Abnormal Cell Surface Antigen Expression in Individuals with Variant CD45 Splicing and Histiocytosis

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ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) and Langerhans cell histiocytosis (LCH) are members of a group of rare heterogeneous disorders, the histiocytoses, characterized by uncontrolled accumulation of pleomorphic infiltrates of leukocytes. The etiology of these diseases is mainly unknown. CD45 is a hemopoietic cell specific tyrosine phosphatase essential for antigen receptor mediated signaling in lymphocytes and different patterns of CD45 splicing are associated with distinct functions. Recently a polymorphism (C77G) in exon 4 of CD45 causing abnormal CD45 splicing and a point mutation affecting CD45 dimerization were implicated in multiple sclerosis in humans and lymphoproliferation and autoimmunity in mice respectively. Here we show that two patients with HLH exhibited abnormal CD45 splicing caused by the C77G variant allele, while a further

21 HLH patients have normal CD45. We have also examined 62 LCH patients and found three to have the C77G mutation. Peripheral blood thymus-derived (T) CD8⁺ cells from *normal* individuals carrying the C77G mutation show a significant decrease in the proportion of cells expressing L-selectin and increased frequency of cells with LFA-1^{hi} expression. It remains to be established whether C77G is a contributing factor in these histiocytic disorders. (*Pediatr Res* 55: 478–484, 2004)

Abbreviations

LCH, Langerhans cell histiocytosis
HLH, hemophagocytic lymphohistiocytosis
PBMC, peripheral blood mononuclear cells
PHA, phytohemagglutinin

The leukocyte common antigen CD45 is an abundant tyrosine phosphatase, essential for lymphocyte antigen receptor signal transduction (1). Both CD45 knockout mice (2, 3) and humans lacking CD45 expression (4, 5) are severely immunodeficient with very few peripheral T lymphocytes and impaired T and B cell responses.

Multiple CD45 isoforms can be generated by alternative splicing of exons A, B, and C of the extracellular domain (6, 7). In humans naïve T cells express high molecular weight CD45

isoforms, recognized by CD45RA MAb (“CD45RA” cells), but activation of the cells results in a change to expression of low molecular weight isoforms, detected by a CD45R0 MAb (“CD45R0” or memory cells) (8). Paragraph deleted. Several polymorphisms of the gene encoding CD45 (*PTPRC* at 1q31–32) have been described and several of these cause altered splicing (9–14).

The C77G polymorphism (9–12) has been linked to the development of multiple sclerosis in German (15) and Italian (16) patient cohorts, although other studies do not support such a correlation (17, 18). An increased frequency of the C77G variant allele has been demonstrated in HIV-1 infected individuals (19), in systemic sclerosis (20) and in autoimmune hepatitis (21). These observations suggest that abnormal CD45

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splicing is associated with altered immunologic function, autoimmunity and viral infections. *Sentence deleted.*

Two patients with a similar defect in CD45 splicing associated with familial hemophagocytic lymphohistiocytosis (22, 23) have been previously described. Hemophagocytic lymphohistiocytosis (HLH) comprises a heterogeneous group of disorders characterized by dysregulated activation of T lymphocytes and macrophages (24–26). Although some patients with HLH harbor perforin mutations (HLH type 2), others show link to a locus on chromosome 9, while the cause of the remaining cases is not known (27–29). Langerhans cell histiocytosis (LCH) is another rare disorder characterized by the dysregulated proliferation of Langerhans cells and subsequent organ infiltration (30, 31). The pathogenesis of the disease is still unknown and the clinical picture is highly variable ranging from a benign course with spontaneous recovery to a very rapidly evolving process with a fatal outcome.

In this study we initially analyzed the two previously described patients with HLH (22, 23) and unusual expression of CD45 isoforms, using flow cytometry and molecular typing. We further typed 21 other HLH patients for the C77G polymorphism. Subsequently we examined the frequency of the C77G polymorphism in a series of Italian patients affected by LCH compared with healthy Italian controls. Finally we analyzed the expression of various cell surface molecules of peripheral blood mononuclear cells from *normal* individuals with the C77G variant. Our results show that individuals carrying the C77G variant display phenotypic differences that *might* affect the migration and function of leukocytes. Further studies are warranted to determine whether CD45 splicing abnormalities play any role in the pathogenesis of histiocytosis.

PATIENTS, MATERIALS AND METHODS

Patients. Fresh blood was obtained from the previously described family W (23) and family G. (with two children with HLH) from the Immunobiology Unit, Institute for Child Health, London, UK. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Paque (Amersham Pharmacia Biotech, Buckinghamshire, UK) density gradient and genomic DNA was extracted by standard procedures (32). Genomic DNA samples from family R (22) together with genomic DNA samples from 19 unrelated Italian HLH patients (9 of which contained *PRF1* mutations as previously described (33)), were provided by MA.

The characteristics of the HLH patients (22, 23) with C77G are as follows:

The patient R. was a first child of consanguineous Belgian Caucasian parents (22). The patient presented at the age of 2 mo with fever, hepatosplenomegaly, neutropenia, thrombocytopenia, hypofibrinogenemia, and hypertriglyceridemia. He responded to initial treatment with etoposide and underwent bone marrow transplantation from his haploidentical half-sibling and remained asymptomatic over 8 y later.

Patient W. was the third child of healthy unrelated British Caucasian parents (23). He presented aged 3 mo with fever, diarrhea, pallor, increasing irritability and marked cervical lymphadenopathy and hepatosplenomegaly. Laboratory in-

vestigations revealed pancytopenia, coagulopathy and hypertriglyceridemia. The diagnosis of HLH was made from the bone marrow aspirate, which showed hemophagocytosis. There was a good response to initial treatment with dexamethasone and etoposide and he underwent allogeneic bone marrow transplantation from his HLA identical brother.

Sixty-two LCH patients were analyzed, and forty-one were diagnosed with LCH when younger than 18 y. Patients' age at time of diagnosis ranged from 5 mo to 17 y with a mean age of 4.73 y and the female to male ratio was 1:1.62. The diagnosis of LCH was biopsy proven, according to current diagnostic criteria established by the Histiocyte Society. The control population consisted of 199 unrelated Italian samples, including medical students, blood donors and university staff, representative of the whole country with a male to female ratio of 1:1.15 (30% being under the age of 18).

The main features of the three patients with LCH and the C77G mutation are as follows.

CASE 1.

S.F., male. At the age of 3.5 y he developed a cranial swelling. Skull x-ray and CT scan revealed an isolated osteolytic region. Surgical biopsy confirmed the diagnosis of CD1a-positive LCH. Complete diagnostic work up ruled out any additional location of disease. One year later the lesion healed without any additional treatment and, after 1 y, the child remains asymptomatic.

CASE 2.

B.S., female. At the age of 5.4 y a skull x-ray performed because of minor trauma revealed an osteolytic region. Surgical biopsy confirmed the diagnosis of CD1a-positive LCH. Complete diagnostic work up ruled out any additional location of disease. One year later the lesion healed without any additional treatment and, after 1 y, the child remains asymptomatic.

CASE 3.

M. F., male. At the age of 17 y he developed polyuria and polydipsia, was diagnosed as affected with diabetes insipidus. Clinical evaluation showed osteolytic lesions and moderate liver dysfunction. Histologic diagnosis of bone lesions was confirmed as LCH by demonstrating CD1a+ cells. A thorough diagnostic work-up showed multisystem LCH with multifocal bone lesions and endocrine defects (diabetes insipidus and GH deficiency with thick pituitary stalk). During the following years he showed recurrent bouts of bony reactivation; since nonsteroidal anti-inflammatory drug therapy was unsuccessful, he underwent treatment with vinblastin and steroids according to the LCH-II study protocol. At the time of writing he is asymptomatic with sub-clinical liver and lung involvement.

Buffy coats bags were obtained from the National Blood Transfusion Service (London, UK), genotyped for the presence of C77G variant and used for flow cytometric analysis as described below.

The patients gave informed consent for the study and approval was obtained from the ethical review board of the Edward Jenner Institute for Vaccine Research.

Genotyping for CD45 Exon A (C77G) Mutation. Genomic DNA was amplified by PCR using forward (5'-GACTACAG-

CAAAGATGCCCAAGTG-3') and reverse (5'-GGGATACTT-GGGTGAAGTA-3') primers as previously described (34). The PCR and digestion products were analyzed on VisiGel Separation Matrix (Stratagene, La Jolla, CA, U.S.A.).

Flow Cytometric Analysis. PBMC were stimulated for 12 d with 1 µg/mL of PHA-P (Sigma Chemical Co., St. Louis, MO, U.S.A.) adding IL-2 on day 10. Flow cytometric analysis of CD45 variant splicing was performed as previously described (34). Briefly, 2×10^5 PBMC were stained with either APC-conjugated CD4 (S3.3, Caltag, Siilverstone, UK) or CD8-APC (clone RPA/T8, Pharmingen, San Diego, CA, U.S.A.) along with FITC-conjugated CD45RA (clone HI10, Pharmingen) and PE-conjugated CD45R0 (clone UCHL1, Pharmingen) mAbs in a single step at 4°C for 20 min and washed with PBS, containing 0.2% BSA. The following reagents and antibodies were also used to stain cell suspensions: CD11a-FITC (G43-25B), CD28-FITC (CD28.2), CD44-FITC (G44-26), CD62L-FITC (Dreg56), CD95-FITC (DX2), CCR7 (2H4) were all from PharMingen, CD62L-FITC (LAM1-116) (Ancell, Bayport, U.S.A.), CD69-FITC (CH14) (Caltag, Silverstone, UK), HLA-DR-FITC (TU36) (Caltag), CD25-FITC (ACT-1) (Dako), CD4-FITC (Dako), CD27-FITC (LT27) (Serotec, Kidlington, UK). Isotype matched mAbs were used as controls. 10,000 or 50,000 events per sample were collected on a FACSCalibur (Becton Dickinson, Mountain View, CA, U.S.A.) and analyzed using WinMDI software.

Statistical Analysis. The Fisher exact test was used to analyze the association of C77G polymorphism and LCH or HLH. For comparison of phenotypic analyses between cell subsets in C77G and control individuals, the *t* test, assuming equal variance, was used.

RESULTS

The C77G variant is the cause of CD45 abnormal splicing in two families with HLH. We obtained material from two patients with HLH, previously described as exhibiting CD45 abnormal splicing, as characterized by the lack of the single CD45R0+ T cell population (22, 23). Subsequently the C77G mutation in exon A of CD45 has been shown to be a common cause for abnormal CD45 splicing in T lymphocytes (10, 34). We therefore re-examined these patients and members of their families to establish the cause for abnormal CD45 splicing.

In the first family reported, the patient R. was a first child of consanguineous Belgian Caucasian parents (22). Genotyping for the C77G polymorphism revealed that the patient and his mother are heterozygotes while his father and grandmother (also the father's sister) carried wild type CD45 (data not shown). Material from two half-siblings, who died from histiocytic disorders, was not available to us. No perforin mutations were detected in this family as analyzed by sequencing of exons 2 and 3.

In the second family six members were analyzed (23). Patient W. was the third child of healthy unrelated British Caucasian parents. Using PCR and *MspI* restriction analysis we found that patient W. his mother and two siblings were heterozygous for the mutant C77G allele, while the father and the oldest brother had wild type CD45 (Fig. 1A). These results

Figure 1. CD45 genotype and phenotype of family W. Family tree indicating the CD45 genotype and phenotype in each member of family W. The patient with HLH (5) is indicated by an asterisk. (A) Identification of the CD45 exon A (C77G) mutation in family W. The C77G transversion introduces a new restriction site for *MspI*, which cleaves the mutant PCR product into two fragments of 72 and 83 bp (lanes 2, 4, 5, and 6). The presence of an undigested band of 155 bp indicates the presence of the wild type allele in the father and older brother (lanes 1 and 3). (B) Expression of CD45 isoforms in human peripheral T cells. PBMC were stained with isoform specific CD45RA-FITC and CD45R0-PE together with CD4-APC mAbs. Analysis was performed on gated CD4⁺ cells. The normal pattern of CD45 isoform expression is characterized by the presence of single CD45RA⁺ and single CD45R0⁺ cells. Abnormal CD45 expression was seen in the patient (5), his mother (2) and two siblings (4 and 6). The father (1) and brother (3) have normal CD45 pattern of expression.

were confirmed by flow cytometric analysis on PBMC from family W. (Fig. 1B). All of the family members genotyped as

having the C77G mutation exhibit phenotypically abnormal CD45 splicing. Members of this family exhibited a novel missense mutation in the Ca^{++} binding domain of perforin, which interestingly co segregated with the C77G mutation (35).

Taken together, these results show that the CD45 exon A (C77G) mutation is the cause for the CD45 abnormal splicing in these two previously described HLH patients.

Analysis of C77G mutation in 21 HLH patients. Since two families with HLH were identified with abnormal CD45 splicing and the C77G mutation we next investigated the pattern of CD45 expression in other HLH patients. Using PCR and *MspI* restriction analysis we genotyped 21 patients with HLH (including the two affected siblings from family G.) for the presence of the C77G mutation. Nine of these cases had previously identified perforin mutations (HLH type 2) (33), while the remaining twelve show an HLH 1 phenotype and the presence of known deleterious *PRF1* mutations has been excluded by sequencing exons 2 and 3. We did not find the mutant C77G allele in any of these patients.

Analysis of C77G variant in LCH patients. LCH is another disorder classified as a histiocytosis and characterized by lesions infiltrated by cells of the dendritic cell lineage. Therefore we investigated the pattern of CD45 expression in a cohort of Italian patients with LCH. Using PCR and *MspI* digestion we identified 3 individuals with the C77G mutation out of 41 LCH infantile patients (frequency of 7.3%), while no mutation was found in patients older than 18. The C77G mutation was observed in 1 out of the 199 controls (frequency 0.5%). Using a two tailed Fisher's exact test to test for the association between C77G mutation and LCH a statistically significant association was demonstrated ($p = 0.043$, on the overall frequency in LCH 4.8% versus 0.5% in control).

Phenotypic analysis of PBMC of individuals with the C77G variant: CD45 isoform expression. We next examined whether the abnormal pattern of CD45 isoform expression affects other aspects of leukocyte phenotype. Cryopreserved PBMCs from healthy individuals known to carry the C77G mutation and cryopreserved normal control cells were analyzed by flow cytometry. No apparent differences in the proportion of CD3, CD4, CD8, CD14, and CD19 cells were observed between the individuals with the C77G variant and wild type CD45 (data not shown). All of the C77G samples showed the previously described typical pattern of CD45 isoform expression on both CD4 and CD8 cells (Fig. 2A). Even after 12 d stimulation with PHA and IL-2 stimulation, neither CD4 nor CD8 cells of C77G individuals were able to switch to expression of only the CD45R0 isoform (data not shown). However, it is noteworthy that the CD8 cells of individuals with the C77G mutation have more CD45RA single positive cells (mean 75%) compared with normal individuals (mean 58%) ($p = 0.001$ for 6 C77G carriers and 6 controls) (Fig. 2A and B). In contrast the proportions of CD45RA versus CD45R0 or CD45RA/R0 double positive cells are similar among CD4 cells from C77G and control samples.

The phenotype of CD8 T cells in individuals with the C77G variant. Because of the strikingly altered proportions of CD45RA positive versus CD45R0 or CD45RA/R0 double

Figure 2. Expression of CD45 isoforms and CD11a on T cells from C77G and control individuals. (A) PBMC were stained with isoform specific CD45R0-PE and CD45RA-FITC antibodies and CD4 and CD8 gated cells were analyzed. Abnormal CD45 splicing can be identified by the absence of the CD45RA-negative population. Data are representative of analyses of six C77G individuals and six controls. (B) The proportions of CD8 T cells from C77G and normal individuals that are CD45R0⁻ or are CD11a^{hi}. Means and standard deviations of data expressed as the percentage of CD8 T cells from six C77G and six normal individuals are shown. (C) Expression of CD11a on CD8 T cells from C77G and normal individuals. Data are representative of analyses of six C77G individuals and six controls.

positive cells among CD8 cells from C77G and control individuals we next examined the expression of various cell surface markers associated with lymphocyte activation, analyzing them in the CD45R0⁺ and CD45R0⁻ subsets. No statistically significant differences were observed in the expression of the adhesion molecule CD44, the costimulatory molecule CD28, cytokine and chemokine receptors CD25 and CCR7, and the activation markers CD69 and HLA-DR (data not shown).

However, an increased frequency of CD8 cells expressing high levels of the adhesion molecule CD11a (CD11a^{hi}) was

detected in the (enlarged) CD45R0⁺ subset ($p = 0.025$) (Fig. 2B and C). In the CD45R0⁺ subset the expression of CD27, CD62L and CD95 was significantly decreased in C77G individuals compared with controls (Fig. 3A and B). The differences in CD62L expression were confirmed with a different CD62L antibody (clone LAM1-116, data not shown), suggesting that the observed variances were not due to differential glycosylation of surface molecules in C77G and control cells.

DISCUSSION

It is becoming clear that many if not all diseases are the result of a complex interaction between individuals and their environment involving multiple host genes. The complexity of this interaction is illustrated by individuals with abnormal CD45 splicing, who have persistent expression of the high molecular weight CD45RA isoforms (9). In this study we analyzed the association between abnormal CD45 splicing and HLH and LCH syndromes. HLH and LCH are classified as histiocytoses, a group of rare diseases, characterized by accumulation of pleomorphic infiltrates of leukocytes into many tissues. A hallmark of the infiltrates is the presence of histiocytic cells such as macrophages and dendritic cells. The etiology of these disorders remains obscure although some cases of

HLH are associated with perforin gene mutations (28, 29, 36, 37) and there is evidence that LCH is a clonal proliferation. Environmental triggers such as viral infections and smoking have also been invoked in the case of LCH (31, 38, 39).

We studied two patients with HLH previously described as exhibiting abnormal CD45 splicing (22, 23) and found that the C77G polymorphism is the cause for the observed variant splicing (35). *Sentence deleted.* We have examined the association between C77G mutation and HLH in an additional 21 DNA samples and none of these were C77G positive. This is, however, a very small group of varied origin (UK, Belgium, Italy) and it is therefore impossible to draw statistically significant conclusions although taken together these results show a frequency of 1:10 in HLH type 2 (with identified *PRF1* mutations), or 2:23 for HLH overall (frequency 8.6%). Published frequencies for normal individuals vary between 0% and 3.6% in different parts of Europe and North America (see below) (40).

We also investigated the distribution of the C77G variant in a group of LCH patients as part of a larger study aiming to identify the genetic factors involved in the pathogenesis of this disorder. Here we show a statistically significant association of the C77G mutation and LCH ($p = 0.043$). We identified 3 individuals with the C77G variant out of 62 LCH patients (frequency 4.8%) compared with one out of 199 healthy controls (frequency 0.5%). The association of C77G and LCH does not result in any distinctive clinical manifestation of LCH as the age of onset of these patients is well within the control range and they include examples of both the most common clinical pattern of single system bone LCH, as well as multi-system disease. Their clinical course was not unusual.

The frequency of the C77G variant in different populations has not been extensively studied. Here we show a frequency of 0.5% individuals with C77G polymorphism among 199 healthy donors from Italy, which is in agreement with another study (16). However another more recent study found frequencies of 2.3 and 1.3% in Northern and Southern Italy respectively (40). These appear slightly lower frequencies compared with 3.6% in North America, 2.8% in Sweden, and 1.76% in the UK. However a study in Germany found less than 1% C77G positive individuals and none were found among 527 African samples (41). One might speculate that the distribution of the C77G variant throughout Europe decreases from North to South, but clearly further studies are required to establish whether these apparent differences are associated with ethnic variations or the health status of the populations tested. More extensive studies need to be performed to establish the frequency of abnormal CD45 splicing and the C77G variant allele in HLH and LCH.

Thorough phenotypic and functional analysis has not been performed on C77G heterozygous individuals so far. Our results indicate that the *normal* carriers of the C77G mutation have normal proportions of T cells, B cells and monocytes in peripheral blood. Monocytes, which normally express the low molecular weight CD45 R0 isoform, express higher amount of CD45RA in C77G carriers compared with normal individuals (data not shown). Further studies of the phenotype of these

Figure 3. CD62L, CD27 and CD95 expression on CD8+ CD45R0+ cells. (A) Proportions of CD8 T cells in the CD45R0 subset that express CD27, CD62L, and CD95. Means and standard deviations of data from six C77G and six control individuals are shown. (B) Flow cytometric dot plots show PBMC samples from C77G and normal individuals stained with CD27, CD62L and CD95 and gated on CD8 T cells. Examples are representative of similar analyses of six C77G and control individuals.

cells are required in relation to the pathogenesis of these two diseases.

However, there were significant differences in the expression of various phenotypic markers associated with activation and differentiation of T cells namely – the adhesion molecules CD11a and CD62L, the co-stimulatory molecule CD27 and the activation antigen CD95. There was also a striking increase in the proportion of CD45RA single positive CD8 T cells in C77G samples; 75% *versus* 58% in controls. The expression of CD11a suggests that these cells may be primed CD45RA “revertants” (42, 43). Decreased expression of CD62L and CD27 in individuals with C77G indicates that these individuals have an increased proportion of CD8 T cells with an activated/effector phenotype capable of migrating into no-lymphoid tissues. This would be consistent with the increased infiltration of lymphocytes found in HLH and LCH.

While there has been considerable success in identifying the genes implicated in Mendelian disorders, the identification of susceptibility genes underlying multifactorial diseases has progressed more slowly. This study illustrates the difficulties involved, particularly in the case of rare diseases such as HLH and LCH. Here we have shown that the frequency of the C77G allele is increased in a cohort of LCH patients compared with controls, we have no evidence that there is an increased frequency in HLH. Separately we have shown evidence for the presence of a population of activated T cell effectors in normal individuals with the C77G allele. We have not established a causal role for C77G in HLH or LCH, but suggest that this merits further investigation.

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