ic differences in the regulation of BLNK or other regulators of B cell development may account for the differences observed between human and murine BLNK deficiencies. However, the present studies in a human and in mice demonstrate a central role for BLNK in relaying signals in the pre-BCR and BCR signaling pathways.

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7. Cells (4×10^6) were prepared with the CytoFix/CytoPerm Kit (PharMingen) according to manufacturer's recommendations. After permeabilization, cells were stained with 2 μg of an antiserum to BLNK (*3*) in buffer containing 50% fetal calf serum (FCS) at 4°C for 30 min. Cells were washed twice with 1 ml of wash buffer and stained with 1 μg of fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (heavy and light chains) (Jackson Research Labs) for 30 min at 4°C. Cells were washed twice with 1 ml of wash buffer, resuspended in 0.4 ml of cold phosphate-buffered saline, and prepared for FACS analysis.
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12. The targeting construct, containing a PGK-neomycin (neo) selection cassette flanked by loxP sites, was provided by T. Ley (Washington University, St. Louis, MO). A 4.5-kb fragment of *BLNK* upstream of the initiation codon and a 1.3-kb fragment downstream of exon 1 were subcloned into the targeting construct. In addition, the initiation codon of BLNK was mutated to ATC, and a green fluorescent protein (GFP) cDNA with its own Kozak and initiation codon was inserted upstream of the short arm. GFP fluorescence was not detected in $BLNK^{+/-}$ splenocytes or bone marrow–derived cells; this may be due to transcriptional silencing of GFP by the PGK-neo cassette. The targeting construct was linearized and electroporated into 129/SVJ ES cells at 250 mV and 500 μF with a Gene Pulser II (Bio-Rad, Hercules, CA). Cells were selected in neomycin (0.2 mg/ml) and clones were expanded after 7 days of selection. Fifteen hundred neomycin-resistant clones were screened, of which three represented correct recombinants.
13. DNA was harvested from mouse tails and digested with Bam HI. A 500–base pair (bp) fragment 6 kb upstream of exon 1 was used as the 5′ probe, and a downstream 300-bp Xba I–Bam HI fragment served as the 3′ probe.
14. R. Pappu and A. Chan, unpublished results.
15. To generate $BLNK^{-/-}$ ES cells, a $BLNK^{+/-}$ ES cell was targeted with a vector containing a puromycin selection cassette. This second vector contained the same targeting arms as the first one. In addition, the vector also contained a herpes simplex virus–thymidine kinase (HSV-TK) gene for negative selection. $BLNK^{+/-}$ ES cells were transfected with this construct under the identical conditions described above (*12*) and were selected in media containing puromycin (1 μg/ml), gancyclovir (2 μM), and neomycin (0.2 mg/ml). Clones were screened and analyzed as described above (*13*). The targeting frequency was ~0.5% (*14*). To ensure that the $BLNK^{-/-}$ ES clone was not contaminated with $BLNK^{+/-}$ ES cells, individual clones were subcloned for one round before injection into blastocysts.
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17. Bone marrow–derived cells (5×10^6) were lysed in 10 mM tris (pH 8.0), 150 mM NaCl, and 1% NP-40 buffer containing protease and phosphatase inhibitors (*3*). Cell debris was clarified at 14,000*g* for 10 min at 4°C. The supernatant fraction was then analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting with antisera to BLNK (*3*) or actin (*14*). The antiserum to BLNK was raised against amino acids 1 through 204 and recognizes amino acids 61 through 204.
18. Cell recoveries from the thymus were $(161 \pm 51) \times 10^6$ ($n = 7$) for $BLNK^{+/+ \text{ or } +/-}$ mice and $(152 \pm 78) \times 10^6$ ($n = 7$) for $BLNK^{-/-}$ mice ($P = 0.334$) (*20*). Recoveries from the bone marrow from two femurs per mouse were $(15.3 \pm 6.5) \times 10^6$ ($n = 12$) for $BLNK^{+/+ \text{ or } +/-}$ mice and $(14.1 \pm 6.4) \times 10^6$ ($n = 11$) for $BLNK^{-/-}$ mice ($P = 0.397$) (*20*). Splenocyte numbers were $(64.6 \pm 32) \times 10^6$ ($n = 10$) for $BLNK^{+/+ \text{ or } +/-}$ mice and $(24.0 \pm 12) \times 10^6$ ($n = 10$) for $BLNK^{-/-}$ mice ($P < 0.001$) (*20*). Cell recoveries from four lymph nodes per mouse were $(4.4 \pm 2.1) \times 10^6$ ($n = 9$) for $BLNK^{+/+ \text{ or } +/-}$ mice and $(4.7 \pm 2.2) \times 10^6$ ($n = 11$) for $BLNK^{-/-}$ mice ($P = 0.388$) (*20*). No statistically significant differences were observed between $BLNK^{+/+}$ and $BLNK^{+/-}$ mice.
19. Supplementary information is available at *Science* Online at www.sciencemag.org/feature/data/1045303.shl
20. Statistical analysis is presented as mean ± SD; *n* represents sample size and *P* values are derived from comparisons of independent sample tests.
21. For each FACS analysis, 1×10^6 cells were examined. All antibodies were purchased from PharMingen. Data were collected with a FACS Calibur (Becton Dickinson, San Jose, CA) and analyzed with Cell Quest Analysis software. All data were collected from live cells within the lymphocyte gate as defined by forward and side scatter values.
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26. Splenocytes (2×10^7) were incubated at room temperature for 20 min in Dulbecco's modified Eagle's medium (DMEM), 10% FCS, and 3.3 μM Fluo-4 (Molecular Dynamics). Cells were diluted 10-fold with DMEM and 10% FCS and were incubated for an additional 20 min at room temperature. Cells were washed twice with media, resuspended at 2×10^7 cells/ml and, stained with antibodies to B220 (PharMingen) according to the manufacturer's recommendations. Changes in $[Ca^{2+}]_i$ were measured on $B220^+$ B cells every 30 *s* by FACS analysis.
27. 10^6 cells isolated from the spleen were cultured overnight at 37°C in media alone or in media containing antibody to $F(ab')_2$ (10 μg/ml, Jackson Labs). The expression of CD69 and CD86 was analyzed by FACS analysis gated on $B220^+$ B cells.
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34. Supported in part by grants from the Human Frontiers Program in Biomedical Sciences (to A.C.C.), the Pew Scholars Program in Biomedical Sciences (to A.C.C.), NIH grants AI42787 (to A.C.C.) and CA71516 (to A.C.C.), and the Burroughs Wellcome Fund (to B.P.S.).

9 September 1999; accepted 26 October 1999

An Essential Role for BLNK in Human B Cell Development

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The signal transduction events that control the progenitor B cell (pro-B cell) to precursor B cell (pre-B cell) transition have not been well delineated. In evaluating patients with absent B cells, a male with a homozygous splice defect in the cytoplasmic adapter protein BLNK (B cell linker protein) was identified. Although this patient had normal numbers of pro-B cells, he had no pre-B cells or mature B cells, indicating that BLNK plays a critical role in orchestrating the pro-B cell to pre-B cell transition. The immune system and overall growth and development were otherwise normal in this patient, suggesting that BLNK function is highly specific.

Cross-linking of the B cell antigen receptor (BCR) results in rapid phosphorylation of the adapter protein BLNK [also called SLP-65 (Src homology 2 domain–containing leukocyte protein of 65 kD) and BASH (B cell adapter containing Src homology 2 domain)], a hematopoietic-specific cytoplasmic protein with homology to SLP-76 (*1, 2*). Once BLNK is phosphorylated by Syk, it serves as a scaffold to assemble the downstream targets of antigen activation, including Grb2, Vav, Nck, and phospholipase C–γ (PLCγ). Hence, BLNK is positioned to coordinate a number of signaling pathways activated by the BCR. Studies in a

BLNK-deficient DT40 chicken B cell line indicate that this adapter is required for the release of intracellular calcium and the activation of the extracellular signal-regulated protein kinase, c-Jun NH_2-terminal kinase, and p38 pathways in response to anti–immunoglobulin M (IgM) stimulation (*3*). If BLNK plays a nonredundant role in mammalian B cell development, mutations in BLNK might result in immunodeficiency.

About 85% of patients with early onset hypogammaglobulinemia and absent B cells are males with X-linked agammaglobulinemia (XLA) (*4*). These patients have mutations in the cytoplasmic tyrosine kinase Btk (*5*). Some of the remaining patients have defects in components of the pre-B cell receptor (pre-BCR) or BCR (*6, 7*); however, the nature of the defect in many patients remains unknown. To determine if mutations in BLNK could give rise to human immunodeficiency, we isolated and characterized a bacterial artificial chromosome clone containing the human genomic BLNK sequence. Fluorescence in situ hybridization demonstrated that BLNK is located on chromosome 10q23.22. The gene consists of 17 exons spread over ~65 kb of DNA. Primers were designed to amplify individual exons by polymerase chain reaction (PCR) for analysis by single-strand conformation polymorphism (SSCP) (*8*). Genomic DNA samples were analyzed from 25 patients with a Btk-deficient phenotype, in whom we had not identified mutations in Btk, μ heavy chain, Igα (mb-1), Igβ (B29), or the surrogate light chain. DNA from one patient, a 20-year-old male with early onset hypogammaglobulinemia and absent B cells, demonstrated a homozygous alteration in the first exon of BLNK and its flanking intronic sequence (Fig. 1). This portion of the gene was cloned and sequenced, and two noncontiguous base-pair substitutions were identified (*9*). The first alteration, a C to A substitution, occurred at the third base-pair position in codon 10, which encodes a proline. This base-pair substitution does not change the amino acid sequence of BLNK. The second alteration, an A to T substitution, was found at the +3 position of the splice donor site for intron 1, 20 base pairs downstream from the alteration in codon 10. SSCP analysis of DNA from 100 unrelated individuals did not reveal any fragments with a migration pattern identical to that seen in the patient (*10*).

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The A to T substitution at the +3 position of the splice donor site occurs at a highly conserved site in the splice consensus sequence; alterations at this site would be expected to result in faulty processing of the BLNK message (*11*). To evaluate this possibility, we derived cDNA from the patient's bone marrow and used reverse transcriptase–PCR (RT-PCR) to examine the abundance of BLNK transcripts (*12*). The results were compared with those obtained from bone marrow of healthy subjects or patients with mutations in Btk or μ heavy chain (Fig. 2). No BLNK transcripts could be amplified from the patient's bone marrow, although BLNK transcripts were easily identified in the bone marrow of the other patients with defects in early B cell development. Other genes expressed in pro-B cells, including Btk, terminal deoxynucleotidyl transferase (TdT), and λ5, were expressed in approximately equal amounts in all of the patients. These results indicate that the base-pair substitutions in BLNK resulted in a marked reduction or absence in BLNK transcripts and therefore in BLNK protein.

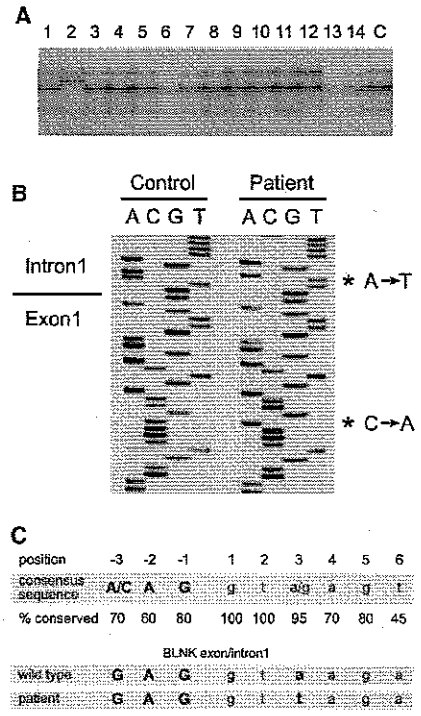

Fig. 1. Characterization of the BLNK mutation in an immunodeficient patient. (**A**) Genomic DNA samples from 14 patients with defects in B cell development and a control (lane C) were analyzed by SSCP for defects in the first exon of BLNK and its flanking sequences. DNA from the patient is shown in lane 2. (**B**) Sequence analysis at the exon-intron border demonstrated two base-pair substitutions, as indicated. (**C**) The consensus sequence for a mammalian 5′ splice donor site is shown with the wild-type and mutant BLNK exon 1/intron 1 sequence. The coding sequence is shown in capital letters; the intronic sequence is in lowercase letters.

The patient with BLNK deficiency demonstrated normal growth and development. At 8 months of age, he had the onset of recurrent otitis. After two episodes of pneumonia, he was evaluated for immunodeficiency at 16 months of age. At that time, he had no detectable serum IgG, IgM, or IgA, and he had <1% B cells in the peripheral circulation. He was started on gammaglobulin replacement, and between 2 and 20 years of age, he did well except for chronic otitis and sinusitis, hepatitis C acquired from intravenous gammaglobulin, and an episode of protein-losing enteropathy in adolescence. Immunologic studies performed when the BLNK-deficient patient was 20 years of age demonstrated serum concentrations of IgM and IgA of <7 mg/dl, normal numbers and percentages of CD4 and $CD8^+$ T cells and natural killer cells, and normal numbers of platelets and myeloid cells. The patient's mother and father, who were heterozygous for both base-pair substitutions in BLNK, were healthy and had normal concentrations of serum immunoglobulins and normal numbers of B cells (*13*). An older brother developed recurrent otitis at 6 months of age and died at 16 months of age of pseudomonas sepsis and neutropenia.

Immunofluorescence analysis of peripheral blood lymphocytes from the patient with BLNK deficiency and an age-matched patient with an amino acid substitution in the pleckstrin homology domain of Btk demonstrated that both patients had <0.01% $CD19^+$ cells in the blood (*14*). To determine the point in B cell differentiation at which the block in develop-

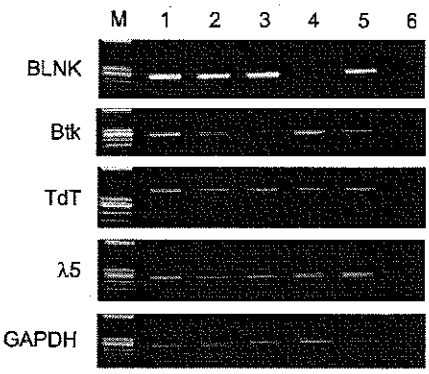

Fig. 2. RT-PCR analysis of B cell–specific transcripts in patients with defects in B cell development. RNA from the following sources was reverse transcribed: the bone marrow of a normal control (lane 1), a patient with an amino acid substitution in codon 113 of Btk (lane 2), a patient with a 4-bp deletion in the coding sequence of Btk (lane 3), the patient with mutations in BLNK (lane 4), and a patient with an amino acid substitution at an invariant cysteine in CH4 of μ heavy chain (lane 5). The cDNA was used as a template for RT-PCR with primers specific for the coding regions of BLNK, Btk, TdT, λ5, and the control transcript, GAPDH. Molecular weight markers are shown on the left (lane M), and a cDNA negative control is shown on the right (lane 6).

ment occurred, we examined bone marrow from both patients using markers that distinguish pro-B cells from pre-B cells and mature B cells. The percentage of $CD19^+$ B lineage cells was less in the patients in comparison to that of the control (0.3% in the BLNK-deficient patient and 1.0% in the Btk-deficient patient versus 15.7% in the control). There were no membrane immunoglobulin-positive (mIg^+) mature B cells in either patient (Fig. 3). In both patients, the block in B cell differentiation occurred at the pro-B cell to pre-B cell transition; >80% of the $CD19^+$ cells from these patients coexpressed the pro-B cell marker, CD34. In contrast, only 22.0% of the $CD19^+$ cells from the control were positive for CD34; the remaining cells from the control were either pre-B cells ($CD34^-$, $CD19^+$, and mIg^-) or B cells ($CD34^-$, $CD19^+$, and mIg^+).

To document that BLNK is expressed in pro-B cells, we indirectly stained permeabilized bone marrow cells from the Btk- and BLNK-deficient patients with a monoclonal antibody to BLNK (*14*). All of the $CD19^+$ pro-B cells from the Btk-deficient patient were positive for BLNK (Fig. 4). By contrast, there was little or no staining for BLNK in the bone marrow of the patient with mutations in BLNK. Because BLNK is expressed in pro-B cells, the possibility that BLNK is required before the expression of the pre-BCR was examined. In previous studies, we have shown that patients with defects in the constant region of the μ heavy chain or the Igα signal transduction component of the BCR have small amounts of transcripts for rearranged μ heavy chain genes in the bone marrow as detected by RT-PCR (*7*). Rearrangement of the μ heavy chain occurs immediately before the pro-B cell to pre-B cell transition. A primer that hybridizes to a conserved sequence within framework region 3 of variable-region genes and a primer within the CH1 domain of μ heavy chain were used to examine cDNA from a control and patients with defects in B cell development (*7*). A small number of rearranged μ heavy chain transcripts could be detected in the bone marrow of the patient with mutations in BLNK as well as in patients that were Btk and μ heavy chain deficient. Thus, BLNK does not play a role in B cell development before the expression of the pre-BCR. This corresponds with earlier studies showing that phosphorylation of BLNK is dependent on cell surface expression of a BCR (*15*).

Cell surface expression of the pre-BCR results in a strong survival signal associated with the cessation of μ heavy chain gene rearrangements, changes in cell surface phenotype, and marked expansion of the pre-B cell population (*16*). The absence of pre-B cells or B cells in the patient with mutations in BLNK demonstrates that BLNK plays a critical role in orchestrating these signals. Like defects in Btk and λ5 (*5, 6, 17*), mutations in BLNK appear to have more severe consequences in the human as compared to the mouse (*18*). This suggests that the requirements for signaling through the pre-BCR and BCR may be more stringent in the human than in the mouse. There may be a reciprocal reliance on signaling through other pathways in murine B cell development. For example, the consequences of defective signaling through interleukin-7 are more severe in the mouse as compared to the human (*19*).

In T cells, the functions performed by BLNK appear to be split between LAT (linker for activation of T cells), which binds to phophatidylinositol 3-kinase, Grb-2, and PLCγ (*20*), and SLP-76, which binds GrpL, Nck, Vav, and FYB (FYN binding protein) (*21*). Mice lacking LAT (*22*) or SLP-76 (*23, 24*) have a block in T cell development at the pro-T to pre-T cell stage of development. In the newborn period, SLP-76–deficient mice also develop a hemorrhagic diathesis, which is related to the requirements for SLP-76 in collagen-mediated platelet activation (*24, 25*). These studies, when coupled with our findings showing that BLNK is required for normal B cell development in the human and the mouse, indicate that adapter proteins play a critical role in highly specific signaling pathways, and they suggest that defects in adapter proteins like LAT or SLP-76 may result in human immunodeficiency.

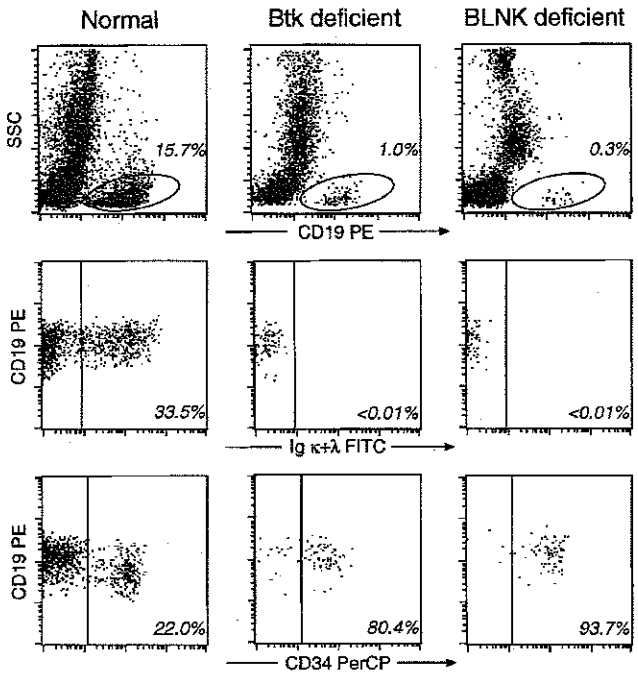

Fig. 3. Immunofluorescence analysis of B lineage cells. Bone marrow mononuclear cells from a normal individual (left column), from a patient with Btk-deficient XLA (middle column), and from the BLNK-deficient patient (right column) were labeled with antibody to CD19 PE, antibody to CD34 PerCP, and antibody to Ig κ and λ light chains FITC. Flow cytometric dot plots in the top row illustrate CD19 staining versus side scatter (SSC); both patients had reduced proportions of $CD19^+$ cells. Gated $CD19^+$ lymphoid cells were then analyzed for expression of mIg light chains (middle row) and CD34 (bottom row). Percentages of mIg^+ and $CD34^+$ among $CD19^+$ cells are indicated.

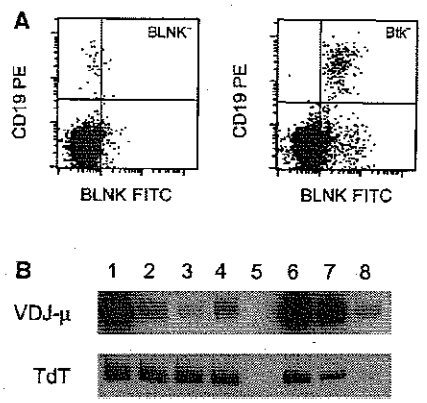

Fig. 4. (**A**) BLNK protein expression in pro-B cells. Bone marrow mononuclear cells from age-matched patients with BLNK (left) or Btk (right) deficiency were labeled with antibody to CD19 (IgM), permeabilized, and labeled with monoclonal antibody to BLNK (IgG2a). Goat antibody to mouse IgM PE and IgG FITC was then added. Dot plots illustrate immunofluorescence staining of lymphoid cells. Quadrants were set at the upper limits of the isotype-matched nonreactive antibody fluorescence. The $BLNK^+$ $CD19^-$ cells seen in the Btk-deficient patient were $CD34^-$ and were similar to monocytes in forward and side light scatter. (**B**) Semiquantitative RT-PCR analysis to evaluate the amount of VDJ-rearranged μ heavy-chain transcripts. Bone marrow cDNA from an age-matched control (lane 1), a patient with XLA (lane 2), the patient with BLNK deficiency (lane 3), a patient with μ heavy chain deficiency (lane 4), and a cDNA negative control (lane 5) and three 10-fold dilutions of control cDNA (1×, 0.1×, and 0.01×) (lanes 6 through 8, respectively) were amplified with primers specific for VDJ-rearranged μ heavy chain and TdT, which was used as a control to demonstrate equal concentrations of pro-B cell transcripts.

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9. The following primers were used to amplify BLNK exon 1 and the associated flanking sequence for both the SSCP analysis and cloning of this region of the gene: 5′-GAACTGCTGACGTGACCA-3′ (5′ untranslated region of exon 1) and 5′-CCCTAAAAGCTCAGTCCAC-3′ (intron 1). The products of two independent PCR reactions were cloned and sequenced.
10. DNA samples from five individuals demonstrated altered migration in comparison to the wild-type pattern. Sequencing showed a T to A substitution at the +113 position in intron 1 in three people, a G to A substitution at the +121 position in intron 1 in one individual, and an A to G substitution 5 base pairs upstream of the start codon.
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13. The father had 6% and the mother had 8% $CD19^+$ B cells (normal is 5 to 22%). Serum IgG in the father was 1110 mg/dl, IgA was 337 mg/dl, and IgM was 93 mg/dl. The mother's IgG was 1060 mg/dl, IgA was 115 mg/dl, and IgM was 127 mg/dl.
14. Bone marrow mononuclear cells were stained with antibody to CD19 conjugated to phycoerythrin (PE) (Dako, Carpinteria, CA), antibody to CD34 conjugated to peridinin chlorophyll protein (PerCP) (Becton-Dickinson, San Jose, CA), and polyclonal antibodies against human light chains conjugated to fluorescein isothiocyanate (FITC) (Southern Biotechnology Associates, Birmingham, AL). Antibodies to nuclear TdT (Supertechs, Bethesda, MD) and cytoplasmic μ heavy chains (Southern Biotechnology Associates) conjugated to FITC and PE, respectively, were applied after cell permeabilization with OrthoPermeafix (Ortho Diagnostics, Raritan, NJ). Monoclonal antibody to BLNK (of IgG2a isotype) was generated against a glutathione S-transferase fusion protein encoding amino acids 4 through 205, as described in (*1*). This antibody was used in combination with an antibody to CD19 of IgM class (BLY3) (Research Diagnostics, Flanders, NJ), with fluorochrome-conjugated secondary antibodies specific for murine IgG and IgM. Antibody to BLNK was applied after cell permeabilization with OrthoPermeafix. Isotype-matched nonreactive antibodies were from Becton-Dickinson. Immunofluorescence staining was analyzed with a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson).
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26. We thank M. D. Cooper for a helpful discussion, E. Boylin for technical assistance, and S. Saucier for administrative assistance. Supported by NIH grants AI25129, AI42787, CA71516, and CA58297; March of Dimes grant FY97-0384; the Assisi Foundation; National Cancer Institute CORE grant P30 CA21765; American Lebanese Syrian Associated Charities; the Human Frontiers Scientific Organization; and funds from the Federal Express Chair of Excellence. A.C.C. is a Pew Scholar in the Biomedical Sciences.

9 September 1999; accepted 27 October 1999

Perforin Gene Defects in Familial Hemophagocytic Lymphohistiocytosis

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Familial hemophagocytic lymphohistiocytosis (FHL) is a rare, rapidly fatal, autosomal recessive immune disorder characterized by uncontrolled activation of T cells and macrophages and overproduction of inflammatory cytokines. Linkage analyses indicate that FHL is genetically heterogeneous and linked to 9q21.3-22, 10q21-22, or another as yet undefined locus. Sequencing of the coding regions of the perforin gene of eight unrelated 10q21-22–linked FHL patients revealed homozygous nonsense mutations in four patients and missense mutations in the other four patients. Cultured lymphocytes from patients had defective cytotoxic activity, and immunostaining revealed little or no perforin in the granules. Thus, defects in perforin are responsible for 10q21-22–linked FHL. Perforin-based effector systems are, therefore, involved not only in the lysis of abnormal cells but also in the down-regulation of cellular immune activation.

FHL is a hemophagocytic lymphohistiocytic disorder in which previously healthy young children present with fever, splenomegaly, hepatomegaly, pancytopenia, coagulation abnormalities, neurological abnormalities, and high serum concentrations of interferon-γ (IFN-γ) and tumor necrosis factor–α (TNF-α). Accumulation of activated macrophages and lymphocytes, mainly $CD8^+$ human lymphocyte antigen DR^+ Fas^+ T cells, as well as hematophagocytosis in the bone marrow, spleen, liver, lymph nodes, and central nervous system, dominate the pathology (*1–3*). Defective T and natural killer (NK) cell cytotoxicity is consistently reported (*4*, *5*). We hypothesized that the primary inherited defect in FHL could be a failure of cytolytic lymphocyte function and that this, together with childhood infections (*6*, *7*), induces the fatal immune deregulation of FHL.

The gene encoding perforin, an important mediator of lymphocyte cytotoxicity, has been mapped to 10q22 (*8*), near one of the previously identified FHL-linked loci (*9*, *10*). Thus, perforin deficiency may play a role in the pathogenesis of FHL. Unlike patients with FHL, perforin knockout mice are generally healthy when maintained in a pathogen-free, controlled environment. However, when infected with lymphocytic choriomeningitis virus (LCMV), similar $CD8^+$ T cell–, IFN-γ–, TNF-α–dependent immunopathology and mortality are seen (*11*, *12*).

We first confirmed the presence of perforin (PRF1) in the candidate region by polymerase chain reaction (PCR) screening a partial yeast artificial chromosome (YAC) contig covering the FHL region on chromosome 10 using prim-

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