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Sequence-Based Typing of the HLA-A10/A19 Group and Confirmation of a Pseudogene Coamplified With A*3401

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ABSTRACT: The strategy for sequencing human leukocyte antigen (HLA)-A was based on separate amplification of exons 2 and 3, followed by forward and reverse heterozygous sequencing of the alleles. Validation of the method was obtained by sequencing 11 individuals carrying alleles from all different HLA-A allele groups, except *43. All alleles could be correctly identified except A*3401. Unexpected polymorphic positions were identified in exon 3, even in individuals homozygous for A*3401. In addition, the pseudogene HLA-COQ or HLA-DEL linked to A*3401 was coamplified and sequenced. The problem was solved by using different amplification primers for exon 3 with mismatches for the two pseudogenes. A total of 252 unrelated individuals with at least one allele belonging to the A10 or A19 group were typed for HLA-A by this strategy. Ten different alleles were identified in the A10 group and 14 in the A19 group. As second allele a further 30 different subtypes from all different groups were sequenced. In 21 individuals, sequencing exon 1 was necessary to distinguish A*7401 from A*7402. The sequencing strategy, with separate amplification of the exons, has proven to be a robust method, resulting in reliable and efficient high-resolution HLA-A typing. *Human Immunology 66, 535–542 (2005).* © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: COQ; DEL; HLA A10; HLA A19; pseudogene; SBT

ABBREVIATIONS

HLA	human leukocyte antigen
PCR-SSP	polymerase chain reaction–with sequence specific primers
SBT	sequence-based typing

INTRODUCTION

The human leukocyte antigens (HLAs) are highly polymorphic cell-surface glycoproteins encoded by the major histocompatibility complex, which play an important role in the immune response. For HLA-A, more than 320 alleles are identified by sequence analysis [1]. The polymorphism is mainly located in exons 2 and 3 encoding the extracellular domains α1 and α2, which are responsible for peptide binding and recognition by both T and natural killer cells [2–4].

The antigens of the HLA-A10 group, A25, A26, A34, and A66 form a so-called cross-reacting group [5]. Most antisera with A10 specificity react with two or more of the subtypes, and monospecific antibodies against A26 and A66 are rarely seen. This implies that the definition of some of these antigens is largely dependent on the interpretation of serologic patterns obtained with different anti-A10 sera. The subdivision of HLA-A10 into the two subtypes A25 and A26 was described in 1971 by Richiardi *et al.* and by Legrand *et al.* [6, 7]. A third specificity belonging to the A10 group was described later and named A34 in 1975 [8–10]. In 1983, a Dutch group described another split of the A10 antigen, later named A66 [11, 12]. Thirty-eight different A10 subtypes are known, giving rise to 36 different proteins. Five A*25 alleles, 23 A*26 alleles, six A*34 alleles, and four A*66 alleles are described [1]. Comparison of the se-

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quences suggests that shared A10 epitopes, which define the family serologically, may be formed by residues of the α2 helix. In particular, the motif of threonine at position 149 together with glutamic acid at 152 is shared by almost all A10 molecules. The only other HLA-A, HLA-B, or HLA-C molecules in which this motif has been found are the A*0203 and A*0238 subtypes of A2 [1, 13].

The HLA-A19 complex is another group of serologically cross-reactive antigens, which was first described in 1973 [14]. The group now includes six subtypes—A29, A30, A31, A32, A33, and A74—with A74 as the last one assigned in 1987 [15]. Sixty alleles for A19 subtypes are known; 14 for A*29, 12 for A*30, nine for A*31, eight for A*32, seven for A*33, and ten for A*74. The 60 alleles represent 55 different proteins [1]. The alleles of A*29,*31,*32,*33, and *74 are serologically and genetically closely related, sharing the unique sequence motifs of leucine at position −11 and phenylalanine and arginine at positions 298 and 307 [1, 16]. The A*30 alleles are genetically related to the A*01/*03/*11 group, implying that they arose from the same ancestral allele [16–18]. The serologic relationship of A*30 with the other members of the A19 group has been proposed to be the result of gene conversion [16].

Several different sequencing protocols for HLA-A have already been established [19–25]. We report a unique sequencing strategy with separate amplification and sequencing of exons 2 and 3, enabling robust heterozygous sequencing of HLA-A. The sequence-based typing (SBT) strategy was validated against a panel of 11 individuals carrying alleles from all different HLA-A allele groups. In addition, 252 unrelated individuals with at least one allele belonging to the A10 or A19 group were typed for HLA-A by this strategy. Coamplification of a pseudogene linked to A*3401 was observed, as previously noted by other groups [26, 27].

MATERIALS AND METHODS

Samples

A panel of 11 individuals carrying HLA-A alleles from 20 different allele groups was selected to validate the SBT protocol. The different HLA-A groups included in the panel were: A*01, *02, *03, *11, *23, *24, *25, *26, *29, *30, *31, *32, *33, *34, *36, *66, *68, *69, *74, and *80. No individuals carrying A*43 were available. Furthermore, the SBT protocol was used to type a total of 252 unrelated individuals for HLA-A. They were selected to possess at least one HLA-A allele of the A10 (A*25, *26, *34, and *66) or A19 (A*29, *30, *31, *32, *33, and *74) group. The individuals typed were laboratory personnel, blood bank donors, patients, organ donors, and quality control samples; in the latter, consanguinity was sometimes unknown. The ethnic origin of the individuals was not always known, but at least half were non-Caucasians. All samples were previously typed by serology or low-resolution polymerase chain reaction–with sequence specific primers (PCR-SSP). The latter was carried out using locally developed A-locus SSP primer mixes with the SSP amplification protocol [28] or the commercially available PCR-SSP HLA-A kit (Dynal, Biotech, Oslo, Norway). Each individual shown to be HLA-A homozygous by SBT was subsequently retyped by low-resolution PCR-SSP to exclude allele drop-out.

DNA Isolation

DNA was isolated from fresh heparinized blood or from lymphocytes stored in liquid nitrogen, either by the "salting out" extraction procedure as described by Miller *et al.* [29] or by using QIA-AMP kits following the supplier's protocol (Qiagen, Westburg, Leuden, the Netherlands). Concentration and purity of DNA samples were measured at optical densities of 260 and 280 nm.

Primers and Approach

The strategy used for SBT of HLA-A included separate amplification and sequencing of exons 2 and 3 using primers located in adjacent exons and introns to obtain complete exon sequences. Locations and sequences of all primers tested are indicated in Table 1. Table 2 shows the final combinations of primers used for the heterozygous sequencing strategy of exons 2 and 3 of HLA-A. All primers were designed locally. The Cy-5 labeled sequencing primers were located internally of the PCR products to ensure specific sequencing.

The amplification of exon 2 was performed using an A-locus–specific 5′ primer (A97107) located in the transition from the 5′ untranslated region and exon 1 combined with a 3′ primer (A97104) located in intron 2. Two forward and one reverse sequencing primer were tested to achieve complete sequencing of exon 2. Exon 3 was amplified with a 5′ primer located in exon 2 (A98009) and an A-locus–specific 3′ primer located in intron 3 (A96090). For correct sequencing of exon 3, three forward and one reverse sequencing primer were tested. To achieve correct amplification of individuals possessing an A*3401 allele, the 5′ amplification primer A00056 was used for exon 3.

Amplification and Sequencing of Exons 2 and 3

The PCR conditions used were identical to the conditions for SBT of HLA-B exons 2 and 3 as previously described [30]. In brief, the PCR reaction was carried out in a final volume of 60 μl, containing 600 ng DNA, 2.0 U Amplitaq DNA polymerase, 20 pmol of biotinylated primer, 40 pmol of unbiotinylated primer, PCR buffer according to Bunce [31] (67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 1.5 mM $MgCl_2$, 6