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Functional Analysis of Peripheral Blood B Cells in Patients with X-Linked Agammaglobulinemia

Shigeaki Nonoyama,* Satoshi Tsukada,† Tomoki Yamadori,‡ Toshio Miyawaki,‡ Yin Zhu Jin,§ Chiaki Watanabe,* Tomohiro Morio,§ Jun-Ichi Yata,§ and Hans D. Ochs* X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disease caused by mutations of Bruton tyrosine kinase (Btk); Btk plays an essential role in the development of mature B cells. However, small numbers of B cells (“leaky B cells”) are present in the peripheral blood of most XLA patients. In this study, we analyzed the function of these leaky B cells obtained from XLA patients. Enough numbers of B cells were available for analysis from five of nine XLA patients originally screened. Sequence analysis revealed missense mutations of Btk in four of the five XLA patients. No mutation was found in the coding region of Btk in one patient. Western blotting and/or flow cytometric analysis failed to detect Btk protein in all five patients. B cells isolated from peripheral blood of these XLA patients were CD19+, CD20+, CD19+, and CD21-. If stimulated with anti-CD40 and IL-4, XLA B cells proliferated normally and produced significant amounts of IgE. Anti-CD40 stimulation of XLA B cells resulted in normal expression of CD23. In addition, three of the five XLA patients studied were immunized with bacteriophage ϕX174 and produced low but detectable levels of antiphage-specific Ab. Similarly, X-linked immunodeficiency mice, which carry a missense mutation in Btk, produced substantial amounts of antiphage Ab. These results indicate that CD40 signaling is intact in B cells lacking demonstrable Btk, and that leaky B cells in XLA patients can proliferate, undergo isotype switching, and differentiate into specific Ab-producing cells. The Journal of Immunology, 1998, 161: 3925–3929.

1 Abbreviations used in this paper: XLA, X-linked agammaglobulinemia; xid, X-linked immunodeficiency; K

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Phenotypic analysis of freshly isolated B cells and B-LCLs was performed by flow cytometry using FITC- or phycoerythrin (PE)-conjugated anti-CD5, anti-CD19, anti-CD20, anti-CD21 (Coulter, Hialeah, FL), and anti-CD23 (Becton Dickinson, San Jose, CA).

Sequence analysis of the Btk gene

Total RNA was isolated from PBMCs obtained from patients with XLA or from B-LCLs established from patients with XLA. First-strand cDNA was synthesized with the Superscript Preamplification System kit (Life Technologies, Gaithersburg, MD). PCR was performed using primer pairs covering the entire coding region of Btk cDNA as described previously (5). Direct sequencing was performed using a modified dideoxynucleotide chain termination method and the Pfu DNA-sequencing kit (Stratagene, La Jolla, CA). The mutations observed in the cDNA of patients were confirmed by sequencing genomic DNA.

Btk protein analysis

Btk protein expression was determined either by Western blot analysis or by flow cytometry using anti-Btk mAb (48-2H) (4). For Western blot analysis, 1 × 10⁵ B-LCLs from normal control subjects and patients with XLA were suspended in 1 ml of lysis buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mmol/L PMSF, 0.5% aprotinin, and 10 μg/ml leupeptin at pH 7.5 and kept on ice for 10 min. Protein concentration was determined in each lysate using a protein assay kit (Bio-Rad, Hercules, CA). From each sample, 20 μg of total protein was loaded onto an SDS-polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride Immobilon-P membrane (Millipore, Bedford, MA). After blocking with 10% nonfat milk, the membranes were incubated with anti-Btk mAb at 2 μg/ml or with anti-actin mAb (Sigma) at 1 μg/ml for 1 h at room temperature. After washing, membranes were incubated with alkaline phosphatase-conjugated anti-mouse IgG (Promega, Madison, WI) diluted 1:7500 for 1 h. Images were obtained with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer-Mannheim, Indianapolis, IN).

Flow cytometric analysis of intracytoplasmic Btk protein expression was performed as described previously (21). Briefly, PBMCs obtained from normal control subjects and patients with XLA were stained with PE-conjugated anti-CD14 mAb (Dako, Kyoto, Japan). Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After washing, cells were reacted with anti-Btk mAb 48-2H for 20 min at 4°C and subsequently stained with FITC-conjugated anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) for 20 min at 4°C. Expression of Btk protein in monocytes was analyzed by gating for CD14 cells using a FACScan (Becton Dickinson).

Stimulation of B cells with anti-CD40 mAb

To induce B cell proliferation, 2.5 × 10⁴ freshly isolated B cells were cultured for 4 days in 96-well flat-bottom microculture plates at a final volume of 200 μl of RPMI 1640 supplemented with 10% FCS (HyClone, Logan, UT), 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml of streptomycin (complete medium) with anti-CD40 mAb G28-5 (1 μg/ml) and IL-4 (100 U/ml). After 16 h of culture, cells were cultured in medium containing 1 μCi of [3H]thymidine. To induce CD23 expression, 10 × 10⁴ purified B cells were cultured in 500 μl of complete medium in the presence of mAb G28-5 (1 μg/ml) and IL-4 (100 U/ml). After 12 days of culture, supernatants were collected and tested for IgE concentrations using an ELISA technique as described previously (19, 20).

Immunization with bacteriophage φX174

Bacteriophage φX174 (phage), prepared as described previously (22, 23), was i.v. administered twice (6 wk apart) to three XLA patients (patients 2, 3, and 4) at the standard dose of 2 × 10⁸ plaque-forming units/kg body weight. A total of 12 healthy, young, male volunteers were immunized similarly; sera were collected from both patients and controls immediately before immunization and at 1, 2, and 4 wk after each immunization.

Flow cytometric analysis of intracytoplasmic Btk protein expression was performed as described in the Methods section. Data were compared using the Student t test and StatView 4.01 software (Abacus Concepts, Berkeley, CA).

Results

Laboratory findings of XLA patients

The five XLA patients selected for this study are from five unrelated families; each family has a history indicating X-linked inheritance. All patients had low or undetectable levels of serum IgG, IgA, and IgM, and the number of circulating CD20⁺ B cells was <1.0% as determined by flow cytometry (Table I). Western blot analysis revealed that B-LCLs established from four patients failed to express Btk (Fig. 1A). FACScan analysis was used to study patient 5, from whom a B-LCL could not be established; Btk protein was not detectable in the monocytes of this patient (Fig. 1B). Monocytes from mother of patient 5 showed a biphasic pattern of Btk expression (21), indicating that the mother was a carrier of XLA (Fig. 1B). These results demonstrated that all five patients lacked Btk protein expression. Sequence analysis of Btk revealed four different point mutations resulting in single amino acid substitutions (Table I). In patient 5, we were unable to identify a mutation within the coding region of the Btk gene.

Flow cytometric analysis of freshly isolated B cells from each of the five XLA patients revealed that the recovered B cells were CD1⁹⁺, CD20⁺, CD21⁺, CD23⁺, and CD5⁻ (data not shown). Similarly, B-LCLs established from four patients (patients 1–4) were CD1⁹⁺, CD20⁺, CD21⁺, and CD5⁻, although they were uniformly positive for CD23 (data not shown).

Proliferative responses of B cells

B cell proliferation was studied by stimulating freshly isolated B cells with anti-CD40 and IL-4. Purified B cells from XLA patients and normal controls showed comparable [3H]thymidine uptake if cultured with anti-CD40 and IL-4 (Fig. 2). The stimulation index

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**Table I. Laboratory findings of the XLA patients studied**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (year)</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>B Cells (%)</th>
<th>Btk Protein Expression</th>
<th>Mutation of Btk</th>
</tr>
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<tbody>
<tr>
<td>Pt. 1</td>
<td>6</td>
<td>80</td>
<td>&lt;7</td>
<td>16</td>
<td>&lt;1.0</td>
<td>—</td>
<td>Arg28→His</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>12</td>
<td>37</td>
<td>&lt;7</td>
<td>5</td>
<td>&lt;1.0</td>
<td>—</td>
<td>Thr13→Pro</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>15</td>
<td>344</td>
<td>&lt;7</td>
<td>33</td>
<td>&lt;1.0</td>
<td>—</td>
<td>Leu408→Pro</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>12</td>
<td>12</td>
<td>&lt;17</td>
<td>17</td>
<td>&lt;1.0</td>
<td>—</td>
<td>Arg325→Gln</td>
</tr>
<tr>
<td>Pt. 5</td>
<td>24</td>
<td>177</td>
<td>&lt;34</td>
<td>34</td>
<td>&lt;1.0</td>
<td>—</td>
<td>No mutation in the coding region</td>
</tr>
</tbody>
</table>

*Serum IgG concentrations were measured before IVIG therapy was introduced.

*Percentages of CD20⁺ cells in peripheral blood are shown.

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**Statistics**

Data were compared using the Student t test and StatView 4.01 software (Abacus Concepts, Berkeley, CA).
observed in normal B cells was 52.1 ± 29.2 (mean ± SD), and the index observed in XLA B cells was 60.3 ± 41.6 (p = 0.73, Student’s t test).

**Induction of CD23 expression by B cells following stimulation with anti-CD40**

B cells obtained from three XLA patients (patients 1, 2, and 5) and from normal subjects were stimulated with anti-CD40 for 16 h, and the expression of CD23 was examined by flow cytometry. B cells from XLA patients expressed anti-CD40-induced CD23 at concentrations comparable with those of normal B cells (patient 1, 18.2%; patient 2, 14.0%; patient 5, 17.7%; and controls, 21.7%). Typical results observed in patient 1 and in a normal control are shown in Figure 3.

**IgE production by B cells stimulated with anti-CD40 and IL-4**

As shown in Figure 4, purified B cells from all normal subjects (n = 5) produced IgE if cultured for 12 days in the presence of anti-CD40 mAb and IL-4. Mean ± SD of IgE was 16,107 ± 5,096 pg/ml. Using the same culture system, XLA B cells were studied for IgE production (Fig. 4). Although the amount of IgE produced varied, B cells from all five XLA patients produced IgE at a concentration of 13,795 ± 9,429 pg/ml (mean ± SD), which is not significantly different from normal controls (p = 0.75, Student’s t test).
patients produced very low but detectable amounts of antiphage Ab, although isotype switching was not observed (% IgG = 0%) after a secondary immunization (Fig. 5).

Following immunization with bacteriophage dX174, xid mice produced substantial amounts of antiphage Ab, although the Ab titers produced by xid mice were significantly lower than those produced by control mice. The peak $K_v$ (mean ± SD) after primary immunization was $0.859 \pm 0.301$ in xid mice and $6.595 \pm 1.979$ in control mice ($p < 0.01$, Student's $t$ test); the peak $K_v$ after secondary immunization was $147.9 \pm 95.9$ in xid mice and $406.7 \pm 165.1$ in control mice ($p < 0.01$, Student's $t$ test). The isotype switching observed after a secondary immunization in xid mice (% IgG = 64%) was comparable with that observed in normal congenic mice (% IgG = 79%).

Discussion

Patients with XLA due to a mutation of Btk may present with variable clinical phenotypes and laboratory findings (24). Although a very low number of circulating B lymphocytes is a hallmark of XLA, a B cell number in the peripheral blood of >0.5% is often associated with a milder phenotype and with the production of limited quantities of specific Ab following immunization (24). Using standard techniques, we were able to isolate sufficient B lymphocytes to perform functional analysis from the peripheral blood of five of nine XLA patients available for study. When stimulated with anti-CD40 mAb and IL-4, B lymphocytes from XLA patients proliferated normally and produced near normal quantities of IgE, although enrichment of B cells from the blood of XLA patients resulted in a cell population consisting of only 50 to 60% CD20 $^+$ B cells as compared with >90% CD20 $^+$ B cells isolated from control blood. Furthermore, CD23 expression by XLA B lymphocytes following stimulation with anti-CD40 mAb was comparable with that of normal control B cells. The demonstration of absent Btk expression in EBV-induced lymphoblasts and in monocytes from all five of the XLA patients studied suggests that signaling through CD40 occurs normally in patient B cells despite the absence of Btk. In contrast, B cells isolated from xid mice do not proliferate (17) and fail to up-regulate CD80 and CD86 expression (18) following ligation of CD40, suggesting that CD40 signaling is defective in xid mice with the Arg28 $\rightarrow$ Cys mutation of Btk. One possible explanation for this discrepancy is a difference in the potency of CD40 stimulation between the anti-CD40 mAb used in xid mice and the anti-CD40 mAb that we used in the human system. The recent observation that xid B cells proliferate normally if stimulated with soluble CD40 ligand (16) or with a different anti-CD40 mAb further supports this possibility (25). Obviously, the precise role of Btk in CD40 signaling pathways in both mice and humans requires further investigation.

Three of the five XLA patients studied were immunized with bacteriophage dX174, a T-dependent neo-antigen; in normal controls, this neo-antigen induces a classic Ab response consisting of immunologic memory, amplification of titers and switch from IgM to IgG (% IgG = 47%) after a secondary immunization. The XLA

In vivo Ab responses to bacteriophage dX174

To measure in vivo Ab responses to a T-dependent neo-antigen, we immunized 3 of the 5 XLA patients (patients 2, 3, and 4) and 12 normal controls with bacteriophage dX174. As shown in Figure 5, normal controls show a characteristic pattern of neutralizing Ab production, including amplification of titers and switch from IgM to IgG (% IgG = 47%) after a secondary immunization. The XLA

![Image 4](http://www.jimmunol.org/)

**FIGURE 4.** IgE production by B cells obtained from patients with XLA. Purified B cells obtained from patients with XLA and from age-matched healthy donors were cultured with or without anti-CD40 mAb (1 µg/ml) and IL-4 (100 U/ml) for 12 days. IgE concentrations in culture supernatants were analyzed by ELISA. –, cultured without anti-CD40 and IL-4; +, cultured with anti-CD40 and IL-4. Horizontal bars indicate geometric means of the IgE concentrations produced.

![Image 5](http://www.jimmunol.org/)

**FIGURE 5.** In vivo production of phage-neutralizing Ab by normal humans and patients with XLA. Normal humans ($n = 12$) and three patients with XLA were immunized twice with bacteriophage dX174. Sera were collected at the time indicated, and antiphage Ab titers ($K_v$) were measured by a neutralizing assay. ○, geometric means of controls; dotted line, ± 1 SD of controls; ▪, patient 2; △, patient 3; □, patient 4.
These observations indicate that B cells can differentiate into specific Ab-producing lymphocytes despite our inability to demonstrate Btk protein. However, ~40% of a group of 36 XLA patients immunized with bacteriophage failed to clear Ag and produce Ab. Those XLA patients that were able to generate phosphate-specific Ab did so at very low titers and failed to switch from IgM to IgG (24). This pattern of in vivo Ab responses may reflect the generation of a very limited number of B cell clones in some XLA patients or the complete absence of Ag-specific B cell clones in others. It is of interest that the four XLA patients that we had to exclude from this study due to insufficient B cell numbers in the peripheral blood failed to clear phage and produce detectable antiphage Ab. The formidable, although depressed, response of xid mice to T-dependent Ags may be related to the relatively high B cell number in xid mice (50% of normal mice).

The circulating B cells obtained from the five XLA patients studied resembled conventional B cells. They were found to have the normal phenotype CD19+, CD20+, and CD5+, similar to circulating B cells in xid mice that also have a conventional phenotype of B cells (10). It is tempting to speculate that the leaky B cells observed in XLA patients have differentiated using Btk-independent pathways. A role of CD40 in this differentiation process is suggested by the finding of Oka et al., who reported that xid mice that were made simultaneously CD40- showed a profound reduction of mature B cells (27). These authors hypothesize that mature B cells are usually generated through a Btk-dependent pathway, but, alternatively, may also be generated through a CD40-controlled pathway. Based on this hypothesis, the cells observed in xid mice would have been generated by a CD40-controlled pathway. Mice that were targeted for both Btk and CD40 gene deletions had a more severely impaired B cell maturation than xid mice (28). Therefore, it is possible that the circulating B cells observed in XLA patients are derived from CD40-controlled, Btk-independent pathways. This hypothesis is consistent with our observation that CD40 signaling is intact in XLA B cells. The difference between the severely depressed B cell numbers observed in XLA patients and the moderately reduced B cell numbers characteristic for xid- or Btk-targeted mice suggests that the CD40-controlled pathway is insufficient for effective B cell differentiation in humans. Alternatively, it is possible that the leaky B cells obtained from XLA patients with missense mutations of Btk have a minimal amount of functional Btk that is not demonstrable with flow cytometry or Western blot analysis. The fact that XLA B cells proliferate normally and undergo isotype switching in vitro supports a novel treatment strategy that involves expanding leaky B cells in vitro by CD40 stimulation that could be injected into the patient. Such a therapy could be of use for a subgroup of XLA patients.

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References