Transgene Expression of *bcl-x_L* Permits Anti-immunoglobulin (Ig)–induced Proliferation in *xid* B Cells

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Summary

Mutations in the tyrosine kinase, Btk, result in a mild immunodeficiency in mice (*xid*). While B lymphocytes from *xid* mice do not proliferate to anti-immunoglobulin (Ig), we show here induction of the complete complement of cell cycle regulatory molecules, though the level of induction is about half that detected in normal B cells. Cell cycle analysis reveals that anti-Ig stimulated *xid* B cells enter S phase, but fail to complete the cell cycle, exhibiting a high rate of apoptosis. This correlated with a decreased ability to induce the anti-apoptosis regulatory protein, Bcl-x_L. Ectopic expression of Bcl-x_L in *xid* B cells permitted anti-Ig induced cell cycle progression demonstrating dual requirements for induction of anti-apoptotic proteins plus cell cycle regulatory proteins during antigen receptor mediated proliferation. Furthermore, our results link one of the immunodeficient traits caused by mutant Btk with the failure to properly regulate Bcl-x_L.

B cell development proceeds as a series of sequential check points designed to ensure the production and coupling of functional heavy and light chains, and the integrity of the Ig signal transduction cascade (1). Mutations which lead to disruption of the Ig receptor complex or downstream signaling components can have dramatic effects on the generation and/or function of developing B cells (2–4). The X chromosome–encoded cytoplasmic Bruton's tyrosine kinase (Btk)[1] is required for efficient signaling through the antigen receptor and B cell development in both mice and humans (5–8). In humans, mutations in *Btk* result in X-linked agammaglobulinemia (XLA), a severe immunodeficiency that manifests as a block in B cell development at the pre–B cell stage, rendering affected males virtually devoid of peripheral B cells (9–11). A spontaneous mutation in *Btk* in mice results in the milder X-linked immunodeficiency, *xid*, which is characterized by reduced numbers of splenic B cells and a failure of these B cells to enter the long-lived B cell pool (12, 13). Additionally, *xid* B cells have an unusual surface phenotype that is not characteristic of either immature or mature B cells (14, 15), suggesting some defect in development after their generation in the bone marrow. Furthermore, peritoneal B1 cells are absent (16) and there are specific Ig isotype deficiencies. However, despite these immunodeficient traits, the mice are robust and have a normal life span. The variability in severity of disease between *xid* and XLA reflects genuine species-specific differences in the requirement for Btk during B cell development rather than differences in site-specific mutations in *Btk* (7, 8, 11).

Although Btk is thought to be a component of several signal transduction pathways, it is almost certainly the disruption of the Ig signal transduction pathway that results in the immunodeficiencies of XLA and *xid*. In vitro cross-linking of surface IgM on *xid* B cells fails to promote cell cycle progression, consistent with the observation that XLA and *xid* phenotypes include a failure to expand specific B cell populations (pre–B cells in humans and B1 cells in mice) whose development is thought to require productive antigen receptor signaling (17, 18).

The molecular control of cell cycle progression involves the sequential activation of a family of serine/threonine kinases known as cyclin-dependent kinases, or cdks, and their subsequent phosphorylation of specific substrates (19, 20). cdks are activated in part by physical coupling with cyclins, a family of regulatory subunits that are induced at phase-specific stages during the cell cycle. The decision to enter S

[1]*Abbreviations used in this paper:* BrdU, bromodeoxyuridine; Btk, Bruton's tyrosine kinase; cdc, cell division cycle; cdk, cyclin-dependent kinases; PI, propidium iodide; Rb, retinoblastoma gene; XLA, X-linked agammaglobulinemia.

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phase occurs late in G1, at the restriction point, R, after which the cells are committed to DNA replication and cellular division. A major component of the restriction point is the phosphorylation and inactivation of the protein production of the retinoblastoma gene, Rb, during G1 by the cyclin D–associated kinase activity (21). However, despite the requirement for Rb phosphorylation for S phase entry, inactivation of Rb does not guarantee cell cycle progression since Rb phosphorylation can be detected in cell lines undergoing apoptosis (22, 23). In such systems, ectopic expression of the antiapoptotic molecule, Bcl-2, permits orderly cell cycle progression. These and other experiments led to the hypothesis that cellular division can only be achieved with the engagement of the proliferative machinery in the presence of antiapoptotic proteins (22, 24, 25).

The Bcl-2–related antiapoptosis regulatory protein, Bcl-x_L, is expressed at different stages during B cell ontogeny (26–29). Intriguingly, Bcl-x_L is expressed at stages of B cell development arrested in XLA (pre–B cells) and *xid* (B1 B cells) (29) and is known to be upregulated as a consequence of antigen receptor cross-linking (27–29). The importance of Bcl-x_L expression during B cell development is demonstrated by the expansion of the pro-pre-B cell compartment in two independently generated *bcl-x_L* transgenic mouse models (26, 27) and the dramatic loss of peripheral lymphoid system in chimeric mice homozygous for a targeted deletion in *bcl-x* (30).

Given that the defects in XLA and *xid* both affect expansion of B cells at developmental stages dependent on Ig signaling, we explored the influence of defective Btk on the induction of cell cycle regulatory proteins after anti-Ig stimulation. Our results indicate that the Ig signal transduction cascade that uses mutant Btk does stimulate the induction of cyclins, cdks, and kinase activity; however, there is a specific failure to normally upregulate Bcl-x_L. The induction of cell cycle regulatory machinery via mutant Btk was sufficient to promote proliferation in the presence of transgene-driven Bcl-x_L expression. These results demonstrate a specific requirement for the antiapoptotic protein Bcl-x_L during antigen receptor–induced B cell proliferation and links certain immunodeficient traits in *xid* with failure to induce this viability-enhancing protein.

Materials and Methods

Mice. Male and female CBA/N (*xid*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used as breeding pairs at DNAX Research Institute. CBA/Ca mice were obtained from The Jackson Laboratory and used as control mice. Males from two previously described and independently derived strains of *bcl-x* transgenic mice (*bcl-x*-87; reference 26, and *bcl-x*-81; reference 25) were used in breedings with female CBA/N (*xid*).

Reagents. Antibodies used in this study were monoclonal rat anti–mouse cyclin D3 and D2, rabbit anti–mouse cyclin E, cyclin A, cyclin B, cdk2, cdk4, and cdk6; these reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti–mouse cell division cycle (cdc)2, mouse anti–human Rb, and anti–Bcl-2 antibodies used for Western blotting were purchased from PharMingen (San Diego, CA). The anti-CD43 FITC, anti-B220 PE, anti-CD5 PE, and anti-B220 FITC used in flow cytometric analysis were purchased from PharMingen. Anti-Bcl-x_L and anti-Bad were purchased from Transduction Laboratories (Lexington, KY). Horseradish peroxidase–conjugated anti-rat, rabbit, and mouse secondary reagents were purchased from Amersham Corp. (Arlington Heights, IL). The anti-cdc2 antibody used for immunoprecipitations was purchased from GIBCO BRL (Gaithersburg, MD). The substrate for the kinase assay, histone H1, was purchased from Boehringer Mannheim (Indianapolis, IN). The affinity purified $F(ab')_2$ goat anti–mouse IgM used to stimulate B cells was purchased from Cappel Labs. (Malvern, PA). The rat anti–mouse monoclonal anti-CD40 antibody has been described previously (31).

Purification of Splenic B Cells. To isolate splenic B cells, single cell suspensions were prepared from spleens of unprimed mice. Red blood cells were lysed using red blood cell lysis buffer (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions. To deplete T cells, splenocytes were stained using anti–Thy-1 (Dupont-NEN, Boston, MA) and anti-CD4 (RL172), followed by incubation in rabbit complement (Cedarlane Labs. Ltd., Hornby, Ontario, Canada) at 37°C for 45 min. These cells were consistently >90% B2201/IgM1 and included both the low and high density B cells.

In Vitro Stimulations and Whole Cell Lysate Preparation. The expression of cell cycle regulatory proteins in anti-Ig–stimulated B cell populations was evaluated using procedures previously described (32). In brief, 50-ml cultures were set up at 10^6 B cells/ml in 75 cm^2 flasks (Becton Dickinson Labware, Lincoln Park, NJ) and stimulated with 25 μg/ml $F(ab')_2$ anti-IgM. Cells were collected at 6, 12, 24, 36, 48, and 72 h after stimulation. All stimulations were carried out in supplemented RPMI containing 10% FCS (JR Scientific, Woodland, CA), 5×10^5 M 2-ME (Polysciences, Inc., Warrington, PA), 2 mM glutamate (JR Scientific), 10 mM Hepes buffer (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific). After stimulation, cells were recovered, washed three times in cold PBS, divided into three pellets, and stored at −80°C for later use in Western blotting and in vitro kinase assays. When all time points were collected, one pellet from each time point was lysed in NP-40 lysis buffer (1% NP-40, 250 mM NaCl, 1 mM Hepes, pH 7.5, and 1 mM dithiothreotol [DTT; United States Biochemical Corp., Cleveland, OH] with protease inhibitors added; final concentration: 5 μg/ml aprotinin [Sigma Chemical Co.], 125 μg/ml Pefabloc [Boehringer Mannheim], 5 μg/ml pepstatin [Sigma Chemical Co.]). Protein concentrations were calculated using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's procedures. An equal volume of 2× SDS sample buffer (Novex, San Diego, CA) with 2-ME was then added to the total cell lysates. 125 μg of lysate was run per time point on 12% Novex reducing gels according to manufacturer's suggested protocol (6% gels were used to detect the Rb protein), and transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA) at 30 V overnight in 4°C. The mouse plasmacytoma line P3X was obtained from the American Type Culture Collection (Rockville, MD) and used as a positive control on all gels.

In Vitro Kinase Assay. cdk2- and cdc2-dependent kinase assays were performed using the histone substrate according to procedures described elsewhere (33). In brief, frozen pellets were lysed in 1 ml 0.1% NP-40 lysis buffer containing 50 mM Hepes, pH 7.0, and 250 mM NaCl in the presence of the above protease inhibitors. Protein concentration was calculated as described

above, and 200 μg of total protein was used for each kinase assay. In brief, lysates were precleared with normal rabbit serum followed by Zysorbin (Zymed, South San Francisco, CA). 1 μg of anti-cdk2, anti-cdc2, or normal rabbit sera was added to the lysate and incubated at 4°C for 4 h with rocking. Protein G beads (Pharmacia Biotech, Inc., Piscataway, NJ) were added and the lysates were rotated in the cold for another hour. Immunoprecipitates were washed 4 times in lysis buffer followed by two washes in kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT). The kinase reactions were carried out in a 50 μl volume containing 2.5 μg histone H1 and 5 μCi [^{32}P]γATP (Amersham Corp.) per reaction. Reactions proceeded for 30 min at room temperature with rocking. Samples were run on 12% Novex precast gels, fixed, dried, and exposed to phosphor screen (Molecular Dynamics, Sunnyvale, CA). The results were read on a Storm 860 phosphorimager (Molecular Dynamics).

Cell Cycle Analysis. Cell cycle analysis was performed using a modified method of Carayon and Bord (34). Purified B cells were cultured at 10^6/ml in 200-μl aliquots in the presence of 50 μM bromodeoxyuridine (BrdU; Sigma Chemical Co.) and an equimolar concentration of cytidine (Sigma Chemical Co.) plus 25 μg/ml anti-IgM for times indicated. Cells were harvested and stored in PBS with 0.5% paraformaldehyde overnight. PBS with 5% Tween 20 was added to permeabilize the nuclear membrane. The cells were then washed and resuspended in a solution of 10 mg/ml DNAse I (Sigma Chemical Co.) plus anti-BrdU (Becton Dickinson, Mountain View, CA). Finally propidium iodide (PI) is added and then analysis is performed on FACScalibur® using Cellquest software (Becton Dickinson).

Thymidine Incorporation. 200-μl aliquots (2×10^5 total cells) of stimulated cells were placed in 96-well flat-bottomed plates at times indicated and pulsed for 45 min in 1 μCi [^{3}H]thymidine (Amersham Corp.). Cells were harvested and incorporated cpms were counted using a PHD cell harvester (Cambridge Technology Corp., Cambridge, MA).

Testing of Serum IgM and IgG3 Levels by ELISA. Sera was collected from the experimental mice. Samples were serially diluted and compared with known standards of purified IgM and IgG3 as previously described (35).

Results

Induction of Complete Complement of Cell Cycle Regulatory Proteins in Anti-Ig–stimulated xid B Cells. Consistent with a previous report (36), purified B cells from *xid* mice showed little [^{3}H]thymidine incorporation in response to stimulation with 25 μg/ml anti-IgM (Fig. 1 *A*). In contrast, the level of [^{3}H]thymidine incorporation in *xid* B cells after stimulation with 25 μg/ml anti-CD40 was comparable to normal B cells (Fig. 1 *B*). Since B cells from *xid* mice have a selective inability to proliferate in response to anti-IgM stimulation, it was possible that the *xid* mutation in *Btk* affected induction of proteins that control cell cycle progression in response to antigen receptor cross-linking. To test this, lysates were prepared from anti-IgM stimulated *xid* and control B cells at different times after stimulation through 72 h and Western blot analysis of cell cycle regulatory proteins was performed. Fig. 2 shows the induction of regulatory proteins that control the entry into G1 (i.e., cyclin D2, cyclin D3, cdk4, cdk6, and Rb). Cyclins D2 and D3 were induced after anti-Ig stimulation of *xid* B cells with the same kinetics as in the control CBA/Ca B cells, the only difference being the slightly greater induction of the faster migrating form of cyclin D3 in the control B cells, the significance of which is not known (Fig. 2, *A* and *B*). Similarly, both cdk4 and cdk6 were present to comparable extents in both the anti-Ig–stimulated control and *xid* B cells (Fig. 2, *C* and *D*). Most notable was the comparable induction and hyperphosphorylation of Rb in both *xid* and control B cells after anti-Ig stimulation (Fig. 2 *E*). Surprisingly, despite the inability of *xid* B cells to proliferate, the induction of cyclins and cdks involved in S phase progression were also detected. Specifically, both cyclins E and A (Fig. 3, *A* and *B*), along with their kinase partners cdk2 and cdc2 (Fig. 3, *C* and *D*) were detected at about half the levels seen in control B cells. The only significant difference in these two populations was found at the level of expression of cyclin B, the regulatory protein that governs the progression through mitosis. Although cyclin B was only weakly induced in anti-Ig–stimulated *xid* B cells, it was easily detected in control B cells (Fig. 3 *C*).

Since both the cyclins and cdk partners required for cell cycle progression were induced in anti-Ig–stimulated *xid* B cells despite the inability of these cells to proliferate, we performed cdk2- and cdc2-associated kinase assays to determine if cyclin/cdk complexes were assembled correctly and that activating posttranslational modifications had occurred. Fig. 4 shows that both cdk2- and cdc2-associated kinase activities are clearly present in anti-IgM–stimulated *xid* B cells at 48 and 72 h after stimulation, albeit at approximately half the levels detected in anti-Ig–treated control B cells. Collectively, these data indicate that anti-Ig–stimu-

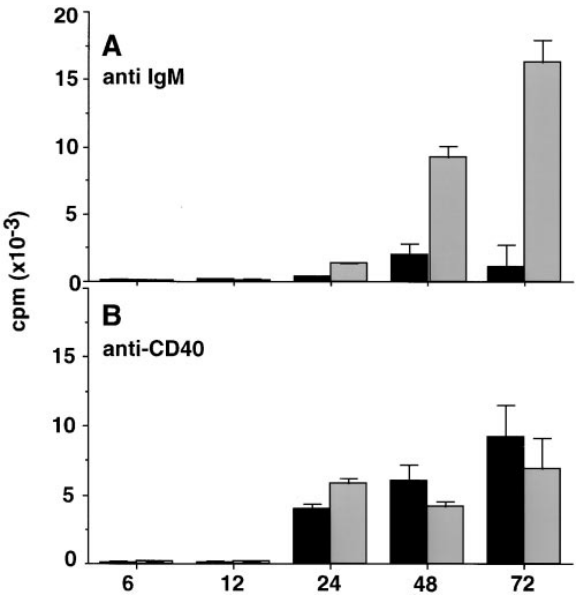
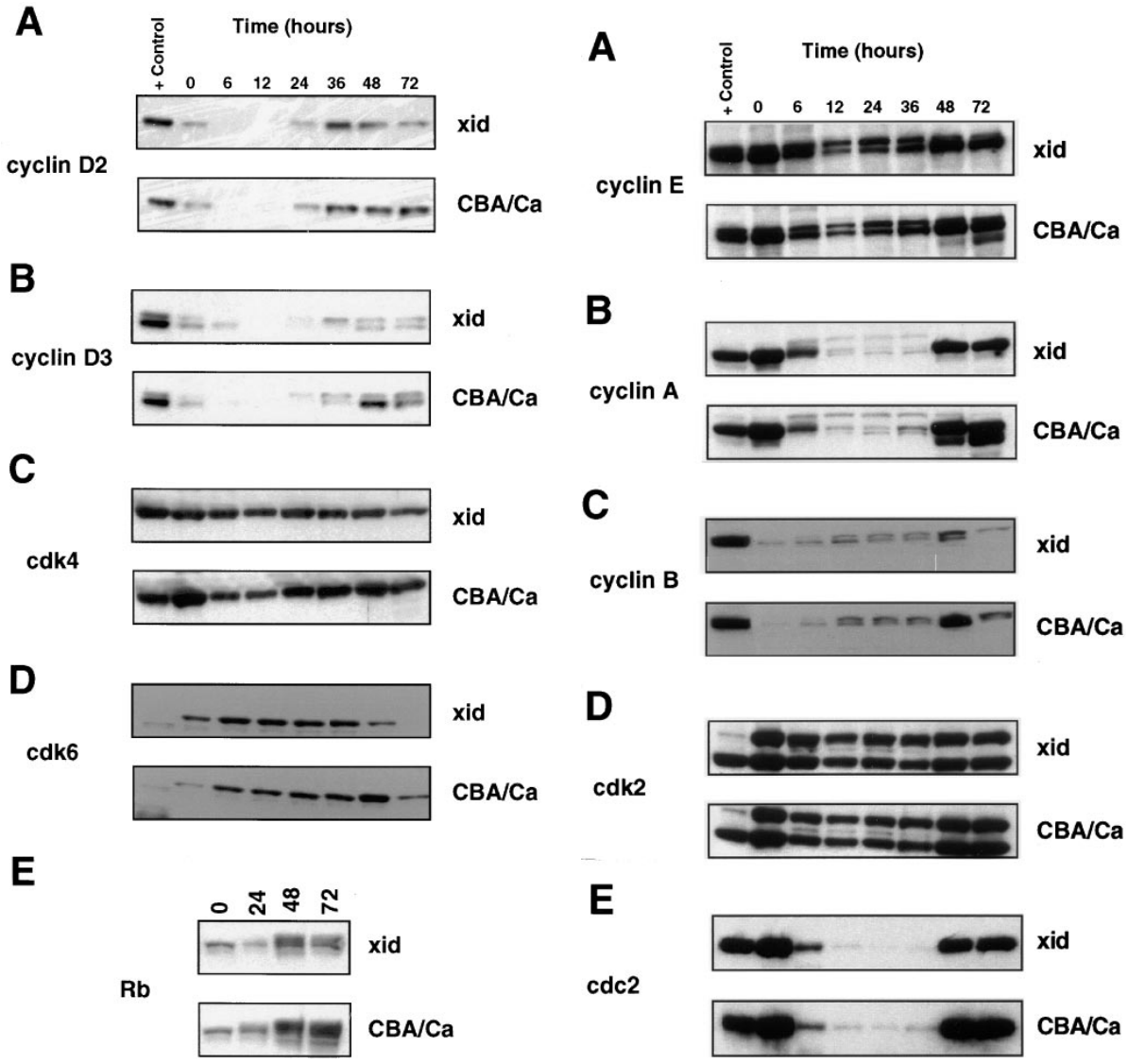


Figure 1. Defective anti-IgM–triggered proliferation in *xid* B cells. B cells from *xid* (*black bar*) and CBA/Ca (*gray bar*) were stimulated with mAbs against CD40 (*A*) or F(ab′)$_2$ anti-IgM (*B*) and pulsed with [^{3}H]thymidine for 1 h at the time indicated. Standard errors are shown and results are representative of three independent experiments.



Figures 2 and 3. Induction of cell cycle regulatory proteins in anti-IgM–stimulated B cells from *xid* and CBA/Ca mice. B cells were stimulated with 25 μg/ml anti-IgM and pellets were collected at the indicated time points after stimulation. Figure 2: Western blots were prepared and sequentially screened with antibodies against cyclin D2 (*A*), cyclin D3 (*B*), cdk4 (*C*), cdk6 (*D*), and Rb (*E*). Figure 3: The same Western blot was sequentially screened with antibodies against cyclin E (*A*), cyclin A (*B*), cyclin B (*C*), cdk2 (*D*), and cdc2 (*E*). Horseradish peroxidase–conjugated secondary reagents were used in combination with enhanced chemiluminescence to visualize results. The cell line, P3X, was used as positive control.

lated *xid* B cells induce substantial levels of active cell cycle regulatory proteins despite their inability to complete the cell cycle in response to this stimulus.

Anti-Ig–stimulated xid B Cells Successfully Pass the G1 Restriction Point, then Enter an Aborted S Phase. Since cell cycle regulatory protein induction and kinase activity were detectable in anti-Ig–stimulated *xid* B cells, we carefully examined the various stages of cell cycle progression using BrdU and PI labeling to distinguish the discrete steps of cell cycle progression. As previously described (34, 37), the combination of long-term BrdU incorporation plus PI staining allows the discrimination of cells that are in (*a*) the original G0/G1 phase; (*b*) the S phase; (*c*) the G0/G1 phase of the second cell cycle; and finally (*d*) apoptotic cells (as illustrated diagrammatically in Fig. 5, *top*). Anti-IgM–stimulated control B cells clearly revealed a characteristic S phase entry that began as early as 24 h after stimulation (Fig. 5, *center*, *arrow*) (for review see reference 38). By 36 h, cells were beginning to successfully accumulate in the second round of the cell cycle and could still be detected in S phase at 48 and 72 h after stimulation. Such detailed analyses revealed that anti-IgM–stimulated *xid* B cells also successfully passed the restriction point and entered S phase at 36 h (Fig. 5, *bottom*, *arrow*), albeit some 12 h later than in the control B cells. These data presumably explain the small amounts of thymidine incorporation that can be detected at 48 and 72 h after stimulation of *xid* B cells with anti-Ig (Fig. 1). However, BrdU and PI costaining convincingly

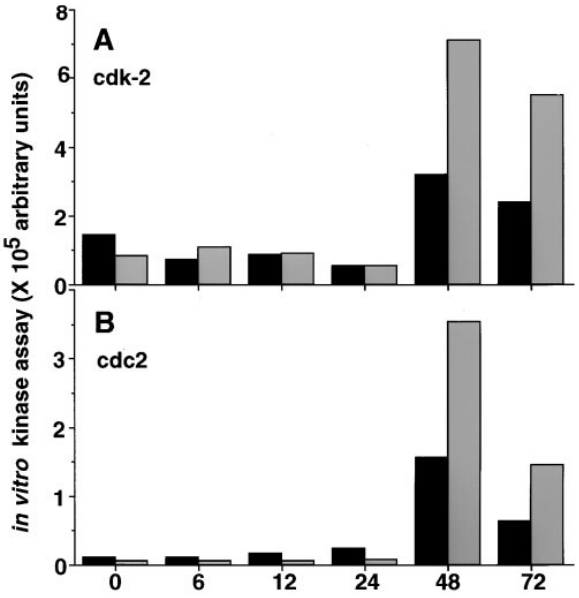


Figure 4. In vitro kinase assays from anti-IgM–stimulated B cells. Stimulated B cells were collected at the time points indicated and used to measure (*A*) cdk2- and (*B*) cdc2-associated specific kinase activity using Histone H1 as the substrate in the presence of [^{32}P]gATP. Kinase activity was quantitated by comparing the amount of ^{32}P incorporated into the substrate by phosphorimager analysis. Kinase activity detected in *xid* (*black bars*) and CBA/Ca (*gray bars*) are shown as arbitrary units. Similar results were obtained in three independent experiments.

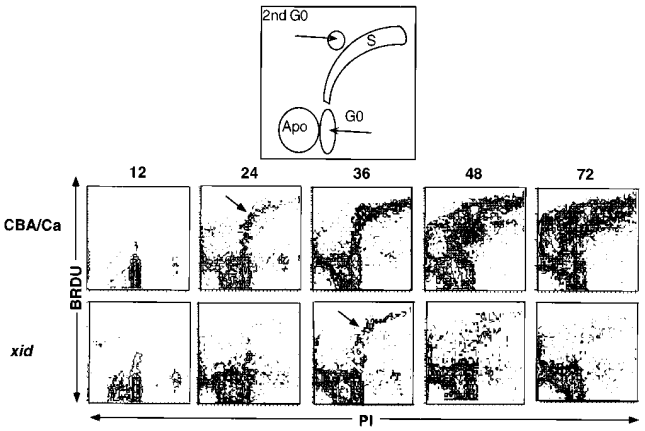


Figure 5. BrdU incorporation and PI analysis of anti-IgM–stimulated B cells from CBA/Ca and *xid* B mice. The diagram at the top indicates the cell cycle position of cells at the time the cells were harvested. This analysis identifies cells in G0 (*G0*), S phase (*S*), and those which have completed S phase and are now in the second cell cycle G0 phase (*2nd G0*), and apoptotic cells (*Apo*). B cells from CBA/Ca (*top row*) and *xid* (*bottom row*) mice were stimulated with 25 μg/ml of anti-IgM in the presence of BrdU. Cells were harvested at time points indicated and fixed to permit intranuclear staining of DNA with PI. The initial entry of B cells from *xid* and CBA/Ca is indicated with an arrow. Analysis was performed on FACSCalibur® using CellQuest software.

established that there was no accumulation of anti-Ig–stimulated cells in the second round of the cell cycle (Fig. 5, *bottom*), indicating that the cells do not successfully complete S phase. Indeed, by 48 and 72 h after stimulation, >85% of the anti-Ig–stimulated *xid* B cells are apoptotic (Fig. 5, *bottom*).

These analyses are also consistent with previous reports showing that *xid* B cells are more prone to undergo apoptosis than control B cells (29, 39). The increased susceptibility of *xid* B cells to apoptosis was evident as early as 12 h after stimulation with anti-Ig, when 20% of the cells were found to be apoptotic by PI staining compared with 5% of the normal B cells (Fig. 5). The increased susceptibility to apoptosis was unrelated to anti-Ig stimulation, since *xid* B cells cultured in media alone died at a significantly faster rate than did their non-*xid* counterparts (data not shown). Even in the presence of the strong mitogen, anti-CD40, that clearly stimulates *xid* B cells to proliferate (see Fig. 1), there is a higher proportion of apoptotic cells in the cultures than in the anti–CD40-stimulated control cells (data not shown). Thus, in addition to the selective inability to proliferate to anti-IgM stimulation, *xid* B cells are more apoptosis-susceptible than are their normal B cell counterparts.

Diminished Induction of Bcl-x_L in Anti-Ig–stimulated xid B Cells. To explore the mechanism underlying the increased apoptosis susceptibility of *xid* B cells, we evaluated expression of the apoptotic regulatory proteins Bcl-2, Bax, and Bcl-x_L in this population. As shown in Fig. 6, *A* and *B*, the levels of Bcl-2 and Bax are comparable in anti-Ig–stimulated *xid* and control B cells. In contrast, there was a striking and consistent decrease in Bcl-x_L induction in anti-Ig–stimulated *xid* B cells compared with normal B cells (Fig. 6 *C*). The induction of Bcl-x_L in anti-Ig–stimulated normal B cells was found to begin as early as 12 h after stimulation, presumably exerting its antiapoptotic effects long before the cells enter S phase.

Bcl-x_L Transgene Permits Anti-Ig–induced xid B Cell Proliferation. To determine whether the diminished induction of Bcl-x_L in anti-Ig–stimulated *xid* B cells contributed to their failure to proliferate, we evaluated the consequence of introducing two independently generated *bcl-x* transgenes into these animals. CBA/N (*xid*) female mice, homozygous for the mutation in the X-linked gene *Btk*, were bred to male mice that were heterozygous for a *bcl-x* transgene (*bcl-x*-87 or *bcl-x*-81). These crosses provided offspring in which all of the males were *xid* while the females were non-*xid*. Additionally, the offspring were either heterozygous for the *bcl-x* transgene or were wild-type. The presence of the *bcl-x* transgene permitted B cells from F1 male (*xid.bcl-x*-87) mice to proliferate to anti-IgM stimulation; these cells successfully completed the cell cycle and could be detected in the G0/G1 stage of second cell cycle (Fig. 7), confirming our observations that mutations in Btk do not block cell cycle machinery induction. However, it should be noted that there was still a delay in entry into S phase that was not detected in the female F1 (*xid.bcl-x*-87) mice, suggesting that mutant Btk continued to effect the rate of entry into cell cycle. Indeed, although splenic B cells from transgene positive male F1 (*xid.bcl-x*-87*)* mice did proliferate to anti-

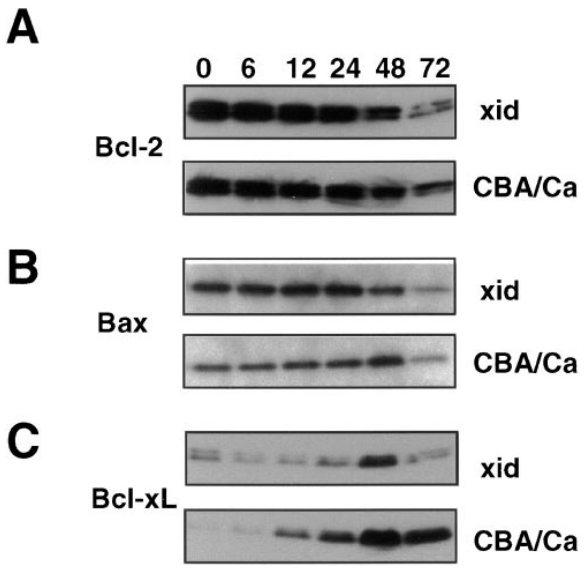


Figure 6. Analysis of Bcl-2 and related proteins. B cells were stimulated as described in the legend to Fig. 2 and were collected at the time points indicated. Western blots were prepared and screened with antibodies specific for Bcl-2 (*A*), Bax (*B*) or Bcl-x_L (*C*). Horseradish peroxidase conjugates were used as secondary reagents followed by visualization with enhanced chemiluminescence.

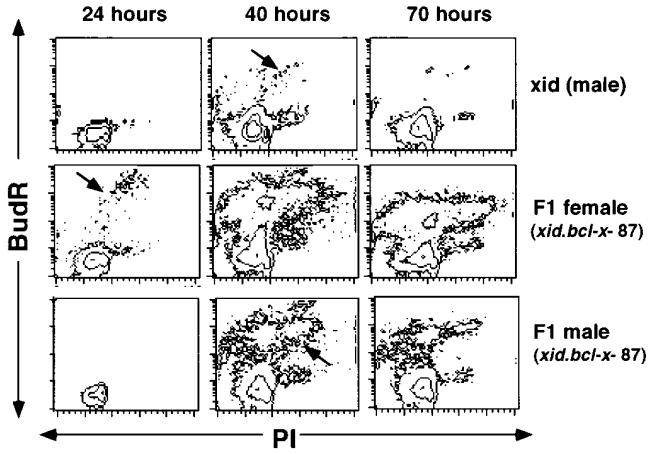


Figure 7. BrdU and PI analysis of stimulated B cells from *xid* and F1 (*xid.bcl-x*-87) offspring. B cells from *xid*, female F1 (*xid.bcl-x*-87), and male F1 (*xid.bcl-x*-87) mice were stimulated with 25 μg/ml anti-IgM in the presence of BrdU and collected at the indicated time points. Cells were prepared and analyzed as described in the legend to Fig. 5. Arrows indicate the initial entry of cells into S phase.

Ig, the proportion of responding cells was only about half of that detected in the female F1 (*xid. bcl-x*-87*)* mice. Consistent with this, we detected only about half the amount of [^{3}H]thymidine incorporation in the male F1 (*xid.bcl-x*-87) mice compared to that in the female F1 (*xid.bcl-x*-87) mice (Table 1).

Partial Correction of Other xid Traits. Since the Bcl-x_L transgene restored the proliferative response in *xid* B cells, we examined the male F1 (*xid. bcl-x*-87) mice to evaluate the overall ability of the Bcl-x_L transgene to complement other immunodeficient traits. The male F1 (*xid.bcl-x*-87) mice, all of which expressed the *bcl-x* transgene along with defective Btk (F1 male Nos. 1, 2, 3, and 4, Table 2) had ~3 times the number of splenic B cells as were detected in normal CBA/Ca mice and 15 times the number of splenic B cells found in the CBA/N (*xid*) mice. The effect of the *bcl-x* transgene was also detected in the female F1 (*xid.bcl-x*-87) mice carrying the *bcl-x* transgene (F1 female Nos. 2 and 4, Table 2) increasing the splenic B cells numbers to 2–3 times that detected in the normal CBA/Ca controls or female F1 (*xid.bcl-x*-87) mice that did not express the transgene. Consistent with these findings, the presence of the *bcl-x* transgene in the male F1 (*xid.bcl-x*-87) mice increased the total number of peritoneal B cells to that detected in the normal CBA/Ca control mice, which is >10 times the number of B cells detected in the peritoneal cavity of *xid* mice (Table 3).

In addition to expanding the peripheral B cell pool, the *bcl-x*-87 transgene provided some correction of the unusually high levels of sIgM expressed on *xid* splenic B cells (Table 2). Transgene positive splenic B cells from male F1 (*xid.bcl-x*-87) mice expressed less IgM than the B cells from

Table 1. *Comparison of [^{3}H]thymidine Incorporation in F1 (xid.bcl-x) and xid Mice*

Mouse	[^{3}H]thymidine* incorporation	Transgene	Btk‡	Phenotype
Female F1 (*xid.bcl-x*-87)	15,489	yes	+/−	non-*xid*
Female F1 (*xid.bcl-x*-87)	12,300	yes	+/−	non-*xid*
Female F1 (*xid.bcl-x*-81)	14,032	yes	+/−	non-*xid*
Female F1 (*xid.bcl-x*-81)	11,089	yes	+/−	non-*xid*
Male F1 (*xid.bcl-x*-87)	7,050	yes	−	*xid*
Male F1 (*xid.bcl-x*-87)	6,500	yes	−	*xid*
Male F1 (*xid.bcl-x*-81)	1,200	yes	−	*xid*
Male F1 (*xid.bcl-x*-81)	1,999	yes	−	*xid*
Male xid 1	2,000	na	−	*xid*
Male xid 2	1,524	na§	−	*xid*

*2×10^5 B cells were stimulated for 48 h with 25 μg/ml anti-IgM and pulsed for the final 4 h with 1 μCi [^{3}H]thymidine.
‡+ indicates presence of wild-type Btk; − indicates presence of mutant Btk. Since Btk is an X-linked gene, males carry only one allele.
§na, not applicable.

Table 2. *Comparison of Splenic B Cell Numbers and Phenotype in CBA/Ca, xid, Female F1 (xid.bcl-x-87), and Male F1 (xid.bcl-x-87) Mice*

Strain and gender	No.	Total B cell number $\times 10^7$	MFI* IgM	MFI* IgD	Percentage of B cells‡	Transgene	Btk‖	*xid*
CBA/Ca males	1	4.7	429	953	43	na§	+	no
	2	3.8	480	974	43	na		
xid males	1	0.6	881	ND	22	na	−	yes
	2	0.9	1015	ND	23	na		
	3	0.9	1220	804	24	na		
	4	0.7	1182	934	22	na		
F1 (*xid.bcl-x*-87) females	1	5.7	427	1,258	36	no	−/+	no
	2	8.0	567	1,023	50	yes		
	3	12.2	440	ND	32	no		
	4	4.2	702	ND	31	yes		
F1 (*xid.bcl-x*-87) males	1	12.5	731	965	50	yes	−	yes
	2	10.5	765	881	50	yes		
	3	14.0	983	1,020	54	yes		
	4	12.8	990	1,005	52	yes		

*Mean fluorescence intensity (MFI) was determined by flow cytometry using FITC-conjugated anti-IgM or PE-conjugated anti-IgD antibodies.
‡Percentage of B cells was determined for lymphoid gate only.
§na, not applicable.
‖+ indicates wild-type Btk; − indicates mutant Btk.

the *xid* mice, whereas the levels of IgD were comparable between these two groups (Table 2). However, the levels of IgM on B cells from the male F1 (*xid.bcl-x*-87) was still higher than that detected on either the CBA/Ca mice or the female F1 (*xid.bcl-x*-87). Additionally, though the peritoneal compartment of the male F1 (*xid.bcl-x*-87) mice was expanded, most of the cells did not express the typical cell surface phenotype of CD5 B cells, which represent the major B cell subset in a normal peritoneal cavity (Table 3). The transgene did increase the number of CD5 B cells to fourfold that detected in the *xid* mice. However, this still reflected a 90% reduction when compared with wild type animals.

To determine the effect of the *bcl-x* transgene on viability, splenic B cells from *xid*, CBA/Ca controls, male and female F1 (*xid.bcl-x*-87) mice that carried the *bcl-x* transgene were cultured in vitro and viability compared. As expected, viability of *xid* B cells was less than B cells from

Table 3. *Number and Phenotype of B Cells in the Peritoneal Cavity of CBA/Ca, xid, and male F1 (xid.bcl-x-87) Mice*

		Total number of B cells $\times 10^5$	Percentage of CD5 B cells‡	Total number of CD5 B cells $\times 10^4$	Transgene	Btk‖	*xid*
CBA/Ca	1	11	58	64	na§	+	no
male	2	13	55	71	na		
xid	1	2	12	2	na	−	yes
male	2	1	15	1	na		
F1 male	1	14	4	5	yes	−	yes
(*xid.bcl-x*-87)	2	14	4	6	yes		

*Normal peritoneal cells were recovered from 8-wk-old mice by gently washing peritoneal cavity with 10 ml of cold RPMI with 10% FCS. More than 9 ml was recovered from each mouse by this procedure.
‡Percentage includes only cells within the lymphoid gate.
§na, not applicable.
‖+ indicates wild-type Btk; − indicates mutant Btk.

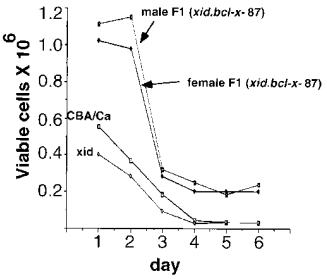


Figure 8. Comparison of in vitro viability. Splenic B cells from CBA/Ca, *xid*, male F1 (*xid.bcl-x*-87), and female F1 (*xid.bcl-x*-87) mice were cultured in 96-well flat-bottomed wells at 10^6 cells/ml in RPMI with 10% FCS for times indicated. From days 1 to 6, viability of spleen B cells was assessed by Trypan blue exclusion. Results are representative of three independent experiments.

normal control CBA/Ca (Fig. 8), however, the *bcl-x*-87 transgene substantially enhances the viability of the B cells in both the male and female F1(*xid.bcl-x*-87) mice.

Although the *bcl-x*-87 transgene increased the number and viability of B cells from *xid* F1 male mice, Bcl-x was incapable of correcting the reduced levels of serum IgM and IgG3 levels in the male F1 (*xid.bcl-x*-87) mice compared with *xid* mice (Fig. 9). Curiously, the levels of IgM in the F1 male (*xid.bcl-x*-87) mice were consistently lower than levels detected in the male *xid* mice. Serum IgM and IgG3 levels in the female F1 (*xid.bcl-x*-87) mice were indistinguishable from that detected in the parent C57BL/6 strain.

Interestingly, only the F1 offspring from the *bcl-x*-87 transgenic subline showed any correction of the *xid* phenotype. Table 1 indicates that anti-Ig–stimulated B cells from male F1 (*xid.bcl-x*-81) mice showed little difference in [^{3}H]thymidine incorporation when compared with the *xid* mice. The *bcl-x*-81 transgene expresses at high levels in developing B cells (26), but at low levels in peripheral B cells (Behrens, T., et al., unpublished data). Additionally, there was minimal increase in the peripheral B cell pool, little enhanced viability, and no difference in serum Ig levels between the F1 male (*xid.bcl-x*-81) in comparison with the *xid* males (data not shown).

Discussion

Our initial experiments focused on the hypothesis that the observed inability of *xid* B cells to proliferate to antigen receptor cross-linking stemmed from a failure to induce components of the cell cycle machinery. However, although there was an ~50% reduction in cyclin, cdk, and kinase activity in anti-Ig–stimulated *xid* B cells compared with normal controls, the complete complement of cyclins and cdks were induced in anti-Ig–stimulated *xid* B cells. Additionally, the kinetics of protein induction and active kinase induction in stimulated *xid* B cells was comparable to that detected in anti-Ig–treated control B cells. In data not shown, we confirm that the destruction of the cdk inhibitor, p27, after stimulation with anti-Ig antibodies was also indistinguishable in *xid* and control B cells, as was the appearance of the INK4 inhibitors, p18 and p19. Together these results suggest that the signals inducing cell cycle machinery after antigen receptor cross-linking in B cells from *xid* mice are intact although insufficiently amplified due to mutations in Btk.

The decision to undergo cellular replication occurs late in G1 at the restriction point, R, after which the cell is committed to undergo DNA replication and cellular division. Despite the diminished induction of cell cycle regulatory proteins in anti-Ig–stimulated *xid* B cells, these cells passed the restriction point and began entry into S phase, albeit some 12 h after control B cells. The ability of anti-Ig–stimulated *xid* B cells to pass the restriction point suggests that the insufficient amplification of the Ig-generated

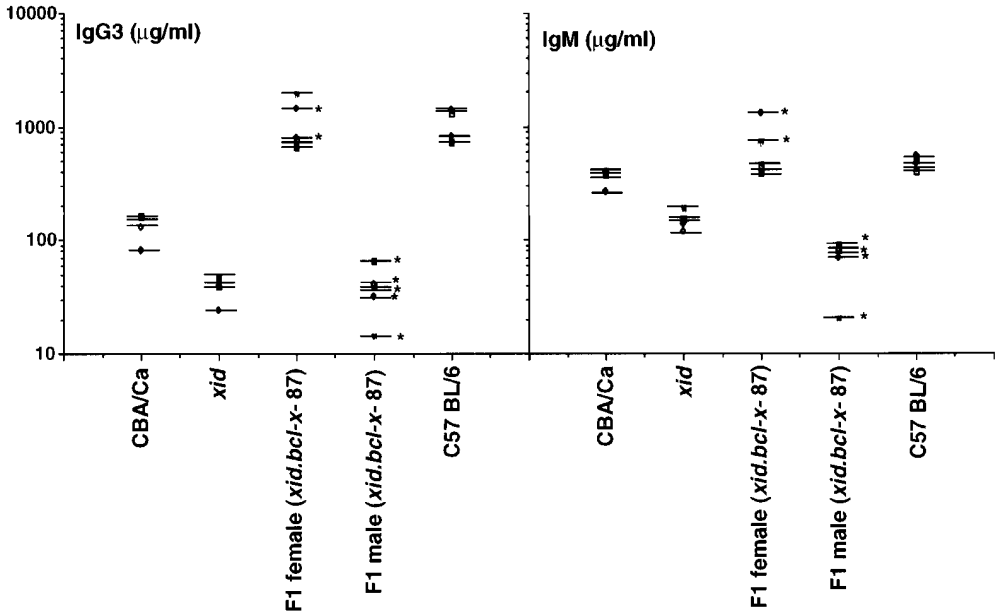


Figure 9. Serum immunoglobulin levels of IgM and IgG3. Sera was collected from 8-wk-old *xid* ($n = 5$), CBA/Ca ($n = 4$), F1 female ($n = 5$), and F1 male ($n = 6$) mice. Biotin-conjugated anti-IgG3 and anti-IgM reagents were used in combination with horseradish peroxidase–conjugated streptavidin in ELISAs to quantitate serum Ig levels. Serial dilutions of each sample were made and compared with standard curves generated by IgM or IgG3 preparations of known concentrations. Asterisks indicate mice carrying the *bcl-x* transgene.

signal simply resulted in a delay in passage through R, rather than an inability to pass R. Typically, unless severe damage to DNA is encountered, the entry into S phase guarantees completion of cell cycle progression. Indeed, there was significant cdk2- and cdc2-associated kinase activity present at time points during which the S and G2/M phases should have occurred; however, at this time at least 85% of the cells were apoptotic, indicating that the cell cycle machinery was still engaged despite the fact that the cells were dying.

Our finding that Bcl-x induction was diminished in anti-Ig–stimulated *xid* B cells is consistent with reports from Anderson et al. (29) and Choi et al. (28). We extend these observations by demonstrating that transgene driven expression of Bcl-x_L substantially corrects the inability of *xid* B cells to proliferate in response to stimulation with anti-Ig antibodies in vitro. It is noteworthy that we could detect some Bcl-x_L induction in anti-Ig–stimulated B cells from *xid* mice, indicating again that the signaling pathway leading to Bcl-x_L induction is not entirely disrupted. It seems likely that some critical level of Bcl-x_L protein is needed to permit anti-Ig–induced proliferation since reconstitution of the proliferative response was only detected in *xid* crosses with the *bcl-x*-87 transgenic mouse, which expresses high levels of Bcl-x_L in the peripheral B cell compartment. Despite the difference in phenotype, both the *bcl-x*-87 and *bcl-x*-81 transgenics are carried on the C57BL/6 background. Thus, it is unlikely that the correction in the *xid* phenotype observed with the male F1 (*xid.bcl-x*-87) mice is due to C57BL/6 background genes.

It is significant that the proportion of anti-Ig–responsive B cells in the male F1 (*xid.bcl-x*-87) mice and the rate of entry into S phase was still reduced compared with female F1 (*xid.bcl-x*-87) mice. We suspect this results from the still incompletely amplified signal through Btk leading to half-maximal induction of cell cycle regulatory proteins. These results suggest that the crippling effect of mutant Btk on the proliferative response is the failure to properly amplify signals generated by the antigen receptor. This is supported by our data showing the induction of both cell cycle regulatory proteins plus Bcl-x_L although at levels far below those detected in wild-type mice. Thus, one would predict that if signals generated at the antigen receptor could be significantly enhanced that perhaps B cells from *xid* mice could be stimulated to proliferate. Indeed, reports from Lindsberg et al. (40) showed that *xid* B cells can proliferate in the presence of anti-Ig reagents, which induces extensive and prolonged antigen receptor cross-linking.

The physiological significance of defective Bcl-x_L induction in relation to other immunodeficient traits is established in our male F1 (*xid.bcl-x*-87) mice, where there was a dramatic increase in the number of splenic and peritoneal B cells plus enhanced in vitro viability. The hyperreconstitution in splenic B cell numbers we observed is comparable to that reported in the original description of the *bcl-x*-87 transgenic line (27). We suspect that the expansion of the splenic B cell pool stems from the demonstrated enhancement of B cell viability coupled with inhibition of cell death rather than from enhancing B cell proliferation. The transgene, however, provided only minimal correction of the unusual phenotype of splenic B cells in the *xid* mice. Splenic B cells expressed IgM at levels intermediate between the *xid* and the CBA/Ca controls suggesting, at most, a partial correction of the atypical *xid* splenic B cell phenotype.

The number of B lymphocytes in the peritoneal cavity of male F1 (*xid.bcl-x*-87) mice was also expanded to numbers comparable to normal control mice. However, there was only a modest increase in the total number of CD5-expressing B cells compared with control mice. Furthermore, the expanded peritoneal B cell population in the male F1 (*xid.bcl-x*-87) mice was not the CD5 "sister" population since the B cells did not express Mac-1 nor the characteristic ratio of IgM^{hi}/IgD^{lo} (data not shown). The inability to reconstitute CD5 B cells is difficult to interpret since it has not been established that the *bcl-x* transgene is expressed in the CD5 B cell compartment; normal CD5 B cells express Bcl-x_L constitutively (29), making the efficiency of transgene-driven Bcl-x_L expression difficult to establish in these cells. Alternatively, we cannot rule out the possibility that there are additional defects in the Btk signal-transduction cascades that effect development of this particular B cell subset. Regardless of the mechanism, the poor reconstitution of the CD5 B cell population may partially explain the failure to correct the serum IgM and IgG3 deficiencies since CD5 B cells have been shown to be a major source of these isotypes in vivo (41).

Our demonstration that anti-Ig–stimulated *xid* B cells can induce the complete complement of cell cycle regulatory proteins while the cells continue to undergo apoptosis indicates that induction of cell cycle machinery alone is insufficient to ensure cellular replication. At least for B lymphocytes, induction of cell cycle machinery must be coupled with antiapoptotic proteins for proliferation. Furthermore, our results suggest a specific requirement for the antiapoptotic protein Bcl-x, since Woodland et al. has shown that transgene driven expression of Bcl-2 in *xid* mice could not support anti-Ig induced proliferation (39). Therefore, Bcl-x_L may have a unique and nonredundant role in maintaining viability during antigen-driven B cell expansion.

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