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8. The sequence flanking each exon of BLNK has been deposited in GenBank (accession numbers AF180740 through AF180756). The primers used to amplify each exon are available upon request. The conditions used in SSCP are as described in work by M. E. Conley, M. E. Fitch-Hilgenberg, J. L. Cleveland, O. Parolini, and J. Rohrer [*Hum. Mol. Genet.* **3**, 1751 (1994)].
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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12. The primers used to amplify BLNK cDNA were from the 5′ untranslated region [shown in (*9*)] and 5′-GTCGCTGTCAAAGTCATCGGA-3′ from exon 4. The Btk-specific primers were from exons 6 and 8 of that gene. The primers used to amplify TdT, λ5, VDJ-μ, and glyceraldehyde phosphate dehydrogenase (GAPDH) were as reported in (*7*).
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

Susan E. Stepp,[1] Rémi Dufourcq-Lagelouse,[2] Françoise Le Deist,[2,3] Sadhna Bhawan,[1] Stéphanie Certain,[2] Porunelloor A. Mathew,[4] Jan-Inge Henter,[5] Michael Bennett,[1] Alain Fischer,[2,3] Geneviève de Saint Basile,[2]*† Vinay Kumar[1]*

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.

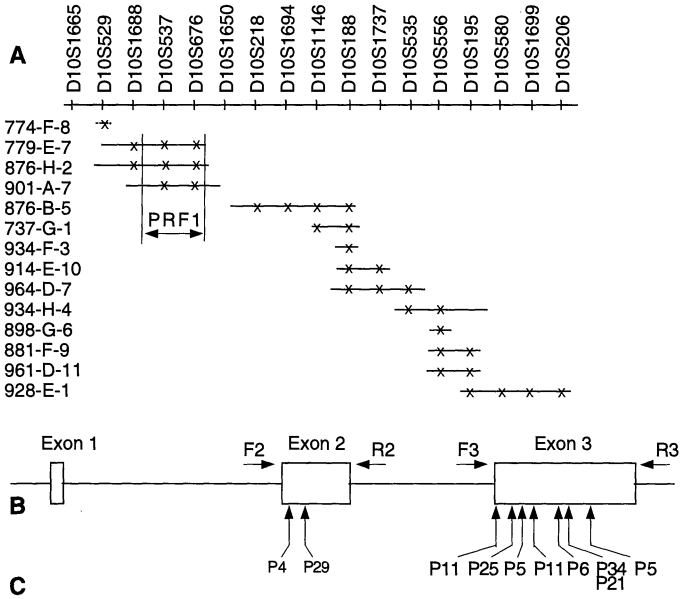
FHL is a hemophagocytic lymphohistiocytic disorder in which previously healthy young children present with fever, splenomegaly, hepatomegaly, pancytopenia, coagulation abnormalities, neurological abnormalities, and high serum concentrations of interferon-γ (IFN-γ) and tumor necrosis factor–α (TNF-α). 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-

[1]Department of Pathology and the Graduate Program in Immunology, University of Texas Southwestern Medical School, Dallas, TX 75235, USA. [2]Unité de Recherches sur le Dévelopment Normal et Pathologique du Système Immunitaire INSERM U429, 75015 Paris, France. [3]Unité d'Immunologie et d'Hématologie Pédiatrique, Hôpital Necker-Enfants Malades, 75015 Paris, France. [4]Department of Molecular Biology and Immunology, University of North Texas Health Science Center, Fort Worth, TX 76107, USA. [5]Child Cancer Research, Karolinska Institutet, Department of Pediatric Hematology and Oncology, Karolinska Hospital, Stockholm S-17177, Sweden.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: sbasile@necker.fr



C

Patient	Nucleotide change	Predicted effect
P4	** 50 del T	L 17 frameshift and **stop**
P21	** 1122 G>A	W 374 **stop**
P29	** 190 C>T	Q 64 **stop**
P34	** 1122 G>A	W 374 **stop**
P5	* 673 C>T	R 225 W
P5	* 1286 G>A	G 429 E
P6	** 1034 C>T	P 345 L
P11	* 836 G>A	C 279 Y
P11	* 548 T>G	V 183 G
P25	* 755 A>G	N 252 S

Fig. 1. Perforin gene mutations are found in FHL patients. (**A**) The perforin gene (PRF1, OMIM 170280) maps to the centromeric portion of the 10q21-22 FHL locus as determined by PCR screening of the partial YAC contig shown. This localized perforin to 10q21. YAC clones are listed in the left column, and the radiation hybrid markers in the 13-centimorgan chromosome region of 10q21-22 are shown across the top. (**B**) Diagram of the perforin gene. Perforin has three exons, two of which (exons 2 and 3) contain coding sequences. The locations of the primers used and mutations found are shown. (**C**) Perforin gene mutations are found in eight FHL patients. Nucleotide and amino acid positions of the mutations are numbered according to National Center for Biotechnology Information accession number M28393. Homozygous mutations are identified by a double asterisk (**), and heterozygous mutations are identified by a single asterisk (*) (*30*). Single-letter abbreviations for the amino acid residues are as follows: C, Cys; E, Glu; G, Gly; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; V, Val; W, Trp; and Y, Tyr.

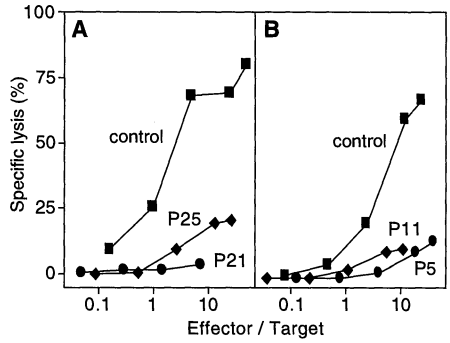


Fig. 2. Anti-CD3–dependent cytotoxicity of FHL patient cells is defective. (**A**) ■, control cells; ●, patient P21 cells; ◆, patient P25. (**B**) ■, control cells; ●, patient P5 cells; ◆, patient P11. Patient or control cells were cultured in PHA (1/700 dilution; Difco, Detroit, Michigan) and IL-2 (20 IU/ml, Valbiotech) for 1 day, followed by IL-2 without PHA for 5 days. $CD8^+$ T cells were enumerated by fluorescence-activated cell sorting analysis [(A): control = 76%, P21 = 23%, P25 = 40%; (B): control = 38%, P5 = 62%, P11 = 17%]. The lysis of Fas-deficient L1210-3 target cells (kindly provided by P. Golstein, Marseille, France) was measured in a standard 4-hour ^{51}Cr-release assay after a 3-hour incubation of the effector cells in the presence of monoclonal anti-CD3 (OKT3; Ortho Pharmaceutical, Rajitan, New Jersey). The cytotoxicity under these culture conditions and in this assay system is CD3-dependent (*31*). The effector to target ratio reflects the ratios of $CD8^+$ T cells to target cells. To abrogate any residual Fas/FasL-dependent cytotoxicity, we used anti-Fas ZB4 (Immunotech, Marseille, France), at 2 μg/ml, to block Fas. The results are expressed as percentage of specific lysis.

ers specific for exon 2 of perforin (Fig. 1, A and B). This allowed us to more precisely map perforin to 10q21. Next we studied eight unrelated 10q21-22–linked FHL patients (*9*) by sequencing the coding regions of the perforin gene in exons 2 and 3. Five of the patients were from consanguineous families (P4, P6, P21, P29, and P34), and the other three patients were from nonconsanguineous families (P5, P11, and P25).

Nine independent mutations in exons 2 and 3 of the perforin gene were detected (Fig. 1B). Patients P21 and P34 had the same homozygous point mutation that led to a premature stop codon in exon 3. Patient P29 had a distinct homozygous point mutation that also led to a premature stop codon. Each parent of patient P29 was heterozygous for this mutation. A single homozygous nucleotide deletion found near the beginning of exon 2 in patient P4 caused a frameshift and introduced a premature stop codon. In the four remaining patients (P5, P6, P11, and P25), six missense mutations were identified, each one leading to a different amino acid substitution (Fig. 1C). A single homozygous missense mutation was seen in patient P6, and two heterozygous missense mutations were seen in each of patients P5 and P11. Only one heterozygous missense mutation was identified in the perforin coding sequence of patient P25.

The premature stop codon of four patients would be predicted to give rise to a truncated, nonfunctional perforin protein. The missense mutations found in the other four patients may affect the synthesis, stability, or function of perforin. Alternatively, unidentified mutations affecting transcription of the perforin gene or the splicing or stability of perforin RNA may exist in the noncoding regions. To confirm that defective perforin can account for FHL disease in these patients, we tested the perforin-mediated cytolytic capabilities of patient cells.

Cytotoxic activity of T cells and NK cells in short-term in vitro assays can be mediated both by perforin and Fas-dependent pathways (*13*). A defect in the Fas/FasL system is unlikely to contribute to the pathogenesis of FHL because Fas and FasL do not map to the FHL locus at 10q21-22 and because Fas/FasL-mediated cell death is normal in FHL (*14*, *15*). To focus on the perforin-dependent component of cytotoxicity, we selected Fas-deficient murine L1210-3 cells as target cells (*13*). In addition, we added ZB4, a monoclonal antibody to Fas (anti-Fas) that interrupts Fas-FasL interactions to ensure that the cytotoxicity assays would detect only perforin-dependent killing. To generate cytotoxic cells, we cultured previously frozen cells from four FHL patients and controls in phytohemagglutinin (PHA) and interleukin-2 (IL-2). CD3-dependent cytolytic activity was measured in a 4-hour ^{51}Cr-release assay. Cells from all patients had greatly reduced cytolytic activity as compared with normal controls (Fig. 2). Cells from a patient with a premature stop codon (P21) displayed no cytotoxicity. Cells from three patients with missense mutations (P5, P11, and P25) showed greatly reduced lysis of the target cells. We conclude that perforin-mediated cytotoxic activity of $CD8^+$ T cells is defective in FHL patients.

To determine whether the reduced cytotoxicity of patients' cells was due to lack of perforin or the presence of nonfunctional perforin protein, we immunostained cytotoxic cells obtained from three patients (Fig. 3). Perforin and granzyme B showed the expected pattern of colocalization in the cytotoxic granules of the controls (Fig. 3, A and C). However, staining of the patient-derived cells revealed a complete (patient P21, Fig. 3D) or nearly complete (patients P5 and P25, Fig. 3, B and E, respectively) absence of perforin. The small amount of perforin in cells from patients P5 and P25 may

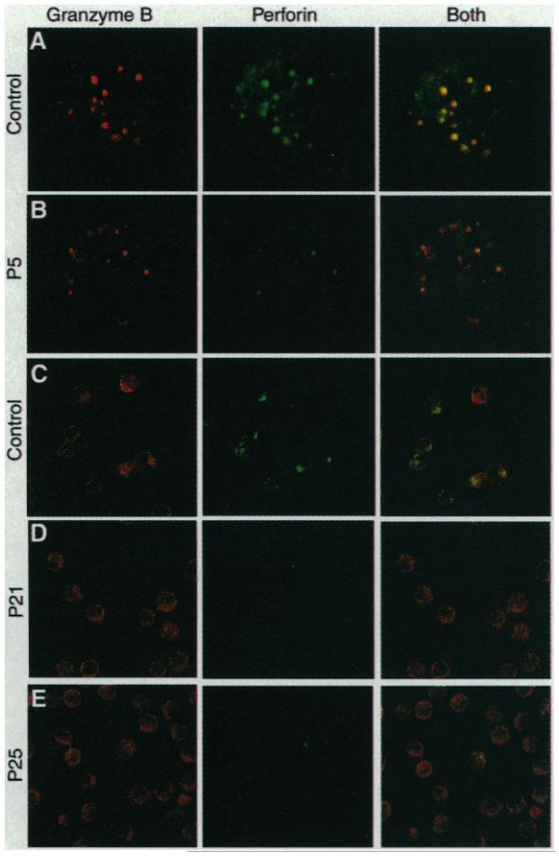


Fig. 3. Perforin protein is deficient in FHL patient cytotoxic cells. Cells were double stained with anti–granzyme B (red, first column), anti-perforin (green, second column), or both (third column) in two experiments. (**A**) and (**B**) show single cells at high magnification; (**C**), (**D**), and (**E**) show several cells at a lower magnification. (A) Control for patient P5. (B) Patient P5. (C) Control for patients P21 and P25. (D) Patient P21. (E) Patient P25. PHA-activated cells from controls and FHL patients cultured 6 days in IL-2 (as described previously in Fig. 2) was fixed on glass cover slips previously coated with poly-L-lysine. They were permeabilized and stained for anti–granzyme B (Chemicon International, Temecula, California) and with secondary stain tetramethyl rhodamine isothiocyanate–coupled goat antibody to mouse IgG, as well as with fluorescein isothiocyanate–coupled monoclonal anti-perforin (Ancell, Bayport, Minnesota). Immunofluorescence was examined by confocal laser microscopy. The frequency of granzyme B in $CD8^+$ T cells was similar in patient and control samples.

explain their low-level cytotoxic activity (Fig. 2). Cells from patient P25 with a single heterozygous missense mutation had greatly reduced cytotoxic activity as well as grossly deficient perforin protein. This could be explained if the single missense mutation acts in a dominant negative manner. However, because patient P25's parents did not have FHL (*9*), this is unlikely. It is more likely that an additional unidentified mutation or mutations exist in the noncoding regions that have led to the reduced perforin protein level and cytotoxic activity. Fas/FasL-mediated apoptosis was also normal in this patient (*15*).

A role for perforin in immune regulation may be inferred from recent studies in mouse models of LCMV infection (*11*, *12*, *16*, *17*), bone marrow transplantation (*18*), immunization (*19*), and autoimmunity (*20*, *21*). However, the physiologic role of perforin in maintaining immune homeostasis could not be determined from these studies in mice. The discovery of defective perforin in 10q21-22–linked FHL provides direct evidence for the physiologic relevance of perforin in down-regulation of human immune responses. Perforin may down-regulate an immune response by the elimination of antigen-presenting cells (APCs) or by perforin-dependent activation-induced cell death (AICD). During the course of a normal immune response, the APCs that activate cytolytic T lymphocytes (CTLs) may be eliminated by perforin-dependent cytotoxicity (*22*–*25*). In the absence of APC elimination, CTLs may continue to receive activation and proliferation signals. Alternatively or additionally, AICD of activated T cells could be impaired in the absence of perforin (*12*, *18*, *26*). However, defective AICD alone is unlikely to be responsible for FHL because partial as well as full engraftment of donor bone marrow can cure FHL (*27*). This suggests that the disease is due to the absence of perforin-competent cells rather than the presence of perforin-defective cells. The critical role of the Fas-FasL pathway of cell death in immune homeostasis is demonstrated by the human disease autoimmune lymphoproliferative syndrome (ALPS) type I, caused by defective Fas/FasL function (*28*, *29*). However, the course and pathology of ALPS type I are quite distinct from those of FHL. Fas deficiency causes a spontaneous and chronic autoimmune immunoproliferative disorder, whereas perforin deficiency gives rise to an acutely fatal immune deregulation. Thus, it appears that the mechanisms that underlie immune homeostasis by the Fas/FasL and perforin pathways are distinct.

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15. To determine the status of the Fas-/FasL pathway of killing, we cultured peripheral blood cells from a control and two patients (P11 and P25) as described in text. Because anti-CD3–mediated apoptosis depends on Fas-FasL interactions, control and patients cells were incubated on plate-bound (1 μg/ml) OKT3 antibody for 24 hours. Cell death was determined as described (*28*) and was equivalent in all three samples (range from 55 to 60%). In addition, the cells were incubated with monoclonal anti-Fas (Apo-1) (250 to 0.25 ng/ml) and a rat antibody to mouse immunoglobulin G (IgG) for 24 hours. Fas-mediated apoptosis was present in both control and patient cells. Percentage of apoptotic cells ranged from 5 to 15% at 0.25 ng/ml and 65 to 80% at 250 ng/ml of antibody.
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30. Perforin has three exons, and the coding region of the gene was PCR amplified from exon 2 and exon 3. Primers used for amplification were as follows: for exon 2, forward (F2) 5′-CCCTTCCATGTGCCCTGATAATC-3′ and reverse (R2) 5′-AGCAGCCTCCAAGTTTGATTGG-3′; and for exon 3, forward (F3) 5′-CCAGTCCTAGTTCTGCCCACTTAC-3′ and reverse (R3) 5′-GAACCCCTTCAGTCCAAGCATAC-3′. Three to five independent PCR reactions were sequenced for each patient sample with the previously listed primers and the additional forward 5′-CAGGTCAACATAGGCATCCACG-3′ and reverse 5′-TTGCATCTCACCTCATGGGAAC-3′ primers.
31. F. Le Deist, unpublished data.

32. We thank Y. Goureau for confocal analysis; R. Winkler-Pickett and J. Ortaldo for technical help; R. McFarland for discussion; B. Stewart for assistance with figures; and P. V. Sivakumar, J. Schatzle, N. Williams, and M. Seigelman for critical reading of the manuscript. This work was supported by grants from NIH (to V.K. and M.B.) and by grants from the Institut National de la Santé et de la Recherche Médicale and the Association Française Contre les Myopathies (to G.S.B.)

7 September 1999; accepted 27 October 1999

References and Notes

[13] **Fas and Perforin Pathways as Major Mechanisms of T Cell-Mediated Cytotoxicity**

David Kagi; Francoise Vignaux; Birgit Ledermann; Kurt Burki; Valerie Depraetere; Shigekazu Nagata; Hans Hengartner; Pierre Golstein

Science, New Series, Vol. 265, No. 5171. (Jul. 22, 1994), pp. 528-530.

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[28] **Mutations in Fas Associated with Human Lymphoproliferative Syndrome and Autoimmunity**

F. Rieux-Laucat; F. Le Deist; C. Hivroz; I. A. G. Roberts; K. M. Debatin; A. Fischer; J. P. de Villartay

Science, New Series, Vol. 268, No. 5215. (Jun. 2, 1995), pp. 1347-1349.

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