INTRODUCTION

Granulocyte antibodies have been implicated in alloimmune neonatal neutropenia (ANN), autoimmune neutropenia of infancy (AINI), febrile non-hemolytic transfusion reaction (FNHTR), and transfusion-related acute lung injury (TRALI)\([1, 2]\). Identification of granulocyte antigens and antibodies is important for diagnosing these disorders\([3]\).

Among human granulocyte antigens, human neutrophil antigen 4a (HNA-4a) and HNA-5a are high-frequency antigens\([4, 5]\). Therefore, it is difficult to find individuals with an HNA-4a-negative or HNA-5a-negative phenotype. Moreover, typing HNA-4a or HNA-5a by serological methods is limited because anti-HNA-4a or anti-HNA-5a typing sera are very rare. Antibodies to HNA-4a cause ANN, and AINI\([3, 6, 7]\). However, the clinical significance of antibodies to HNA-5a is not known\([3]\).

The molecular basis of HNA-4a and HNA-5a has been elucidated\([4]\), and genotyping methods have been devel-
oped[4, 5]. These genotyping techniques avoid the use of extremely rare typing sera and are more convenient than serotyping methods. HNA-4a and HNA-5a are located on the leukocyte adhesion molecules (β2 integrin) and are caused by single nucleotide mutations in the αM (CD11b) and αL (CD11a) subunits[2, 3]. The HNA-4a-negative phenotype is due to a single nucleotide substitution (G-302A) and the subsequent Arg61His amino acid substitution on the αM chain of β2 integrin. The HNA-5a-negative phenotype is due to a single nucleotide substitution (G2466A) and the subsequent Arg766Thr amino acid substitution on the αL chain of β2 integrin.

In Asia, antigen frequencies of HNA-1a, HNA-1b, and HNA-2a have been determined using a serological method [8, 9], and gene frequencies of HNA-1a and HNA-1b have been determined using a genotyping method[10-13]. However, no published studies have used a genotyping method to assess the gene frequencies of HNA-4a and HNA-5a among Asians. In this study, we determined HNA-4a and HNA-5a gene frequencies among the Korean population using a polymerase chain reaction with sequence-specific primers (PCR-SSP) for HNA-4a genotyping and an allele-specific restriction enzyme analysis (ASRA) for HNA-5a genotyping.

**MATERIALS AND METHODS**

1. **Specimens**

   From February 2004 to June 2005, CPDA-1 anticoagulated blood (2-3 mL) was collected from 110 healthy and unrelated Korean blood donors (40 female, 70 male: aged 18 to 52 years [mean±SD 25±6.9 years]) at the blood bank in Sanggye Paik Hospital. The donors gave informed consent for the use of their blood.

2. **Isolation of DNA and RNA**

   Granulocytes were isolated from donor blood using the PMN isolation medium (Robbins Scientific, Sunnyvale, CA, USA). DNA and RNA were extracted from isolated granulocytes using the QiAamp DNA blood Mini Kit (Qiagen GmbH, Hilden, Germany) and the Ribo Pure RNA isolation kit (Ambion, Austin, TX, USA), respectively.

3. **HNA-4a genotyping by PCR-SSP**

   To genotype HNA-4a, PCR-SSP was performed according to the protocols described by Clague et al[5]. Two PCR-SSPs were performed per sample, one for the HNA-4a-positive allele and the other for the HNA-4a-negative allele. Pos-R (5′-AGTGACTCACCTGATGC-3′) was used as an antisense primer specific for the HNA-4a-positive allele, and Neg-R (5′-AGTGACTCACCTGCATGTC-3′) was used as an antisense primer specific for the HNA-4a-negative allele. HNA-4a-F (5′-CTCCCCACAGGTTGTG-3′) was used as a sense primer common to the HNA-4a-positive and HNA-4a-negative alleles. For internal control purposes, two primers (HGH I and HGH II), which amplify a 439 bp fragment of the human growth hormone (HGH) gene, were used.

   Amplification was performed in a 20 μL reaction mixture containing: 0.2 μM of each primer; 200 μM dATP, dCTP, dTTP, and dGTP; 10 mM Tris-HCl (pH 9.0): 1.5 mM MgCl2: 40 mM KCl: 1 unit of Taq polymerase (Perkin-Elmer, Cetus, CT, USA); and 1 μL of DNA sample. Amplification was performed in a DNA thermal cycler (iCycler Thermal Cycler, Bio-Rad Laboratories, Hercules, CA, USA). Each cycle comprised predenaturation at 95°C for 3 min and 30 amplification cycles (denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min, and extension at 72°C for 1 min). The amplified DNA fragments for the HNA-4a-negative and HNA-4a-positive alleles were 124 bp long[7](Fig. 1).
4. HNA-5a genotyping by reverse transcription and PCR-ASRA

To type HNA-5a, reverse transcription and PCR-ASRA were performed according to the protocols described by Simsek et al.[4]. Reverse transcription of 0.5 μg of total RNA was performed in a final volume of 20 μL containing 5 μM random hexamer, 1 mM of each dNTP, 2 units of RNase inhibitor, and 9 units of reverse transcriptase (Bioneer, Daejeon, Korea). After incubation at 42°C for 60 min, samples were heated for 5 min at 94°C to end the reactions. The primers L5 (5’-ATTTCTCTCTTTGGAGGAGG-3’) and L5A (5’-TGGGTATGTGTTG-TGTTG-3’) were used to amplify the coding region of the α chain of β2 integrin cDNA. The PCR product (709 bp) was treated with the restriction endonuclease Bsp1286I (Takara Biotechnology, Ostu, Japan), size-separated on a 2% agarose gel with ethidium bromide, and visualized with UV light.

In HNA-5a-positive homozygote samples, three fragments of 297 bp, 217 bp, and 195 bp were generated, whereas in HNA-5a-negative homozygote samples, two fragments of 412 bp and 297 bp were generated, and in HNA-5a heterozygote samples, four fragments of 412 bp, 297 bp, 217 bp, and 195 bp were generated[4](Fig. 2).

5. Statistical analysis

The HNA-4a and HNA-5a gene frequencies were estimated by the gene counting method. Agreement of the observed and expected genotype, based on the Hardy–Weinberg equilibrium, was determined using the chi-square test.

RESULTS

Table 1 shows the HNA-4a and HNA-5a gene frequencies. The genotype frequencies for HNA-4a were as follows: HNA-4a-positive homozygotes, 0.973 (107/110); HNA-4a heterozygotes, 0.027 (3/110); and HNA-4a-negative homozygotes, 0 (0/110). The genotype frequencies for HNA-5a were as follows: HNA-5a-positive homozygotes, 0.927 (102/110); HNA-5a heterozygotes, 0.064 (7/110); and HNA-5a-negative homozygotes, 0.009 (1/110). The gene frequencies of HNA-4a and HNA-5a were 0.986 and 0.959, respectively, and were higher than those in the Caucasian population (Table 2). However, the gene frequency of HNA-5a was significantly higher \( (P < 0.001) \) than in Caucasians. The deviation of the observed numbers of genotype from those expected on the Hardy–Weinberg equilibrium was statistically insignificant.

DISCUSSION

The nomenclature for Human neutrophil antigens (HNA)
was established by an International Society of Blood Transfusion (ISBT) Working Party[14]. The HNA system comprises seven antigens (HNA-1a, 1b, 1c, 2a, 3a, 4a, and 5a), which are assigned to five glycoproteins[14-16]. Antibodies to HNA-1 antigens cause ANN, AINI, and TRALI. Antibodies to HNA-2a antigens cause ANN, and TRALI. Antibodies to HNA-3a antigens are often implicated in TRALI.

HNA-4a and HNA-5a are also expressed on non-granulocytic leukocytes[2, 15] and there was some debate about whether HNA-4a and HNA-5a should be included in the HNA nomenclature[14, 16]. However, Fung et al. reported a case of neonatal alloimmune neutropenia that was caused by anti-HNA-4a[6], thus, justifying the inclusion of HNA-4a in the HNA system. In addition, anti-HNA-4a antibodies are implicated in autoimmune neutropenia[7]. However, there are no confirmed clinical reports on anti-HNA-5a-related disorders and controversy remains about the clinical significance of anti-HNA-5a[3, 15].

It has been reported that the gene frequencies of granulocyte antigens differ among ethnic groups[6-13]. The gene frequencies of HNA-1a, 1b, 1c, 2a, 3a, 4a, and 5a among ethnic group are summarized in Table 2. Our results show that the gene frequency of HNA-4a in the Korean population is not significantly higher ($P>0.05$) than in Caucasian populations. However, the gene frequency of HNA-5a in the Korean population is significantly higher ($P<0.01$) than in Caucasian populations (Table 2).

In conclusion, we have determined the gene frequencies of HNA-4a and HNA-5a in the Korean population for the first time. Moreover, we found an HNA-5a-negative individual for the granulocyte panel that could be used for anti-HNA-5a antibody identification.

요 약

배경: 한국인의 HNA-1a, HNA-1b, HNA-2a 항원(혹은 유전자) 빈도에 대한 연구는 이루어졌으나 HNA-4a와 HNA-5a의 유전자빈도는 아직 연구되지 않았다. 항-HNA-4a 항체는 동종면역성중성구감소증과 자가면역성중성구감소증과 관련이 있는 보고가 있으나 항HNA-5a 항체는 이런 질환들과 관련이 있는 보고는 아직 없다. 이런 연구에서 저자들은 이번 연구에서 한국인의 HNA-1a와 HNA-5a의 유전자빈도를 확인하고자 하였다.

대상 및 방법: 저자는 110명의 건강한 비혈연 공혈자의 HNA-4a와 HNA-5a 유전자형을 중합효소연쇄반응(polymerase chain reaction with sequence-specific primers)과 대립유전자 독이 제한효소법(allele-specific restriction enzyme analysis)을 이용하여 검사하였다.

결과: 한국인의 HNA-4a와 HNA-5a의 유전자빈도는 각각 0.99와 0.96이었고 이 중 HNA-5a만이 백인보다 통계적으로 유의하게 높았다 ($P<0.01$).

결론: 저자들은 한국인에서 HNA-4a와 HNA-5a 유전자 빈도를 확인하였고 HNA-5a 음성인 동형접합자 1인을 확인할 수 있었다. 이 공혈자의 과립구를 항-HNA-5a 항체 동정에 이용될 수 있을 것으로 판단하였다.

REFERENCES

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