Further Evidence for Genetic Heterogeneity in Familial Hemophagocytic Lymphohistiocytosis (FHLH)

GAIL E. GRAHAM, LISA M. GRAHAM, PETER J. BRIDGE, LINDA D. MACLAREN,
JOHANNES E.A. WOLFF, MAX J. COPPES, AND R. MAARTEN EGELER

*Departments of Medical Genetics [G.E.G., L.M.G., P.J.B., L.D.M.], Oncology [J.E.A.W., M.J.C., R.M.E.],
and Pediatrics [J.E.A.W., M.J.C., R.M.E.], Alberta Children's Hospital and University of Calgary,
Calgary, Alberta T2T 5C7, Canada*

ABSTRACT

Familial hemophagocytic lymphohistiocytosis (FHLH; MIM #267700) is an autosomal recessive disorder of immune regulation characterized by fever, hepatosplenomegaly, and cytopenia that is fatal without bone marrow transplantation. Recent studies have suggested the existence of FHLH loci at 9q21.3–22 and 10q21–22 in Asian and European/African/Australian families, respectively. We studied two unrelated Canadian families in which first cousins were affected with FHLH. In an effort to localize the causative gene, we completed a genome-wide screen for homozygosity by descent by using an automated system to genotype 400 highly polymorphic dinucleotide repeat markers covering the genome with an average resolution of 10 centiMorgans (cM). We identified a total of three candidate loci that met the combined criteria for homozygosity by descent in one family and shared maternal alleles in the other family. One of these, D9S1690, had a cytogenetic localization (9q22.33) proximal to a previously reported inversion of chromosome 9 in an FHLH patient. However, additional closely linked flanking markers within 1–2 cM of all three candidates did not conform to the

criteria for linkage in either family. Similarly, we excluded the linked 9q21.3-q22 and 10q21–22 regions recently reported in Asian and European/African/Australian families, respectively. The two families were then analyzed independently to encompass the possibility that they were segregating separate genes. Six additional candidate loci were identified on the basis of homozygosity for the same allele in all affected members of one family, but further analysis of closely linked flanking markers did not demonstrate similar homozygosity. Our data provide further evidence of genetic heterogeneity in FHLH and suggest the existence of at least a third locus for this disease. (***Pediatr Res 48: 227–232, 2000***)

Abbreviations
FHLH, familial hemophagocytic lymphohistiocytosis
cM, centiMorgan
NK, natural killer cells
YAC, yeast artificial chromosome
EBV, Epstein-Barr virus

The term histiocytosis identifies a group of disorders that have in common the proliferation and accumulation of macrophages and/or dendritic cells (1). Familial hemophagocytic lymphohistiocytosis demonstrates the typical pattern of autosomal recessive inheritance with unaffected parents having one or more affected children. A genetic etiology is accepted on the basis of sibling clustering, a high rate of consanguinity among the parents of affected individuals, concordance of the phenotype in monozygotic twins, and the results of segregation analysis (2). Consequently, FHLH has been recognized as an

autosomal recessive condition in the compendium of single gene disorders known as Online Mendelian Inheritance in Man (3).

Despite recognition that FHLH is an inherited disorder, the diagnosis is difficult and often not made until a second affected child is born into a family. When we embarked on this study, the responsible gene(s) had not been localized, isolated, or characterized. Consequently, there was no carrier or prenatal testing available to those at risk of having an affected child. During the course of this study, two reports localizing putative FHLH genes in Asian (9q21.3–22) and European/African/Australian (10q21–22) families were published (4, 5). Recently, Stepp *et al.* (6) demonstrated that the chromosome 10q21 locus for FHLH is *PRF1*, a gene encoding the protein perforin, known to be an important mediator of lymphocyte cytotoxicity. They found *PRF1* mutations in eight patients, all of whom previously mapped to this locus by linkage analysis.

The only constitutional chromosome rearrangement offering an additional clue to the localization of FHLH genes was a pericentromeric inversion of chromosome 9 with break points at 9p23 and 9q31 in an infant with FHLH (7). The authors of this report speculated that the patient may have inherited one FHLH mutation from a carrier parent, with disruption of the second locus by the inversion. However, coexistence of the inversion with FHLH in this patient could also have been coincidental.

The objective of the current study was to identify candidate regions on which to focus future efforts to localize and characterize the defect causing FHLH.

METHODS

Families. We studied two Canadian kindreds in which first cousins were affected with FHLH according to the diagnostic criteria proposed by the FHLH Study Group of the Histiocyte Society (8). These families were of Anglo-Saxon (English and Irish) origins. The Newfoundland family (kindred 1) consisted of three affected cousins, two of whom are alive (Fig. 1). The diagnosis of FHLH was confirmed by autopsy in the third. These three affected children were born to sisters. The fathers of these children have their origins in a small area of the province and are thought to be distantly related to each other as well as to their wives. All three children developed cytopenia and hepatosplenomegaly with neurologic symptoms in their first year of life. Histologic examination of bone marrow

and/or liver biopsy specimens demonstrated hemophagocytosis. After induction chemotherapy, patient A was treated intermittently with a maintenance regimen of cyclosporin A, corticosteroids, and intrathecal methotrexate until he received an unrelated bone marrow donor transplant at the age of 14. He is currently 15 y of age and his condition is stable without any signs of FHLH. Patient C followed a similar clinical course and at the age of 11 remains on the maintenance regimen. She is currently being considered for an unrelated bone marrow donor transplant. She is developmentally delayed and is being treated for hypoparathyroidism in addition to FHLH. Patient B was the first child diagnosed in the family, and he died at the age of 9 mo.

The Nova Scotia family (kindred 2) consisted of two cousins affected with autopsy-confirmed FHLH who were also born to sisters (Fig. 2). The fathers of the affected children are not known to be related to the mothers or to each other. These two children had a classic early-onset and rapidly fatal clinical course. Patient E was diagnosed before 6 mo of age and died at 8 mo. Her cousin, patient D, was diagnosed at 2 mo of age and died at 32 mo.

With informed consent and institutional ethics approval, we obtained skin biopsies for cultured fibroblasts from the two living affected children and pathologic tissue specimens from the three deceased children. In lieu of blood, skin biopsies were taken from the living children to ensure that their DNA would not be contaminated by their previous transfusions. We also obtained peripheral blood samples from all 10 parents. Genomic DNA was extracted from blood and tissue samples by standard methods (9).

Genotyping. The fact that the parents of the affected children in kindred 1 and the mothers of the affected children in kindred 2 were related, suggested that a common (healthy) ancestor must have carried a single copy of an altered FHLH gene in each family. In kindred 1, the altered gene would have segregated independently through the various branches of the pedigree, and been inherited by all six parents. (The presence of a second normal copy of the gene in each of them would have
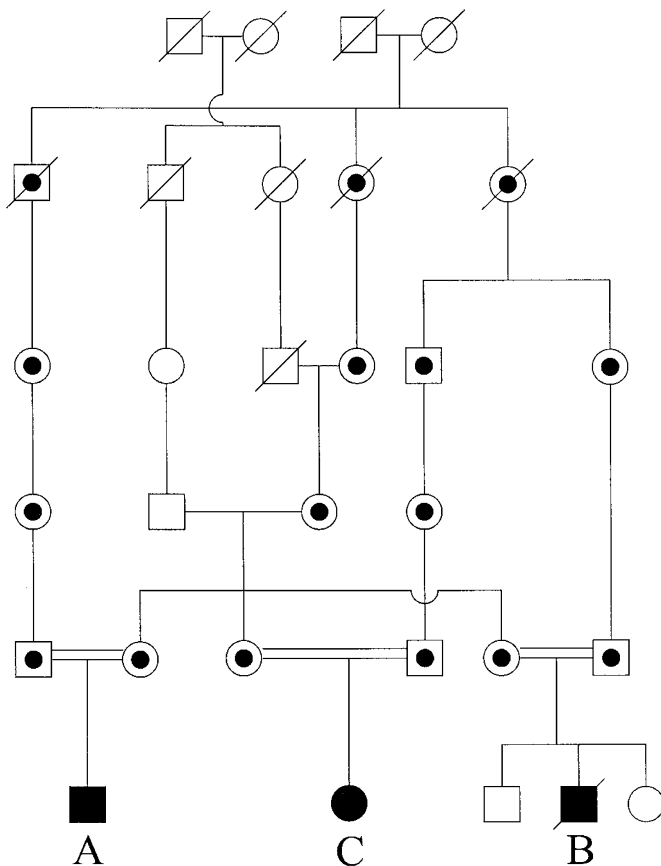

Figure 1. The Newfoundland family consisting of three affected first cousins born to consanguineous parents. Carrier status is inferred.
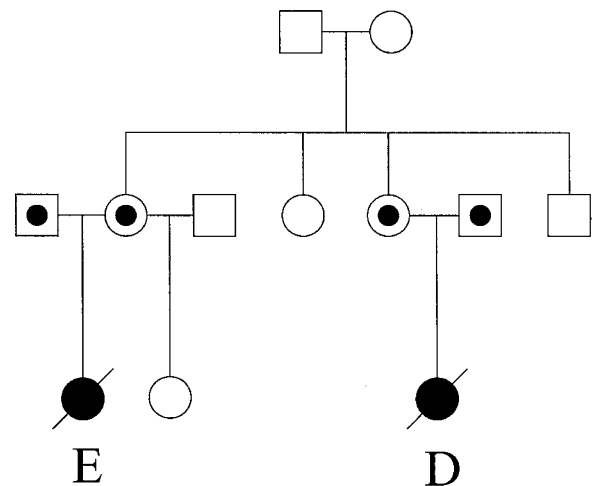

Figure 2. The Nova Scotia family consisting of two affected first cousins. The fathers of these children are not known to be related to their spouses or each other.

prevented them from developing this recessive condition; hence, they would have been healthy carriers.) FHLH would have resulted when their children inherited two altered copies of the gene, one from each parent. A similar logic could be used for kindred 2, the primary difference being that only the mothers were assumed to have a common ancestor.

When the altered genes that are present in each of the affected children are derived from a common ancestor, a specific strategy can be used to find polymorphic markers that are in close proximity to the gene. The rationale is that any polymorphic marker that is close enough to the causative gene to be of interest will demonstrate homozygosity by descent. This is defined as the presence of identical alleles at a given marker locus in each of the affected individuals in a family.

We embarked on a search for a closely linked marker locus by using a commercial multiplex PCR genotyping system (Prism Linkage Mapping Set version 2, Perkin Elmer Applied Biosystems, Inc.) capable of typing 400 highly polymorphic markers located at an average interval of 10 cM throughout the genome. Using this system and an ABI 377 automated sequencer with Genescan 2.1 and Genotyper 2.0 analysis software, we genotyped all 15 individuals (five affected children and 10 parents) for the 400 ABI markers.

We performed simultaneous parental genotyping to identify shared maternal alleles in the Nova Scotia family as well as to confirm paternity and exclude sample mix-up in all cases. For the small number of markers in which amplification of the child's sample was suboptimal, the availability of parental genotypes also allowed us to infer the child's genotype and/or rule out homozygosity by descent.

Candidate loci were selected based on the presence of alleles that were homozygous and identical in all three affected children from the Newfoundland family. In the Nova Scotia family, any marker demonstrating shared maternal alleles in the two affected children was considered a candidate. Thirty-four additional dinucleotide PCR markers not included in the ABI kit and flanking promising candidate loci were amplified and genotyped for all individuals. The additional markers were selected from the summary maps available from The Genetic Location Database at the Wessex Human Genetics Institute

(http://cedar.genetics.soton.ac.uk) (10), and primer sequences were located in the Genome Database (http://gdbwww.gdb.org). Fluorescent end-labeled primers were amplified in 15-μL reactions with 10 mM Tris-HCl pH8.3, 50 mM KCl, 250 μM dNTP, 0.33 μM primers, 0.75 U of AmpliTaq Gold (Perkin-Elmer), 50 ng of genomic DNA, and $MgCl_2$ optimized for each primer set at a concentration between 1.5 and 3.5 mM. Amplification was performed in an ABI 9600 thermocycler. DNA strands were denatured at 95°C for 12 min and subjected to 10 amplification cycles consisting of 95°C for 15 s, 52–58°C for 15 s, and 72°C for 30 s. This was followed by 24 amplification cycles consisting of 89°C for 15 s, 52–58°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 30 min. Products were run on 4.25% polyacrylamide gel on an ABI 377 autosequencer and analyzed with GeneScan 2.1 and Genotyper 2.0 software as for the linkage kit markers.

RESULTS

In our initial genome scan, we assumed a common locus and combined the two families to maximize the power of the analysis. All 400 paternal genotypes were consistent with stated paternity for each of the five affected children, ruling out nonpaternity with a probability of error of less than 10^{-99}. We identified a total of three candidate loci that met the combined criteria for homozygosity by descent in kindred 1 and shared maternal alleles in kindred 2 (boldface in Tables 1, 2, and 3). One of these, D9S1690, had a cytogenetic localization (9q22.33) proximal to one of the break points (9q31) of a previously reported inversion in an FHLH patient (5). We genotyped a total of 17 additional closely linked flanking markers mapping within 1–2 cM of either side of all three candidates. These markers were chosen from the Genetic Location Database (10) for their close proximity to the candidates, though it should be acknowledged that these marker localizations are based on a small number of families and may not be as precise as stated. None of the haplotypes from these additional markers demonstrated a pattern suggestive of homozygosity by descent despite the very small recombination distance between the markers and the candidates (Tables 1–3). In addition, the observed

Table 1. *Genotypes for candidate locus D9S1690 and flanking markers*

Marker	Cytogenetic localization	Genetic distance (cM)†	Kindred 1 patients*			Kindred 2 patients*	
			A (mat/pat)‡	B (mat/pat)	C (mat/pat)	D (mat)	E (mat)
D9S272	9q22.32	1.91	202/204	206/204	200/200	**206**	**206**
D9S176	9q22.33	1.24	130 + 140§	132/134	130/138	136	130
D9S1857	9q22.33	1.08	264/268	264/270	264/260	268	264
D9S1690	**9q22.33**	**0.00**	**231/231**	**231/231**	**231/231**	**231**	**231**
D9S750	9q31.1	1.64	267/269	269/261	255/271	251	263
D9S109	9q31.1	1.78	224/218	220/220	—¶/220	224	—¶
D9S277	9q31.1	3.26	198/172	182/166	—¶/—¶	196	—¶

* A–E refer to the affected children in Figures 1 and 2.

† Averaged from male and female recombination distances in The Genetic Location Database at the Wessex Human Genetics Institute (http://cedar.genetics.soton.ac.uk) (Ref. 9) or taken from Prism Linkage Mapping Set version 2, Perkin Elmer Applied Biosystems, Inc.

‡ Mat and pat designate maternally and paternally derived alleles.

§ + Designates that maternal and paternal alleles could not be distinguished.

¶ — Designates failed PCR or uninformative genotypes; parental alleles rule out locus as candidate.

Bolded alleles meet criteria for homozygosity in kindred 1 or 2.

Table 2. *Genotypes for candidate locus D14S276 and flanking markers*

Marker	Cytogenetic localization	Genetic distance (cM)†	Kindred 1 patients* A (mat/pat)‡	Kindred 1 patients* B (mat/pat)	Kindred 1 patients* C (mat/pat)	Kindred 2 patients* D (mat)	Kindred 2 patients* E (mat)
D14S288	14q21.1	7.8	196/204	196/196	206/206	196	204
D14S562	14q22.1	0.94	192/204	192/200	200/196	195	—¶
D14S276	**14q22.1**	**0.00**	**247/247**	**247/247**	**247/247**	**247**	**—¶**
D14S282	14q22.1	1.45	169/167	167/171	171/169	167	—¶
D14S63	14q23.3	14.3	190/186	186/186	186 + 190§	**186**	**186**

See Table 1 for footnotes.

Table 3. *Genotypes for candidate locus D19S414 and flanking markers*

Marker	Cytogenetic localization	Genetic distance (cM)†	Kindred 1 patients* A (mat/pat)‡	Kindred 1 patients* B (mat/pat)	Kindred 1 patients* C (mat/pat)	Kindred 2 patients* D (mat)	Kindred 2 patients* E (mat)
D19S226	19p13.13	12.6	252/242	251/261	254/258	**252**	**252**
D19S882	19q12	0.88	269/281	269/281	281/277	**281**	**281**
D19S414	**19q12**	**0.00**	**189/189**	**189/189**	**189/189**	**189**	**189**
D19S245	19q13.11	2.00	194/202	194/198	206/206	**202**	**202**
D19S220	19q13.13	7.90	280/286	280/284	282/280	280	274

See Table 1 for footnotes.

marker patterns did not conform to homozygosity by descent when a small number of recombination events were inferred.

The two families were then analyzed independently to encompass the possibility that they were segregating different genes. Kindred 2, consisting of affected first cousins born to sisters, was of limited use in the independent analysis because the only criterion for identification of candidate loci was the demonstration of shared maternal alleles. First cousins share, on average, one eighth of their genes; therefore, at least one eighth of the tested markers (approximately 50 of the 400) would be expected to meet these criteria on the basis of chance alone. In fact, a total of 112 markers demonstrated shared maternal alleles in kindred 2, and an additional 17 markers may have been shared but were not fully informative. Linkage analysis to further evaluate these markers as candidates was not possible because of the paucity of unaffected children in each of the two sibships. These would be considered candidate markers for future exploration in any additionally ascertained families that are suitable for homozygosity by descent or traditional linkage approaches.

Instead, we focused on kindred 1 with three affected first cousins and consanguineous parents. Six additional candidate loci were identified on the basis of shared homozygous alleles in this family (D1S2697, D3S1278, D3S1565, D8S549,

D9S1826, and D16S516), but further analysis of closely linked flanking markers did not demonstrate this pattern (data not shown). Once again, the observed marker alleles did not conform to homozygosity by descent even if a small number of recombination events were inferred.

During the course of this study, two reports localizing the FHLH gene were published (4, 5). We examined these 9q21.3-q22 and 10q21–22 regions in both of our families by using the same markers with which linkage was established in these reports (D9S175, D9S1867, D9S264, D9S167, D9S152, D9S1877, D9S1865, D9S1790, D9S283, D10S537, D10S1650, D10S556, D10S206, and D10S1686). D9S175 and D9S283 had previously been tested as part of the ABI Prism Linkage Set. Our results allowed us to eliminate these regions as candidates on the basis that none of these markers produced a pattern consistent with homozygosity by descent in our families (Tables 4 and 5).

DISCUSSION

FHLH is the most common HLH seen in infancy and childhood, with an estimated incidence of 0.12 cases per 100,000 children per year (11). It has been reported from all continents and from many ethnic groups. An increased inci-

Table 4. *Genotypes for candidate locus 9q21.3–9q22 and flanking markers*

Marker	Cytogenetic localization	Genetic distance (cM)†	Kindred 1 patients* A (mat/pat)‡	Kindred 1 patients* B (mat/pat)	Kindred 1 patients* C (mat/pat)	Kindred 2 patients* D (mat)	Kindred 2 patients* E (mat)
D9S1867	9q21.33	5.1	192/204	192/192	190/211	**206**	**206**
D9S264	9q22.1	2.23	153/166	152/152	164 + 166§	164	166
D9S167	9q22.1	0.69	316/313	318/316	320/318	325	323
D9S152	9q22.1	0.00	— /—¶	127/127	129/133	127	125
D9S1877	9q22.1	0.59	125/125	125/123	123/127	**123**	**123**
D9S1865	9q22.1	4.85	154/148	148/145	148/148	154	153
D9S1790	9q22.1	4.92	176/190	192/190	176/190	190	176

See Table 1 for footnotes.

Table 5. *Genotypes for candidate locus 10q22.2–10q22.3 and flanking markers*

Marker	Cytogenetic localization	Genetic distance (cM)†	Kindred 1 patients*			Kindred 2 patients*	
			A (mat/pat)‡	B (mat/pat)	C (mat/pat)	D (mat)	E (mat)
D10S537	10q22.2	0.07	162/150	164/150	164/164	152	158
D10S1650	10q22.2	**0.00**	134/138	138/118	138/144	**134**	**134**
D10S556	10q22.2	1.10	191/195	197/197	197/193	**197**	**197**
D10S206	10q22.2	0.84	265/265	255 + 265§	265/265	265	262
D10S1686	10q22.3	9.11	261/274	274/266	270/268	**276**	**276**

See Table 1 for footnotes.

dence has been noted in ethnic groups in which consanguineous matings are part of the cultural tradition (2). Although 70% of patients experience onset of symptoms in the first year of life, the disease may present itself as late as the eighth year of life. Within a given family, a tendency toward similar ages of onset has been reported (12).

The signs and symptoms may vary widely, from intermittent fever, pronounced hepatosplenomegaly, and bone marrow suppression to progressive cerebromeningeal symptoms. Hypertriglyceridemia and coagulation abnormalities, particularly hypofibrinogenemia, are common findings. Histopathologically, FHLH is characterized by a nonmalignant mixed lymphohistiocytic accumulation in the monophagocytic system. Finally, most patients have low or absent NK activity and T-cell cytotoxicity (13).

Without treatment, FHLH is usually rapidly fatal with a median survival of approximately 2 mo (14). Although the combination of steroids and epipodophyllotoxins prolongs survival (15), hematopoietic stem cell transplantation remains the only cure (16). Localization of the gene for FHLH would be helpful in the early identification of at-risk siblings in families with a previously affected child. In addition, it would represent the first step toward isolation and characterization of the gene and its protein product.

Using the homozygosity-by-descent strategy in these two unique Canadian families, we completed a genome screen at an average resolution of 10 cM. This technique has been used to localize a wide variety of genes and has proven successful for other conditions in which DNA from as few as four (17) or five (18) affected individuals was available. In our combined and independent analyses, we detected a total of nine candidate loci; however, closely linked and highly polymorphic dinucleotide repeat markers flanking each candidate did not demonstrate a similar pattern of identical alleles. This suggests that the original observations were due to chance alone.

There are at least four explanations for our failure to identify a candidate locus by use of this strategy. The first and most likely is that the gene of interest lies in an interval between the ABI markers. These are spaced at average distances of 10 cM but range from as little as 2.4 cM to as large as 24.1 cM. If the gene is located in the center of one of the large intervals, recombination between the gene and one or more flanking markers may have taken place. Assuming a common founder for the carrier haplotype in the first generation of kindred 1, there have been a total of 22 meioses between the founder and the three affected cousins in the fifth generation. Each of these meioses would be associated with a small opportunity for recombination, and this was a possibility that we acknowledged at the outset of the study.

Inaccurate pedigree information represents the second possible source of error. Unfortunately, there is no population or church registry by which we were able to confirm the family relationships reported to us by the parents of kindred 1. We have reexamined the data to consider the possibility that one of the fathers was unrelated to the family (described below).

The third explanation is that of inaccurate diagnosis: namely, that our patients did not have primary (familial) hemophagocytic lymphohistiocytosis. Recently, Janka *et al*. (19) reviewed the literature with respect to secondary (infection- and malignancy-associated) hemophagocytic syndromes. Over a period of 16 y (1979–1995), 219 children ranging from newborns to 18 y of age had been described in the literature. A triggering organism was identified in 163 of 219 cases. In approximately 75%, EBV infection was the cause. Other viruses identified included human herpes virus 6, cytomegalovirus, adenovirus, parvovirus, and *Varicella zoster* virus. Herpes simplex virus, Q-fever virus, and measles virus were found in a few cases. A bacterial infection in approximately 7% and a fungal or a parasitic infection in 1 to 2% led to the hemophagocytic syndrome. These causes were all excluded in the patients described here.

All of our affected cousins met the existing criteria for the diagnosis of FHLH (8), and, in three of the five individuals, the diagnosis was confirmed by autopsy findings. Of potential relevance, however, are the unusual clinical courses in the two living affected cousins in kindred 1, patients A and C, who are surviving at 15 and 11 y of age. Because the presence of a different disease allele from the father of child B may explain the different course in this child, we reexamined the data from kindred 1, making the assumption that the father of patient B was unrelated to the family. Candidate markers in this reanalysis had to demonstrate identical alleles in patients A and C and a shared maternal allele in patient B. This analysis yielded eight additional loci on six different chromosomes. Three of these eight loci were on the same chromosome, but none of them contiguous or in close proximity, and the intervening loci did not conform to these criteria. In addition, none of the eight loci demonstrated shared maternal alleles in kindred 2. An alternate and more likely explanation for the more severe course in patient B is that he was the first affected individual in his family. The diagnosis was not made as quickly as it was in his cousins, who were the beneficiaries of very early treatment.

A fourth possible source of error is that the disease in either or both families is the result of digenic inheritance. This

explanation would require that the parents of one or more of the affected children were carriers of mutations in different FHLH genes. Although possible, this seems unlikely, particularly in kindred 1 in which parental consanguinity would favor a single founding mutation.

Previous reports have localized putative genes for FHLH to the long arms of chromosome 9 and 10 in families of Asian and mixed European/African/Australian origins (4, 5). The chromosome-10 locus is now known to encode the perforin protein, and both nonsense and missense mutations in the *PRF1* gene have been demonstrated in eight FHLH patients (6). All of these patients were from families that had previously been linked to 10q21–22. The newly revised localization of the *PRF1* gene at 10q21 places it on the centromeric side of our markers. However, it remains close enough to D10S537, our most proximal marker, to be contained within the same YAC clone (6), so the probability of recombination between these loci would not exceed 1% (6). Given that our two kindreds analyzed independently and together failed to demonstrate homozygosity by descent and shared maternal alleles for the same chromosome-10 markers used to establish linkage in other families (5), it is very unlikely that our patients have mutations in this gene. We were not surprised that our eastern Canadian families, who have English and Irish origins, do not appear to map to these loci. Our results suggest the existence of additional FHLH loci and confirm previous reports of genetic heterogeneity in this condition.

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