

Hemochromatosis-Related Mutation Detection

To the Editor:

In her recent letter to BLOOD, Dr Lynas reports the development of a “cheaper and more rapid polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) technique”¹ for detection of mutations in *HFE*,² the candidate gene for hemochromatosis. The PCR-RFLP method discussed by Dr Lynas uses *Rsa* I and *Bcl* I to cleave products amplified using primers designed by Feder et al.² However, many authors, including Jouanolle et al³ in November 1996, Roberts et al⁴ in February 1997, Martinez et al⁵ in the March 1997 issue of this journal, and Carella et al⁶ and Merryweather-Clarke et al⁷ in April 1997, have already published PCR-RFLP methods incorporating the use of *Rsa* I for detection of the C282Y mutation, and Roberts et al⁴ and Martinez et al⁵ have already published the application of *Bcl* I for detection of the H63D mutation. Dr Lynas¹ considers this technique to be superior to other RFLP-PCR methods because these two enzymes are cheaper than *Sna*BI (used by Jazwinska et al⁸ for C282Y RFLP-PCR) and *Mbo* I (used by Jouanolle et al,³ Carella et al,⁶ Merryweather-Clarke et al,⁷ and Jazwinska et al⁸ for H63D RFLP-PCR). In reply, we point out that our reasons for using the more frequent 4-bp cutter *Mbo* I instead of the 6-bp cutter *Bcl* I are particularly pertinent for the diagnostic applications referred to by Dr Lynas and afforded greater accuracy in a worldwide study of the *HFE* H63D/C282Y genotype of 5956 chromosomes.⁷ We redesigned one of the primers used to amplify the DNA fragment encoding the H63D mutation (H63DF primer, 5'ACA TGG TTAAGG CCT GTT GC; H63DR primer, 5'CTT GCT GTG GTT GTG ATT TTC C)⁷ to produce a 294-bp fragment instead of the 208-bp fragment amplified by Feder et al.² The extra 86 bp incorporated in this fragment contain an additional *Mbo* I recognition site, which provides an internal control for restriction digestion.^{7,9} Following agarose gel electrophoresis, it is therefore possible to distinguish between a partially or nondigested product of 294 bp, which may be of either genotype, and a digested product containing the H63D mutation, which yields fragments of 57 bp and 237 bp on *Mbo* I digestion. (The wild-type PCR product yields *Mbo* I digestion products of 138 bp, 99 bp, and 57 bp.) This internal control against misdiagnosis due to partial or nondigestion is not possible in the method discussed by Dr Lynas, as the fragment amplified from an H63D allele remains uncleaved and is thus indistinguishable from a nondigested fragment from either allele.¹

Dr Lynas places considerable emphasis on cost. We suggest that instead of using the QIAmp kit for extracting DNA from blood (Qiagen, Crawley, UK; £1362 for 1,000 extractions from 200 µL of blood), the more economical Nucleon Kit (Amersham Little Chalfont, UK; £95 for sufficient reagents for 2,500 extractions from 200 µL of blood) may be suitable. We have found the Nucleon Kit perfectly adequate for producing DNA for PCR,¹⁰ a procedure taking only 30 minutes. Another rapid method of DNA extraction¹¹ enables one to process 20 samples in 1 hour at a total consumables cost of 5 pence per sample. We have also found that the H63D PCR works using whole blood as a template if it is first subjected to 3 cycles of 95°C for 5 minutes followed by 30°C for 5 seconds, as suggested by Rees et al.¹² Another cheap and rapid method for C282Y typing is mutagenically separated (MS)-PCR.¹⁰ This allele-specific technique is ideally suited to routine analysis of blood samples in a diagnostic laboratory, as whole blood may be used as the template in a single PCR reaction per sample and restriction enzymes are not required. Other methods more suited to automation include heteroduplex analysis¹³ and PCR-SSP.¹⁴

The availability of many different methods for the diagnosis of *HFE* mutations C282Y and H63D means that each will have its own particular advantage in different situations and that diagnostic labs can confirm diagnoses by the simultaneous use of independent methods.

However, while we congratulate Dr Lynas on the adaptation of PCR-RFLP for use in her laboratory¹ and applaud her focus toward the diagnosis of hemochromatosis patients, we contest her claim to have developed a new method.

Finally, there has been a plethora of nonpeer-reviewed material on the subject of *HFE*, and we welcome the interest this stimulates in the field of hemochromatosis. However, we encourage editors and authors alike to take more care over both the nomenclature used (the gene is correctly called *HFE*,¹⁵ not *HLA-H*) and the emphasis given to nonpeer-reviewed literature over peer-reviewed publications. Perhaps this would help to avoid the confusion and duplication of methods observed in this case.

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Fc-εRII/CD23 Receptor on Circulating Human Eosinophils

To the Editor:

The expression of receptors for IgE on human eosinophils remains controversial, as reviewed in a recent comprehensive editorial.¹ Although human eosinophils from subjects infected by *Schistosoma mansoni* were recently reported to express different types of receptors for IgE (high-affinity Fc-ε receptor [Fc-εRI],² low-affinity Fc-ε receptor [Fc-εRII],³ and Mac-2/εBP),⁴ murine eosinophils purified from hepatic granulomas of mice affected by *S mansoni* parasitosis were shown not to express IgE receptors either by flow cytometric or by reverse transcriptase polymerase chain reaction analyses.⁵ In this respect, further studies were encouraged by Kita and Gleich¹ to solve the existing controversies concerning the expression of Fc-εRII in human hypereosinophilic diseases.

We investigated the expression of CD23 antigen on peripheral blood eosinophils from 14 patients affected by vernal keratoconjunctivitis (VKC) with mild-to-moderate eosinophilia and from 10 matched normal controls. All patients showed skin reactivity to grass (n = 5), *Dermatophagoides pteronyssimus* (n = 5), *Parietaria officinalis* (n = 2), or multiple allergens (n = 2). In brief, 150 μL of unfractionated peripheral blood were incubated for 30 minutes at 4°C with the following fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies (MoAbs): CD23 (EBVCS-5 clone, derived from BALB/c mice immunized with in vitro-transformed EBV cell line; IgG₁), CD16 (NKP15 clone; IgG₁; Becton Dickinson, Mountain View, CA), and CD9 (MM2/57 clone; IgG_{2b}; Ylem, Rome, Italy) or isotype-matched FITC- or PE-conjugated irrelevant MoAb as negative controls; erythrocytes were lysed by adding NH₄Cl-EDTA (Ortho Diagnostic, Raritan, NJ). Cells were subsequently fixed with 4% paraformaldehyde (F) and permeabilized with n-octyl-β-D-glucopyranoside (OG; Sigma, St Louis, MO) for 6 minutes at 20°C. The sequential fixation and permeabilization of unfractionated peripheral blood allows the identification and electronic gating of eosinophils based on high side scatter signals, surface staining for CD9, and lack of CD16 expression, without altering surface antigen expression.⁶⁻⁸ After FOG treatment, cells were incubated with the EG2 MoAb (IgG₁; Kabi Pharmacia Diagnostics, Uppsala, Sweden), recognizing the secretory form of eosinophilic cationic protein (ECP) and were subsequently stained with FITC-conjugated F(ab')₂ anti-mouse IgG. Unlabeled mouse IgG₁ MoAb served as negative control. All samples were run through a FACScan flow cytometer (Becton Dickinson) equipped with an argon laser emitting at 488 nm. A minimum of 3,000 events were acquired in list mode using CellQuest software (Becton Dickinson). The purity of eosinophilic gate (>98%) was confirmed by sorting of CD9⁺

cells and subsequent microscopic analysis. Results were expressed as percent values obtained after channel-by-channel subtraction of test and control histograms; antigenic density was expressed as mean fluorescence intensity (MFI) ratio (MFI of test histogram: MFI of control histogram). The concentration of ECP in serum was measured by means of specific radio-immunoassays (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). Data were presented as median values and interquartile ranges. Statistical analyses were performed with Mann-Whitney U test for unpaired determinations. Correlations were examined with Spearman rank analysis. The criterion for statistical significance was defined as $P < .05$.

Fc-εRII/CD23 receptor could not be detected on eosinophils from normal subjects above background fluorescence, as previously reported by Hartnell et al.⁹ The eosinophil count in patients affected by VKC ranged from 100 to 800 cells/μL (median = 300/μL; interquartile range, 200 to 540). CD23 could be seen in 6 of 14 (43%) patients on 28% (10 to 31) of circulating eosinophils. The percentage of CD23-expressing eosinophils positively correlated with the MFI ratio of the EG2 MoAb ($r = .55$; $P = .042$), recognizing the secretory form of ECP. Conversely, no correlation could be found between eosinophil absolute count and either the percentage of CD23⁺ eosinophils or CD23 staining intensity or between the percentage of CD23⁺ eosinophils and the degree of serum ECP level, total serum IgE, and conjunctival hyperemia.

Eosinophilic Fc-εRII is homologous to CD23 differentiation antigen, expressed on activated B lymphocytes; low-affinity receptors for IgE (Fc-εRII/CD23) can be detected on a variety of cell types, including macrophages, monocytes, and platelets.¹⁰ Fc-εRII expressed on activated, low-density cells of hypereosinophilic patients might be involved in IgE-dependent cytotoxicity of helminths and in IgE-dependent release of eosinophilic granules and mediators, although no correlation between protein expression, as measured by flow cytometry, and Northern blot analysis of RNA, could be found by some investigators.^{11,12} In a recently published report, eosinophils from patients with hay fever were found to express Fc-εRII/CD23 at moderate levels, as detected by the BB10 MoAb, but failed to degranulate in response to anti-IgE.¹³

VKC is a severe allergic disease, characterized by activation of eosinophils in tears, conjunctival biopsies, and peripheral blood and by a dysregulated production of IgE.^{14,15} In our cohort of patients presenting with mild-to-moderate eosinophilia, the expression of CD23, restricted to a subgroup of patients and to a discrete subpopulation of circulating eosinophils, correlated with that of the secretory form of ECP, a well-established marker of eosinophilic activation in allergic