RP antigen itself might be, in some ways, implicated in this transition. Further studies are ongoing to elucidate these issues.

Mice with XID (9) lack a mature B cell population that normally develops late in ontogeny and expresses low IgM and high IgD (20). B cell maturation seemed to be arrested at a stage between immature and mature B cells. Taking the expression pattern of the RP antigen into consideration, we were interested in investigating the RP antigen on *xid* B cells. Although B220-positive B cells with *xid* does express the RP antigen comparably to wild-type B cells (Fig. 6), B cells with *xid* do not respond to the signal with RP/14. Recently a gene involved in X-linked agammaglobulinemia was identified (30, 31). The gene, *btk* which encodes a member of the *src* family of protein tyrosine kinases was mapped to the *xid* region of the mouse X chromosome. A single conserved residue within the NH_2-terminal unique region of Bruton's tyrosine kinase (Btk) was mutated in *xid* mice (32). Btk, for which the *btk* gene code is supposed to work in signal transmission which is indispensable for B cell maturation in human and mice. The results in the present study might suggest an indispensable role of Btk in transducing the signal with RP/14

mAb. However it is also possible that a mutation in Btk indirectly affects a signaling complex used by activation with RP/14 mAb.

While B cell production is intact (33, 34), mice with *xid* have a difficulty in maintaining a stable B cell pool in peripheral lymphoid organs especially in competition with wild-type B cells (35). Maturation into a long-lived mature B cell would be prerequisite for joining a stable B cell pool. The acquisition of the RP antigen by B cells would occur at a transitory state from an immature to a mature state. Taken together with these issues, it would be possible that the signal through the RP antigen is involved in maturation itself or in maintaining a long-lived B cell pool. In this regard, the study of the RP antigen and the signal transduction through the RP antigen would be able to contribute to further understanding of B cell maturation and activation in peripheral lymphoid organs.

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