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# Detection of Microchimerism by Minor Histocompatibility Antigen HA-1 Allele–Specific Nested Polymerase Chain Reaction

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## ABSTRACT

**Minor histocompatibility antigens (mHags) can induce T-cell reactivities with important consequences for the graft-versus-leukemia effect and the development of graft-versus-host disease in HLA-matched stem cell transplantation settings. Recently, mHag-specific T cells were also demonstrated in multiparous woman and in solid organ transplant recipients. Microchimeric cells have been detected in the latter settings. To study whether microchimerism is instrumental in the induction and/or maintenance of mHag T cells, we developed an HA-1 allele–specific nested polymerase chain reaction. To optimize and validate the reliability of this method at different levels of microchimerism, serial dilutions of HA-$1^H$ cells titrated into HA-$1^R$ cells were tested. We demonstrated that the HA-$1^H$ allele can be reliably and consistently detected at concentrations as low as $1:10^5$ without losing specificity. The developed HA-1–specific nested polymerase chain reaction is an important tool that facilitates the detection of HA-1 microchimerism in various clinical specimens and that promotes investigation of the effects of microchimerism on induction of mHag-specific T cells in the various settings of immunization.**


## KEY WORDS

Minor histocompatibility antigen • Microchimerism • Nested PCR

## INTRODUCTION

Minor histocompatibility antigens (mHags) are immunogenic peptides, derived from polymorphic self-proteins, that are presented on the cell surface in the context of HLA molecules. In an HLA-matched, mHag-mismatched transplantation setting, presentation of the mHag by antigen-presenting cells can induce a powerful T cell–mediated alloimmune response in vivo and in vitro [1,2]. The mHag HA-1 consists of a nonameric peptide with amino acid sequence VLHDDLLEA, which is presented in the context of HLA-A2 [3]. Its allelic counterpart VLRDDLLEA is nonimmunogenic. In the case of mHag HA-1 disparity, T cells derived from an HA-$1^R$ individual will recognize the HA-$1^H$ peptide as foreign and mount an immune response. Evidence has been obtained for multiparity-induced T cells to paternal mHags [4,5]. Reciprocal priming seemed to occur as well. Namely, umbilical cord blood contains cytotoxic T cells specific for maternal minor antigens [6]. Recently, we demonstrated circulating HA-1–specific T-regulatory and T-cytotoxic cells in a renal transplant patient [7]. It is interesting to note that in both the solid organ recipient and the multiparous women, HA-1 microchimerism was detected. To study the role and causal relationship between microchimerism and the induction and maintenance of mHag-specific T cells, we developed a sensitive technique to detect low numbers of HA-$1^H$ and HA-$1^R$ chimeric cells.

## MATERIALS AND METHODS

### Cell Culture and Isolation of Genomic DNA

Epstein-Barr virus–transformed B-lymphoblastoid cell lines (EBV-BLCL) were cultured in RPMI-1640 containing 3 mmol/L L-glutamine and 10% fetal calf

**Table 1.** *Nucleotide Sequence of Primers Used in the HA-1 Allele–Specific Nested PCR*

| Primer Name | Nucleotide Sequence |
|---|---|
| FI | 5′-GACGTCGTCGAGGACATCTCCCATC-3′ |
| RI | 5′-GCATTCTCTGTTTCCGTGTT-3′ |
| $FII^H$ | 5′-CTTAAGGAGTGTGTGCTGCA-3′ |
| $FII^R$ | 5′-CTTAAGGAGTGTGTTTGCG-3′ |
| RII | 5′-CTGTGCATGGGACATTTCCT-3′ |

serum. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by Ficoll Hypaque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). Informed consent was obtained for the collection of material, and sample collection was part of an approved protocol according to the guidelines of the local ethics committees. Cell subsets were isolated from the PBMC pool with a FACSVantage flow cytometer (Becton Dickinson, San Jose, CA) as described previously [7] by using CD3 for the T-cell fraction and CD19 for the B-cell fraction. The 2 dendritic cell (DC) subsets were sorted by using the Miltenyi beads magnetic sorting system (Miltenyi Biotech, Auburn, CA) with bead conjugated monoclonal antibodies directed against blood dendritic cell antigen (BDCA)-1 and BDCA-3 for DC1 and BDCA-2 for DC2. DC1 populations were judged to be >98% pure as determined by CD11c expression by flow cytometry, and DC2 populations were found to be >92% pure as judged by the expression of CD123. Both subsets were determined by gating on the lineage-negative (CD3, CD14, and CD19), HLA-DR positive population. Genomic DNA from PBMCs, fluorescence-activated cell-sorted fractions, and EBV-BLCLs was isolated with the High Pure Template Purification Kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's description. For each DNA isolation, $2 \times 10^6$ cells were used.

### Genomic Nested Polymerase Chain Reaction

For each first non–allele-specific polymerase chain reaction (PCR), 750 ng of genomic DNA was used. Amplifications were performed with 20 pmol of primer (see Table 1 for specific primers used in this study) in 100 μL of a reaction mixture containing 10 mmol/L Tris/HCl (pH 8.4) buffer, 50 mmol/L KCl, 4 mmol/L $MgCl_2$, 0.06 mg/mL bovine serum albumin, 0.5 mmol/L deoxynucleotide triphosphates, and 2.5 units of *Taq* polymerase (Roche Diagnostics, Almere, The Netherlands). The first non–allele-specific PCR reaction started with a denaturation step of 2 minutes at 95°C. The cycling conditions, which were repeated 25 to 30 times, were 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. The cycling was followed by 72°C for 5 minutes. The obtained product with a size of 397 base pairs (bp) was diluted 100 times, and 2 to 10 μL of this dilution was used in the second PCR. The second allele-specific PCR started with a denaturation step of 2 minutes at 95°C followed by 1 minute at 95°C and 1 minute at 72°C for 10 cycles. A second cycling step was performed (30 to 35 cycles) with the following cycling conditions: 1 minute at 95°C, 1 minute at 69°C, and 1 minute at 72°C. The reaction was finished with a 5-minute incubation step at 72°C. A 10-μL nested PCR product with a size of 304 bp was subjected to DNA electrophoresis on a 1.5% agarose gel with the 100-bp DNA ladder (New England Biolabs, Beverly, MA) for PCR fragment size estimation.

## RESULTS

### Development of a Nested PCR for the Detection of mHag HA-1 Microchimerism

A nested PCR was developed on genomic DNA by using 2 sets of primers. The first primer set (FI and RI) amplifies a part of the HA-1 gene surrounding the HA-1 immunogenic polymorphic T-cell epitope. The resulting PCR fragment size is 397 bp. The second primer set (RII and $FII^H$ or RII and $FII^R$) is used for the allele-specific PCR and amplifies a 304-bp fragment of either the HA-$1^H$–encoding gene or the HA-$1^R$–encoding gene (Figure 1). The primers used in this study are listed in Table 1.

An optimized PCR reaction should give high sensitivity and no background signals. Because the 20-nucleotide-long allele-specific primers $FII^H$ and $FII^R$ differ in only 2 nucleotides, suboptimal PCR conditions can lead to background signals. This background signal appears in the homozygous HA-$1^R$ samples with the $FII^H$ primer and/or in the homozygous HA-$1^H$ samples with the $FII^R$ primer. In the latter situation, the PCR should be performed either with fewer cycles in the first non–allele-specific PCR or with fewer cycles for the allele-specific second PCR. The use of more template DNA is not advisable because it increases the background signal, whereas the use of less template DNA decreases the sensitivity (data not shown).

### Sensitivity of the HA-1 Nested PCR

To determine the sensitivity of the developed HA-1 allele–specific nested PCR, different numbers

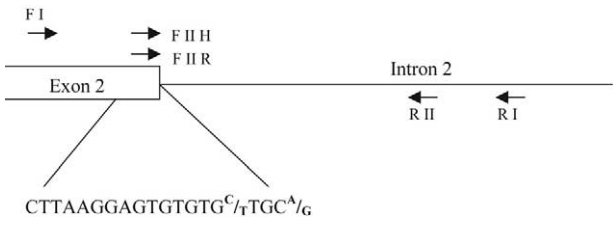


**Figure 1.** Scheme of part of the HA-1–encoding locus, indicating primer positions used for the development of the HA-1 allele–specific nested PCR.

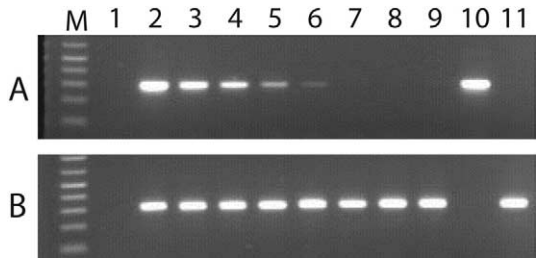


**Figure 2.** Sensitivity determination of the HA-1 nested PCR. A, $FII^H$ as the forward primer in the second PCR step. B, $FII^R$ as the forward primer in the second PCR step. M indicates marker; lane 1, no template control; lanes 2 to 9, PCR results of serial dilutions of cells homozygous for HA-$1^H$ diluted in cells homozygous for HA-$1^R$ in a ratio of 1:10 to 1:$10^8$; lane 10, homozygous HA-$1^H$ control sample; lane 11, homozygous HA-$1^R$ control sample.

of EBV-BLCL cells homozygous for HA-$1^H$ were titrated in EBV-BLCL cells homozygous for HA-$1^R$. The serial dilution series consisted of a ratio of HA-$1^H$ to HA-$1^R$ from 1:$10^1$ to 1:$10^8$. After DNA isolation of these cell mixtures, PCR fragments could consistently be obtained from 750 ng of genomic DNA isolated from the cell batch with a ratio of HA-$1^H$ to HA-$1^R$ of 1:$10^4$ (Figure 2). The $10^5$ dilution sample showed a PCR fragment in approximately 50% of the PCR reactions performed. Thus, analysis of triplicates of each sample is sufficient to detect 1 HA-$1^H$ cell in $10^5$ HA-$1^R$ cells.

### Detection of HA-1 Microchimerism in Blood Samples from mHag-Immunized Individuals

Two sources of samples with assumed microchimerism were tested. One sample source was derived from an HLA-A2 HA-$1^R$ multiparous healthy female donor who delivered 1 HLA-A2 HA-$1^R$ and 1 HLA-A2 HA-$1^H$ child. Using the developed HA-1 allele–specific nested PCR, we could detect the HA-$1^H$–encoding gene in DNA isolated from PBMCs from the mother, most likely derived from the second child. The other sample source was derived from renal allograft recipients who displayed T-cell reactivity against the mismatched mHag HA-1 [7]. The recipients were typed HA-$1^R$ and received an HLA-matched HA-$1^H$ kidney graft. T cells, B cells, dendritic cells, and monocytes were isolated from PBMCs of these individuals, and the presence of HA-$1^H$ microchimerism was analyzed by using the nested PCR. In the PBMCs of renal allografted patients, HA-$1^H$ microchimerism was observed in the T-cell fraction, the dendritic cell population, or both (Figure 3).

## DISCUSSION

We developed a nested PCR for the determination of mHag HA-1 microchimerism. With this newly developed nested PCR, we are able to consistently detect 1 HA-$1^H$ cell in $10^4$ HA-$1^R$ cells without losing specificity. To reach a 10-fold higher sensitivity, ie, 1 HA-$1^H$ cell in $10^5$ HA-$1^R$ cells, triplicates of the same sample need to be performed. The amount of DNA per human diploid cell is 6 pg, which translates into 125 000 copies of the desired template DNA when 750 ng is used per reaction. Thus, the sensitivity of the developed nested PCR reaction approximates the number that it is theoretically possible to detect. This automatically implies that the detection limit of the nested PCR is 1 HA-$1^H$ cell in $10^5$ HA-$1^R$ cells. The amount of template DNA that can be used in the reaction is the limiting factor because an increase in template DNA, with more than 750 ng per reaction, decreases the specificity. The lack of PCR product in higher dilutions, 1:$10^6$ to 1:$10^8$, underlines the specificity of the developed nested PCR.

Microchimerism is defined as the presence of low numbers of foreign cells in an individual. It occurs as a result of pregnancy wherein, in mutual directions, cells flow from the fetus to the mother, from the mother to the fetus, or both. Blood transfusion, solid organ transplantation, and stem cell transplantation also lead to the establishment of microchimerism. Applying the developed nested PCR, we showed the presence of mHag HA-1 microchimerism in multiparous healthy women and in renal allografts recipients.

Nested PCR and real-time quantitative PCR, both

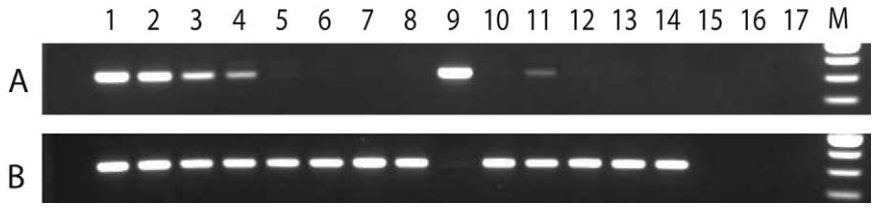


**Figure 3.** Analysis of a clinical sample for the presence of HA-1 microchimerism. Nested PCR results of a sample derived from an HA-$1^R$ kidney transplantation patient receiving an HA-$1^H$ kidney graft. A, $FII^H$ as the forward primer in the second PCR step. B, Positive control PCR using $FII^R$ as the forward primer in the second PCR step. Lanes 1 to 8 indicate PCR results of serial dilution of HA-$1^H$ cells in HA-$1^R$ cells in a ratio of 1:10 to 1:$10^8$; lane 9, homozygous H control sample; lane 10, homozygous R control sample; lane 11, T-cell fraction; lane 12, DC1 cell fraction; lane 13, DC2 cell fraction; lane 14, B-cell fraction; lanes 15 and 16, no-template control of the first PCR; lane 17, no-template control of the second PCR; M, marker.

highly sensitive methods, are used to detect mixed chimerism [8]. One example is the use of Y chromosome–specific sequences for the detection of remaining male cells after sex-mismatched allogeneic stem cell transplantation involving a male patient and a female donor [9]. Naturally, analyses of microchimerism should be performed longitudinally. Especially in transplantation settings, determination of pretransplantation microchimeric levels in both the donor and the patient is needed. These values are especially relevant in female individuals: HA-1 microchimerism was demonstrated in 2 of our cases. Both these cases were female renal allografted patients with a history of parity (also see Verdijk et al. [5]).

In stem cell transplantation settings, detection of decreasing donor chimerism is used as a marker indicative of relapse [8]. Another relevant area for determining mHag HA-1 microchimerism is in patients who undergo stem cell–based mHag HA-1–specific immunotherapy for hematologic and nonhematologic malignancies [10]. The time of the mHag immunotherapeutic intervention in the HA-1–mismatched patient/donor pairs is important. Namely, significant levels of the patient's remaining hematopoietic cells can cause graft-versus-host disease [11]. The exact level of mixed chimeric status in the mHag immunotherapeutic phase I/II trials is as yet unknown. It is interesting to note that in a murine model of donor lymphocyte infusion, the administration of donor lymphocyte infusion mixed chimeras showed a powerful graft-versus-leukemia effect when compared with the administration of donor lymphocyte infusion full chimeras [12].

In conclusion, we have developed a highly sensitive allele-specific nested PCR that allows detection of mHag HA-1 microchimerism. This tool will aid in screening transplantation patients for the risk of relapse and in the timing of HA-1 immunotherapy.

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