DNA from urine sediment or buccal cells can be used for blood group molecular genotyping

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Accurate blood group antigen typing of red blood cells with a positive direct antiglobulin test or from a recently transfused patient has been a long-standing problem. To overcome this problem, we evaluated the feasibility of using somatic cells as a source of DNA for molecular genotyping. Two sources of cells that could be obtained by noninvasive procedures were chosen for analysis: urine samples, which were already available in the clinical laboratory, and buccal epithelial cells collected with cotton wool swabs. DNA, prepared using a commercial kit, was subjected to polymerase chain reaction amplification and followed by digestion with the appropriate restriction enzyme. Genotyping was performed for three alleles encoded by polymorphic genes on three different chromosomes, namely *KEL1/KEL2, JKA/JKB*, and *FYA/FYB*. Genotyping results were compared to the results of typing performed on red blood cells using standard hemagglutination techniques. Results given by samples freshly collected from volunteer donors were concordant. Although results obtained with samples collected from hospital patients were initially not in agreement with the phenotyping results, adjustments to the test protocol resulted in concordance. DNA from blood, urine sediment, or buccal cells can be used for blood group molecular genotyping. *Immunohematology* 15;2:61–65.

Key Words: blood group genotyping, buccal cells—DNA, DNA preparation, urine—DNA

Accurate phenotyping of red blood cells (RBCs) from recently transfused patients is problematic because of the presence of transfused donor(s) RBCs in the circulation of the recipient. This problem is particularly true for patients who have been recently transfused with multiple units of RBCs. Accurate phenotyping of antigens using reagents that only react by the antiglobulin test is also problematic when the patient's RBCs have a positive direct antiglobulin test (DAT). In these situations, it would be valuable to have an alternative to hemagglutination tests to determine the patient's antigen profile as part of an antibody identification process.

In recent years, technological advances have led to the understanding of the molecular basis of many blood group antigens. The majority of blood group polymorphisms are associated with a single-point mutation in the gene encoding the protein carrying the blood group antigen.[1-3] Thus, blood typing at the molecular level using DNA can be used to circumvent the phenotyping difficulties mentioned above. If DNA is prepared from a transfused patient's blood sample, donor leukocytes could, at least theoretically, interfere with genotyping results.[4-7] To avoid this potential problem, we evaluated the feasibility of using sources of DNA other than RBCs to determine the blood group genotype. Because the genetic code is contained within all somatic cells, it is theoretically possible for molecular determination of blood group antigens in multitransfused patients to be performed on alternative sources of DNA, thereby avoiding the difficulties caused by transfused RBCs in currently used RBC phenotyping methods. We collected two sources of cells using noninvasive procedures, namely urine samples that were already available in the clinical laboratory, and buccal epithelial cells collected with cotton wool swabs. To test our approach, we performed genotyping for three pairs of alleles encoded by polymorphic genes on three different chromosomes, namely, *KEL1/KEL2, JKA/JKB*, and *FYA/FYB*. In the Duffy genotype, it has been reported that a mutation in the erythroid promoter motif (GATA-1 box) silences the *FYB* gene expression.[8] Therefore, we also performed restriction fragment length polymorphism (RFLP) analysis for this point mutation and confirmed that it is critical to analyze the associated GATA-1 box for accurate correlation between Duffy genotype and phenotype.

Material and Methods

Sample collection

Institutional Review Board approval in the authors' institutions was obtained prior to the collection of samples. Blood samples were obtained from patients or donors whose RBC phenotype had been determined on the current or previous sample. Urine samples were either surplus to the needs of the clinical chemistry laboratory or collected specifically. We obtained buccal epithelial cells by swiping the mouth mucosa with a cot-

ton wool swab. The swab was placed in a tube and stoppered for transport. Whenever possible, blood samples, urine samples, and buccal smears were obtained from each person. RBC antigen typing was performed by standard hemagglutination tube tests using antisera from several commercial sources. The genotyping tests were performed and interpreted without knowledge of the phenotyping results. The results obtained on DNA from different cell sources were compared to each other, followed by comparison with the K/k, Jk^a/Jk^b, and Fy^a/Fy^b antigen typing.

DNA extraction

DNA was extracted from whole blood samples using the Easy DNA Kit (Invitrogen, Carlsbad, CA), protocol 2 (page 6 in the instruction manual). Urine samples were centrifuged to pellet nucleated cells (presumed to originate from urinary tract epithelia and/or white blood cells), and DNA was extracted from this pellet using the Easy DNA Kit protocol 3 (page 7 in the instruction manual). Buccal cells trapped in the external layer of the cotton swab were placed into a tube containing 200 μL of sterile phosphate-buffered saline, and DNA was extracted using the Easy DNA Kit protocol 3. Extracted DNA was analyzed for quality (degradation/integrity) by examining the pattern obtained after agarose gel electrophoresis, and the quantity was estimated by spectrophotometrical reading of the optical density at 260 nm.

Amplification conditions and profile

Polymerase chain reactions (PCR) were performed with approximately 200 ng of DNA using specific primers flanking the polymorphism for each blood group allele. Primers with high melting temperatures were preferentially chosen in order to allow high stringency. Concentrations of primers and magnesium chloride were adjusted so that the same PCR profile could be used for each allele. The amplification profile was 94°C for 5 minutes followed by 35 cycles of amplification consisting of denaturation at 94°C for 20 seconds, annealing at 62°C for 15 seconds, and elongation at 72°C for 20 seconds. This was followed by 10 minutes at 72°C. PCR buffers and *Taq* DNA polymerase were purchased from Promega (Madison, WI) or Perkin-Elmer (Branchburg, NJ). Amplified products were subjected to electrophoresis in 1.2% agarose gel in tris-acetate-EDTA (TAE) to verify amplification efficiency before restriction enzyme treatment for genotype determination. The primer sequences[8-12] and amplicon sizes are given in Table 1.

Table 1. Sequence of primers and PCR product size

Allele	Primer sequence	Amplicon size
KEL1/KEL2	KEL-S 5'-AAG CTT GGA GGC TGG CGC AT –3' KEL-R 5'-CCT CAC CTG GAT GAC TGG TG –3'	156 bp
JKA/JKB	JKIS 5'-TGA GAT CTT GGC TTC CTA GG –3' * JK2 5'- ATT GCA ATG CAG GCC AGA GA 3'	210 bp
FYA/FYB	FYAB1 5'- TCC CCC TCA ACT GAG AAC TC –3' FYAB2 5'- AAG GCT GAG CCA TAC CAG AC –3'	392 bp
GATA	FYN1 5'- CAA GGC CAG TGA CCC CCA TA –3' FYN2 5'- CAT GGC ACC GTT TGG TTC TC –3'	189 bp

*Primer based on intronic sequence determined in the authors' laboratory

PCR product analysis for genotype determination

The PCR-amplified products were analyzed by appropriate restriction enzymes to identify specific point mutations. Enzymes were purchased from New England Biolab (Beverly, MA) and used according to manufacturer's instructions. The enzymes *Bsm* I, *Mnl* I, and *Ban* I were used to determine, respectively, *KEL1/KEL2* (C698T), *JKA/JKB* (A838G), and *FYA/FYB* (A306G).[9-12] Furthermore, the *Sty* I enzyme was used to distinguish between functional and nonfunctional GATA-1 box (T-46C)—a functional GATA-1 box being critical for expression of Fy^b protein in the red cell membrane.[8,13,14] All digestion incubations were performed overnight. After digestion, RFLPs were analyzed by electrophoresis in 3% agarose in TAE stained with ethidium bromide and compared with molecular standards. For the GATA box analysis, electrophoresis in 8% acrylamide gel in tris-borate-EDTA with ethidium bromide was required.

Results

Tests on samples from volunteer donors

Blood, urine, and buccal swab samples were collected from 16 healthy volunteers. The molecular genotyping results on DNA prepared from blood, urine, and buccal cells for Kell and Kidd alleles were concordant with the hemagglutination results. Two samples that genotyped as *KEL1/KEL2* had been typed as K+k+, and 14 samples that genotyped as *KEL2/KEL2* had been typed as K–k+. Six samples that genotyped as *JKA/JKA* had been typed as Jk(a+b–), seven samples that genotyped as *JKA/JKB* had been typed as Jk(a+b+), and three samples that typed as Jk(a–b+) were genotyped as *JKB/JKB*.

Molecular genotyping results for Duffy were concordant with the hemagglutination typing in the 12 samples that had a double dose of the functional erythroid GATA-1 box (GATA+/+; see Table 2). The other four samples

typed as Fy(a–b–), genotyped as *FYB/FYB*, and had a mutation in the GATA-1 box (GATA–/–). This apparent pseudo-disagreement of results is consistent with previously reported observations[8] that the *FYB* gene can be silenced by a mutation in the GATA-1 box.

Table 2. Results of Duffy testing on 16 volunteer donors

Genotype	RBC Phenotypes			
	Fy(a+b–)	Fy(a+b+)	Fy(a–b+)	Fy(a–b–)
FYA/FYA GATA (+/+)	3	0	0	0
FYA/FYB GATA (+/+)	0	6	0	0
FYB/FYB GATA (+/+)	0	0	3	0
FYB/FYB GATA (–/–)	0	0	0	4

Tests on samples from patients

In order to evaluate collection and performance in the actual clinical setting, we collected urine and/or buccal swab samples from 20 patients whose RBCs had been phenotyped previously. For a variety of reasons, we were unable to collect both urine and buccal smear samples from all 20 patients. Initially, when genotyping results were compared with phenotyping results, several discrepant results were noted. For this reason, the following adjustments were made to our testing protocol as given in Materials and Methods. We used hot-start PCR combined with high stringency annealing temperatures in order to decrease primer-dimer formation, we designed primers flanking shorter genomic sequences to facilitate amplification of small quantities or of poor-quality DNA, and we replaced 4% agarose gels with 8% polycrylamide gel electrophoresis to resolve small differences in the sizes of the digestion products. Once these adjustments had been made, genotyping was repeated in a blinded fashion. In all cases, the genotyping results obtained on analysis of DNA from the different sources were in agreement.

The molecular genotyping on DNA prepared from urine and/or buccal cells was concordant with the hemagglutination results on record for Kell and Kidd alleles. All 20 samples were genotyped as *KEL2/KEL2* and had K–k+ RBCs. Seven samples that were genotyped as *JKA/JKA* had been typed as Jk(a+b–), nine samples that were genotyped as *JKA/JKB* had been typed as Jk(a+b+), and four samples that genotyped as *JKB/JKB* had been typed as Jk(a–b+).

Molecular genotyping results for Duffy were concor-

dant with hemagglutination typing in 13 samples that had a double dose of the functional erythroid GATA-1 box (Table 3). One sample (CB) with Fy(a–b–) RBCs had the *FYB/FYB* genotype with one copy of the functional and one copy of the nonfunctional GATA-1 box. This sample was shown also to have heterozygous mutations at nucleotides 271 and 304, which also silence the *FYB* allele.[15] Another sample (BR) had the *FYA/FYB* genotype with one copy of the functional GATA-1 box and one copy of the nonfunctional GATA-1 box but Fy(a–b–) RBCs; unfortunately, insufficient DNA was available for further study and it was not possible to obtain a fresh sample. Thus, we could not investigate the cause of the silencing of the *FYA* allele; we presume that the nonfunctional GATA-1 box silenced the *FYB* allele in BR. The three other Fy(a–b–) samples were from African American patients and had the expected homozygous mutation in the GATA-1 box. One Fy(a+b–) patient (YM) had the *FYA/FYB* genotype and was heterozygous for a GATA-1 mutation that silenced the *FYB* allele. One Fy(a–b+) patient (CQ) had a *FYB/FYB* genotype and was also heterozygous for the GATA-1 mutation that silenced one *FYB* allele.

Table 3. Results of Duffy testing on 20 patient samples

Genotype	RBC Phenotypes			
	Fy(a+b–)	Fy(a+b+)	Fy(a-b+)	Fy(a–b–)
FYA/FYA GATA (+/+)	1	0	0	0
FYA/FYA GATA (+/–)	0	0	0	0
FYA/FYB GATA (+/+)	0	5	0	0
FYA/FYB GATA (+/–)	1 (YM)	0	0	1 (BR)
FYB/FYB GATA (+/+)	0	0	7	0
FYB/FYB GATA (+/–)	0	0	1 (CQ)	1 (CB)
FYB/FYB GATA (–/–)	0	0	0	3

Discussion

Our results show that DNA from leukocytes, urine sediment, and buccal epithelial cells can be used for blood group genotyping. This is of particular value in genotyping patients who have been recently transfused (when donor white blood cells may be circulating)[4,5] or whose RBCs are positive in the DAT. The ability to use DNA from somatic cells also may be useful during preg-

nancy when the maternal peripheral blood may contain nucleated cells from the fetus.[16] However, because 11 of the patient samples were collected from women on their first or second day postpartum, when fetal cells would be expected to be present in the circulation, our data show that the PCR-RFLP protocols that we used did not detect interference by fetal cells. Our data also confirm that analysis of the GATA box is critical for accurate correlation between Duffy genotyping and phenotyping.[4,13,14]

Although phenotyping of DNA from somatic cells can be performed for any blood group for which the molecular basis is known, this approach has limitations. One unforeseen limitation was the effect of small quantities or poor quality of DNA from specimens collected in an in-patient setting compared with volunteer donors. The former may require adjustments in PCR technique or in sample preparations. In addition, it is possible that, whereas analysis of genomic DNA indicates the presence of an apparently normal gene, the RNA may not be transcribed or correctly spliced. Furthermore, the encoded product may not be expressed in the RBC membrane. Although these events are considered to be rare in the general population, even our small sample of 20 patients revealed variant forms of the *FY* gene in Hispanics as well as in African Americans. Nevertheless, pseudo-disagreement of genotype and phenotype would apply regardless of the source of DNA used for the molecular genotyping, and we have clearly shown that molecular genotyping for blood groups can be performed on DNA prepared from white blood cells, urine sediment, or buccal epithelial cells.

Acknowledgments

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