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Impaired Ca^{2+} mobilization by X-linked agammaglobulinaemia (XLA) B cells in response to ligation of the B cell receptor (BCR)

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SUMMARY

XLA bone marrow samples were shown to contain B cells expressing IgM, and pre-B cells that express the μ-surrogate light chain ($\mu\psi$LC) complex, albeit at a reduced frequency to that found in normal bone marrow. Antibody ligation of μ heavy chain on these cells and an XLA B cell line did not induce a Ca^{2+} flux, whereas ligation of μ heavy chain on normal bone marrow cells, $\mu\psi LC^+$ pre-B cell lines and an IgM^+ B cell line did. The block in XLA B cells was not due to a defect in the basic mechanism of Ca^{2+} flux generation, as the cells responded well to thapsigargin. In addition, the defect did not affect T cells, which were shown to respond to CD3 antibody with a Ca^{2+} flux. Ligation of μ heavy chain on XLA bone marrow cells did, however, activate tyrosine kinases, resulting in tyrosine phosphorylation of a cellular protein with a molecular weight of approximately 115 kD. These results indicate that Btk may be necessary for the generation of the Ca^{2+} flux in response to ligation of μ heavy chain on B cells and $\mu\psi LC^+$ pre-B.

Keywords X-linked agammaglobulinaemia pre B cells Btk calcium mobilization signal transduction

INTRODUCTION

XLA is an inherited immunodeficiency characterized by a lack of circulating B cells and serum immunoglobulin [1]. The phenotype appears to result from a block in pre-B cell development and affected boys have pro- and pre-B cells in the bone marrow, but few or no mature B cells [2,3]. The gene responsible for XLA was recently cloned [4,5] and found to be a Src-like protein tyrosine kinase named Bruton's tyrosine kinase (Btk). It is expressed in B lineage cells from the pro-B to the B cell stages, but not in plasma cells, and is necessary for normal B cell development. It is also expressed in myeloid cells [6–8].

There is some evidence that Btk is part of the B cell receptor (BCR) signalling pathway. Stimulation of surface μ-chain on human B cell lines results in phosphorylation of Btk and an increase in its kinase activity [9,10]. Similarly, ligation of BCR on B cells from xid mice, which have a mutation in the PH domain of Btk but still produce B cells, gives rise to a transient increase in intracellular Ca^{2+} which is 50% lower than the normal response and is insufficient to bring about the normal subsequent synthesis of DNA [11]. These experiments show that Btk is activated following ligation of surface IgM and may be required for normal Ca^{2+} mobilization during BCR signalling.

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Btk may also be important for signalling through the μ-surrogate light chain ($\mu\psi$LC) complex on late pre-B cells and subsequent maturation into sIgM-bearing B cells [12]. In the mouse, targeted disruption of either the $\lambda 5$ gene that codes for a pseudo light chain component of the $\mu\psi$LC complex or the immunoglobulin heavy chain gene prevents surface expression of the $\mu\psi$LC complex and results in arrested or delayed B cell development [13–15]. Moreover, the expression of mutant μ-chain in the $\mu\psi$LC complex abrogates immunoglobulin heavy chain gene allelic exclusion and possibly also immunoglobulin light chain gene rearrangement [16–18]. Other work has shown that ligation of the $\mu\psi$LC complex on human pre-B cell lines generates a Ca^{2+} signal and intracellular protein tyrosine phosphorylation consistent with an important signalling function during B cell development [19].

We show here that B lineage cells in the bone marrow of patients with XLA express μ heavy chain. Some of these B cells co-express ψLC while others co-express light chain. Antibody stimulation of μ heavy chain on XLA bone marrow cells and an XLA B cell line did not induce an intracellular Ca^{2+} flux, whereas stimulation of normal control bone marrow B cells, a B cell line, and $\mu\psi LC^+$ pre-B cell lines did. Ligation of μ heavy chain on XLA bone marrow cells did, however, activate a tyrosine kinase signalling pathway. Our results indicate that Btk is required for Ca^{2+} mobilization during BCR signalling, but not for tyrosine kinase signalling.

SUBJECTS AND METHODS

Subjects

Two XLA patients were included in this study. Patient 1 gave bone marrow at 10 years of age and patient 2 gave bone marrow samples twice, at ages 9 and 12 years. The diagnosis of XLA was made in each case at Great Ormond Street Hospital for Children, London. Both patients have been shown to have mutations in the *Btk* gene [4,20]. Patient 1 has a G→T point mutation at position 2038 causing a premature stop codon at residue 636. This gives rise to a predicted truncated protein lacking the C terminal 24 amino acids which includes several highly conserved residues. Patient 2 has a deletion of approximately 500–600 base pairs affecting the N terminal segment of the protein.

Bone marrow aspirates were taken from the iliac crest of XLA patients and an X-linked hyper-IgM patient who were undergoing necessary medical treatment under general anaesthetic at Great Ormond Street Hospital. A control sample was also obtained from a child providing marrow for transplantation. Further control bone marrow samples were obtained from normal adult volunteers under local anaesthetic (1% lignacaine; AAH Hospital Services, South Ruislip, UK). In all cases, bone marrow was taken with informed consent and ethical approval. The marrow was taken into 1 U/ml of preservative-free heparin and mononuclear cells isolated by density centrifugation over Ficoll–Paque (Pharmacia LKB, Milton Keynes, UK), then washed in RPMI 1640 medium (GIBCO, Paisley, UK).

Cell lines

B lineage cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), 2 mM L-glutamine (Flow Labs, Irvine, UK) and 50 μg/ml of gentamicin (Roussel Labs Ltd., Uxbridge, UK) at 37°C in 5% CO_2 in air. The expression of selected B lineage markers and immunoglobulin gene rearrangements of most of the lines have been described previously [8]. BLCL-276 is an Epstein–Barr virus (EBV)-transformed B cell line expressing surface IgM ($\mu_2\lambda_2$) that was derived from a patient with XLA [21] (kindly provided by Dr M. de Weers, Leiden, The Netherlands). Lines 697, Blin-1 and Nalm-6 are human pre-B cells lines expressing the $\mu\psi$LC complex on the surface, and Daudi is a Burkitt's lymphoma B cell line [22]. The identity and lineage specificity of the lines were confirmed throughout the study by minisatellite restriction fragment length polymorphism (RFLP) analysis [23].

Antibodies and flow cytometry

Antibodies used for flow cytometry were PE-conjugated F(ab′)$_2$ goat anti-human immunoglobulin μ heavy chain (Tago Inc., TCS, Botolph Claydon, UK); CD19-PE (4G7; Becton Dickinson, Oxford, UK); anti-ψLC antibody (SLC-1) kindly supplied by M. Cooper (Birmingham, AL) [12]; and mouse anti-human κ (A8B5) and λ (N10/2) (Dako, High Wycombe, UK) light chain. Fluorochrome-, species- and isotype-matched controls were purchased from the same suppliers.

For flow cytometric analysis, $2{\cdot}5\times10^5$ cells were incubated with SLC-1 or anti-light chain antibody for 20 min at 4°C, washed twice in PBS supplemented with 2% FCS and 0·02% (w/v) sodium azide (Sigma, Poole, UK), and then incubated for 20 min at 4°C with FITC-conjugated F(ab′)$_2$ goat anti-mouse IgG (Tago) in the presence of 2% FCS and 0·5% normal goat serum. Cells were then incubated with anti-μ-PE or CD19-PE, and washed twice before analysis. When CD19-PE was used, the binding sites of the anti-mouse IgG reagent were blocked with 20% normal mouse serum before and during incubation with CD19-PE. Analysis was performed using a FACScan (Becton Dickinson) using Lysis II or DataMATE 2.2 software (Applied Cytometry Systems, Dinnington, UK).

Detection of intracellular calcium ion fluxes

The method for detection of intracellular Ca^{2+} fluxes was essentially as described by Rijkers *et al*. [24]. Briefly, cells were loaded with the Ca^{2+}-sensitive dye, Fluo-3 (Calbiochem-Novabiochem, Nottingham, UK) and then stained for 15 min at room temperature with CD19-PE to gate on B lineage cells (excluding plasma cells). CD19 MoAb has previously been shown not to interfere with B cell signalling in response to BCR ligation [25].

Data were acquired for 30 s or 60 s and the cells then stimulated with 25 or 50 μg/ml of μ-chain-specific F(ab′)$_2$ goat anti-human IgM antibody (Jackson Labs, Stratech Scientific, Luton, UK) in order to ligate surface IgM or the $\mu\psi$LC complex. The same concentration of F(ab′)$_2$ goat IgG was used as a negative control antibody (Jackson). The positive control stimulus was 1 μM thapsigargin (Calbiochem-Novabiochem) which crosses the cell membrane and releases intracellular Ca^{2+} stores by inhibition of the endoplasmic reticulum Ca^{2+}-ATPase [26,27]. CD3 antibody (OKT3) was used to stimulate bone marrow T lymphocytes. Data acquisition and analysis were performed on a FACScan with Chronys software (Becton Dickinson). Intracellular Ca^{2+} ion concentrations were expressed as mean fluorescence channel number and plotted against time.

Detection of tyrosine phosphorylation

To detect tyrosine phosphorylation of intracellular proteins in response to μ heavy chain ligation, 5×10^6 cells in 50 μl of medium were incubated for 5 min with 100 μg/ml of F(ab′)$_2$ goat anti-human IgM or with 100 μg/ml of F(ab′)$_2$ goat IgG as an unstimulated control. A cell lysate was prepared by adding 50 μl of boiling non-reducing Laemmli loading buffer and boiling for 5 min. DNA was sheared by drawing the sample repeatedly through a 25 G needle. Cell lysates were reduced before loading on the gel by boiling with 2·5% 2-mercaptoethanol for 8 min. Samples were electrophoresed on a 10% SDS–PAGE gel and electroblotted onto PVDF membrane (Millipore, Watford, UK) in CAPS buffer (Sigma) [28]. The membrane was then blocked for 20 h with 5% (w/v) non-fat milk protein (Marvel, Premier Brands UK Ltd., Stafford, UK) in PBS and 0·02% thimerosal (Sigma). Tyrosine phosphorylated proteins were detected by incubation with 1 μg/ml of anti-phosphotyrosine antibody (4G10; UBI, TCS) for 2 h, followed by 3·65 μg/ml of horseradish peroxidase-conjugated rabbit anti-mouse IgG2b (Serotec, Oxford, UK). Antigen–antibody reactions were visualized by enhanced chemiluminescence (ECL; Amersham International, Aylesbury, UK) according to the manufacturer's instructions, with exposure time periods ranging from 5 s to 15 min.

RESULTS

A population of XLA bone marrow cells expresses the $\mu\psi$LC complex

Analysis of XLA bone marrow showed that a population of cells co-expressed μ and ψLC on the cell surface (Table 1). XLA bone marrow had a smaller proportion of B lineage (CD19$^+$) cells in the

Table 1. Expression of surface ψLC on B cells in bone marrow of XLA patients and normal donors

	$CD19^+$*	$CD19^+$ ψLC^+*	μ^+*	μ^+ ψLC^+	μ^+ LC^+*
XLA (patient 2, exp. 1)	6	0·7 (12)	5	0·8 (16)	5
XLA (patient 2, exp. 2)	6	0·6 (11)	8	2·0 (24)	1.4
XLA (patient 1)	2	0·4 (17)	8	0·9 (12)	4
Normal adult 1	34	9·8 (29)	41	3·9 (10)	27
Normal adult 2	21	5·5 (26)	20	1·1 (6)	16
Normal child	26	6·0 (23)	36	1·6 (4)	22

Bone marrow cells from controls and patients with XLA were double-stained with anti-ψLC-FITC and CD19-PE or anti-μ-PE to identify populations with the phenotypes $CD19^+$, $CD19^+$ ψLC^+, μ^+, μ^+ ψLC^+ and μ^+ LC^+. The sizes of these populations are expressed as a percentage of the bone marrow lymphoid cells and in parentheses as a percentage of the $CD19^+$ or μ^+ bone marrow cells.

* The difference between normal and XLA bone marrow samples was significant ($P < 0{\cdot}05$).

FACScan gated lymphoid population than did normal marrow (2–6% of XLA cells were $CD19^+$, compared with 21–34% for normal donors). The $CD19^+$ ψLC^+ population comprised a smaller percentage of the total lymphoid cells in XLA (0·4–0·7%) than in normal samples (5·5–9·8%). When the size of the $CD19^+$ ψLC^+ population was expressed as a percentage of total B lineage ($CD19^+$) cells, this population was still under-represented in XLA bone marrow (11–17% in XLA compared with 23–25% in control bone marrow).

The proportion of μ^+ cells was also lower in XLA than in normal individuals (5–8% and 20–41%, respectively), but the percentage of μ^+ ψLC^+ cells was similar in XLA and control bone marrow. Table 1 also shows that the percentage of mature B cells expressing surface IgM was greatly reduced in XLA bone marrow (1·4–5% in XLA compared with 16–27% in controls).

Absence of Ca^{2+} mobilization by XLA B cells and pre-B cells in response to BCR cross-linking

Antibody ligation of μ heavy chain elicited no Ca^{2+} response in B lineage cells from the fresh bone marrow of two XLA patients (Fig. 1c,d). In contrast, the same experiment performed with the bone marrow of a child with X-linked hyper-IgM syndrome or

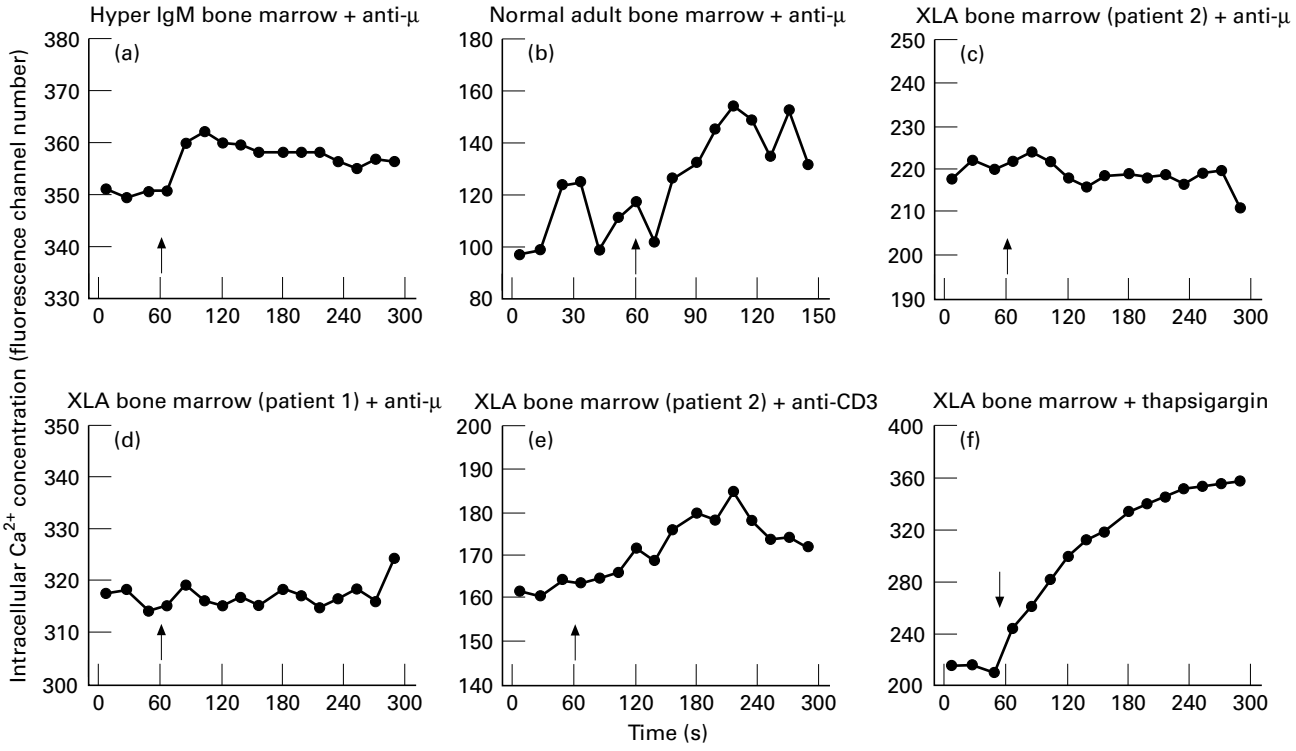

Fig. 1. B lineage cells from XLA patients did not make a Ca^{2+} flux in response to antibody ligation of μ heavy chain. Bone marrow cells from a patient with hyper-IgM syndrome (a), a normal adult (b) and two XLA patients (c–f) were loaded with the Ca^{2+}-sensitive dye Fluo-3 and stimulated at 60 s (arrow) with antibody to cross-link surface μ heavy chain (a–d), CD3 antibody (e) or 1 μM thapsigargin (f). Note: (a, b, c, d, f), events gated to include only B lineage ($CD19^+$) cells. (e) Events gated to exclude B lineage cells.

from normal adults gave a clear-cut Ca^{2+} response (Fig. 1a,b). XLA B lineage cells responded with a Ca^{2+} flux to 1 μM thapsigargin (Fig. 1f) and the T cells of XLA bone marrow were shown to respond with a Ca^{2+} flux to CD3 antibody (Fig. 1e). This suggests that Btk is required for the generation of a Ca^{2+} signal on ligation of IgM on B cells and the μ-chain of the $\mu\psi$LC complex of pre-B cells.

Ligation of the $\mu\psi$LC complex expressed on the surface of pre-B cell lines 697 and Nalm-6 and on a mature B cell line (Daudi) gave a Ca^{2+} signal (Fig. 2), confirming that the complex can signal on pre-B cells. The pre-B cell line 697 and B cell line Daudi gave a similar rapid Ca^{2+} transient, peaking around 60–80 s after addition of the stimulating antibody at 30 s. In agreement with previous work, Nalm-6 made a response of smaller amplitude, reaching a peak after about 140 s [19]. The weaker response of Nalm-6 may be due to the absence of CD45, which is important for signalling through the B cell receptor [29]. In contrast, when a $sIgM^+$ EBV-transformed B cell line derived from the bone marrow of an XLA patient (BLCL-276) was tested for signalling in response to ligation of the B cell receptor, no Ca^{2+} flux was detected, although it responded normally to thapsigargin (Fig. 3).

BCR cross-linking of XLA B lineage cells activates protein tyrosine kinases

Bone marrow cells from one XLA patient (patient 1) were tested for tyrosine kinase activation in response to ligation of μ heavy chain. Controls in this experiment were the pre-B cell lines 697 and Blin-1 (Fig. 4). A band of approximately 115 kD was tyrosine phosphorylated after 5 min of stimulation of XLA bone marrow with antibody. A band of approximately the same size (112 kD) was seen on stimulation of the pre-B cell lines 697 and Blin-1. Phosphorylation of a band of 76 kD was also observed on stimulation of the pre-B cell lines, and 697 had an additional substrate of 70 kD. This experiment shows that pre-B cells or B cells in XLA bone marrow can respond to ligation of the μ heavy chain by tyrosine phosphorylation of a substrate of approximately 115 kD, and that this response is shared by the pre-B cell lines 697 and Blin-1.

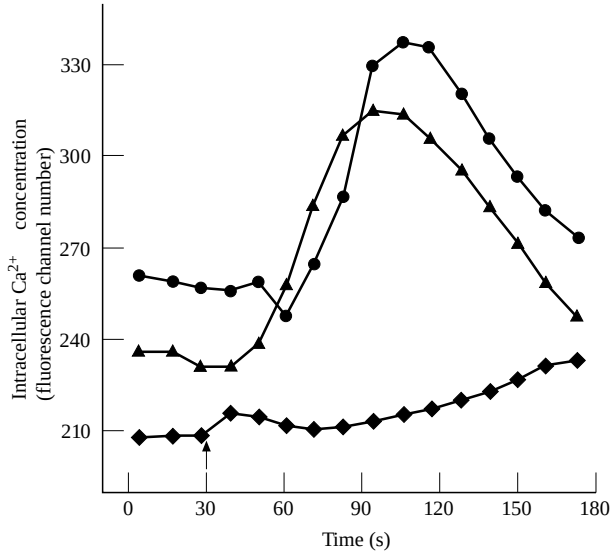

Fig. 2. Human B cell and pre-B cell lines generated a Ca^{2+} flux on antibody ligation of IgM or $\mu\psi$LC complex. The B cell line Daudi (●) and the pre-B cell lines 697 (▲) and Nalm-6 (◆) which express the $\mu\psi$LC complex were loaded with the Ca^{2+}-sensitive dye Fluo-3 and stimulated with antibody to ligate μ heavy chain after 30 s (arrow).

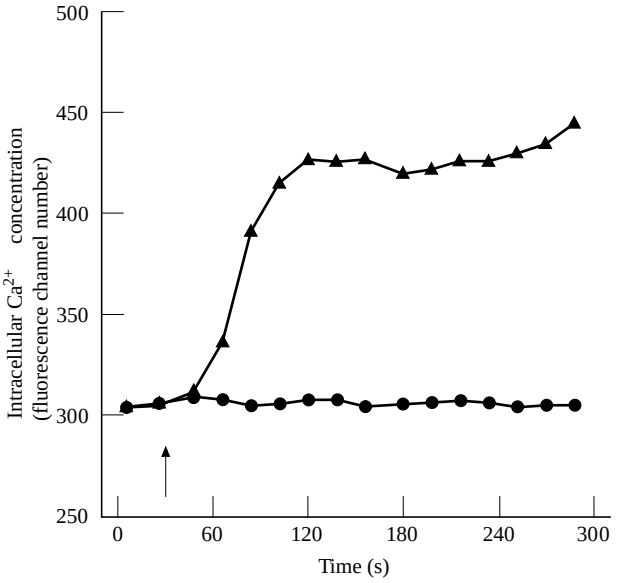

Fig. 3. The XLA Epstein–Barr virus (EBV) line BLCL-276 did not make a Ca^{2+} response to antibody ligation of IgM. BLCL-276 was loaded with the Ca^{2+}-sensitive dye Fluo-3 and was stimulated after 30 s (arrow) either with antibody to cross-link surface IgM (●) or with 1 μM thapsigargin (▲).

DISCUSSION

Btk is a cytoplasmic protein tyrosine kinase that has been implicated in signalling through the BCR and several cytokine receptors [9,10,30–40]. It is phosphorylated and its kinase activity increased on ligation of the BCR on murine and human cell lines [9,10,30,31]. BCR signalling is defective in the murine immunodeficiency xid which is caused by a point mutation in the pleckstrin homology domain of the *Btk* gene [41]. Ligation of the BCR on xid B cells results in a Ca^{2+} flux that is 50% smaller in amplitude than that obtained with normal B cells, and the signal is insufficient to bring about the normal subsequent synthesis of DNA [11].

In the present study, we show that surface μ^+ cells in XLA bone marrow were unable to mobilize Ca^{2+} in response to BCR

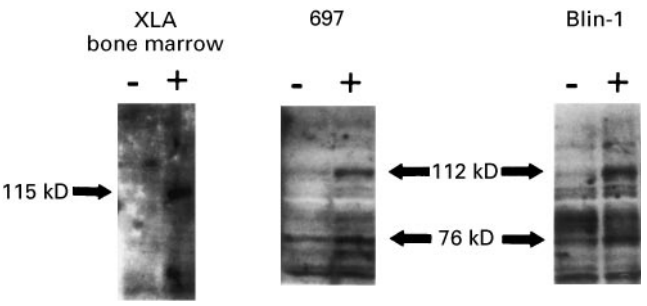

Fig. 4. Cross-linking of μ heavy chain resulted in tyrosine phosphorylation of a protein of about 115 kD in bone marrow cells from a patient with XLA. Tyrosine phosphorylation was analysed by Western blotting with the anti-phosphotyrosine antibody 4G10. The pre-B cell lines 697 and Blin-1 are shown as positive controls.

ligation, whereas significant responses were obtained with μ^+ cells in normal bone marrow and in bone marrow from X-linked hyper-IgM syndrome. In addition, no calcium flux was observed in an XLA B cell line on BCR cross-linking. In contrast, two pre-B cell lines Nalm 6 and 697 expressing surface $\mu\psi$LC gave detectable calcium responses. These lines have been shown by us to express $\mu\psi$LC and cytoplasmic Btk, but no light chain [8]. Nalm 6 has been reported previously to give a calcium response on cross-linking of $\mu\psi$LC [19], but in another study no calcium response was obtained with 697 [42]. The reason for this discrepancy with 697 is not known.

In contrast to the calcium responses, XLA bone marrow cells responded to BCR ligation by tyrosine phosphorylation of a 115-kD cellular protein. Similar phosphorylated proteins have been described in pre-B cell lines and shown to correspond to the p85 and p110 subunits of phosphatidylinositol 3-kinase [42]. Our results provide evidence that Btk is required for the normal generation of Ca^{2+} fluxes by human B cells in response to cross-linking of the BCR, but is not necessary for protein tyrosine kinase activation. This suggests that the Ca^{2+} transient may be dependent upon and subsequent to Btk activation, while activation of other tyrosine kinases occurs independently of both Btk and the Ca^{2+} flux. Consistent with this suggestion, ligation of the BCR in murine B cell lines is known to activate Btk after the Src-family tyrosine kinases Lyn and Blk [30]. The inability to detect a BCR-induced Ca^{2+} signal in XLA B cells suggests that the defect is more severe than in xid B cells, in which a Ca^{2+} signal is detectable, albeit severely reduced. This finding is consistent with the very few mature B cells typically found in XLA, whereas xid mice do have circulating B cells. Our findings are in agreement with the recent study by Takata & Kurosaki, who showed that phosphorylation of phospholipase C (PLC)-γ2 and subsequent Ca^{2+} calcium mobilization in response to BCR ligation were greatly reduced in a Btk-deficient cell line [43].

Bone marrow from our XLA patients was found to contain $CD19^+$ and μ^+ B lineage cells that co-express ψLC on the surface, presumably as part of the $\mu\psi$LC complex, although the proportion of this population with respect to the total number of lymphoid cells or $CD19^+$ cells was markedly lower than that seen in bone marrow from normal donors. Our data suggest that the ψLC^+ stage of pre-B cell differentiation is under-represented in XLA. This observation is in agreement with other studies showing that the pre-B cell compartment is reduced in XLA [2,3] and is consistent with a requirement for Btk for optimal generation of ψLC^+ pre-B cells.

In our experiments with bone marrow from XLA patients and normal controls it was not possible to distinguish between BCR-induced Ca^{2+} mobilization in B cells and pre-B cells. Accordingly, it was not possible to show unequivocally that ligation of the $\mu\psi$LC complex on pre-B cells in normal bone marrow generated a Ca^{2+} flux. Nevertheless, it is likely that normal bone marrow pre-B cells expressing the $\mu\psi$LC complex mobilize Ca^{2+} on ligation of μ heavy chain, because ligation of the BCR clearly generates a Ca^{2+} signal in pre-B cell lines [19]. The complete absence of a Ca^{2+} signal in XLA bone marrow in which approximately 15% of the gated cells were ψLC^+ pre-B cells suggests that Ca^{2+} mobilization by the $\mu\psi$LC complex on pre-B cells as well as the BCR on more mature IgM^+ B cells is defective in XLA.

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