Original Paper

Fumi-ichiro Yamamoto[a]
Patricia D. McNeill[a]
Miyako Yamamoto[a]
Sen-itiroh Hakomori[a]
Teresa Harris[b]
W. John Judd[c]
Robertson D. Davenport[d]

[a] The Biomembrane Institute and
University of Washington, Seattle, Wash.;
[b] Pacific Northwest Regional Blood Services,
Portland, Oreg.;
Departments of
[c] Immunohematology and
[d] Pathology, The University of Michigan
Hospitals, Ann Arbor, Mich., USA

Molecular Genetic Analysis of the ABO Blood Group System: 1. Weak Subgroups: A^3 and B^3 Alleles

Abstract

We have determined the nucleotide sequences of the coding region in the last two coding exons of ABO genes (which occupy 91% of the soluble form of A^1 transferase) from 7 individuals with weak subgroup phenotypes. Four of the individuals had an A_3 phenotype and 3 individuals had a B_3 phenotype. We determined the nucleotide sequences based on PCR followed by subcloning and DNA sequencing of the amplified fragments. Two cases of the A^3 allele and 1 case of the B^3 allele were found to contain a single-base substitution which resulted in an amino acid substitution. However, no other cases of A^3 and B^3 alleles were found to contain differences in this region. This finding demonstrates for the first time heterogeneity among these weak subgroups at the nucleotide level.

Introduction

Since the discovery of the histo-blood group ABO system by Landsteiner in 1900 [1], almost a century passed until we were successfully able to elucidate the molecular genetic basis of the three major alleles (A^1, B, and O) at the blood group ABO gene locus by cloning A^1 transferase cDNA [2], and by cloning B and O alleles [3] followed by nucleotide sequencing. O alleles are characterized by the presence of a single-base deletion close to the N-terminal of the coding sequence, which shifts the reading frame of codons and results in the translation of an entirely different protein thereafter without any activity of A or B transferases. A^1- and B-allelic cDNAs are identical except for seven nucleotide substitutions resulting in four amino acid substitutions between A^1 and B transferases.

We have subsequently extended our study to subgroups. Subgroups of A, A_1 and A_2 were first identified by von Dungern in 1911 when he found two kinds of group A erythrocytes. Sera from blood group B individuals contain two types of antibodies against A antigens: anti-A and anti-A_1. Group A erythrocytes, which react with both anti-A and anti-A_1 were named A_1, and those which react only with anti-A, were named A_2. Dolichos biflorus lectin has been used as an anti-A_1 reagent. We analyzed A^1/A^2 differences at the nucleotide level and found a single-base substitution and a single-base deletion in the coding sequence of the last coding exon of A^2 alleles [4]. These two differences were found present in all the A^2 alleles tested (8 specimens). The single-base deletion is located close to the carboxyl terminal of the transferase in the coding sequence. As a result of frame shifting, A^2 transferase has an

additional domain composed of 21 amino acids. We constructed an A^2 transferase expression construct and performed DNA transfection experiments. We found this single-nucleotide deletion to be responsible for the weak A transferase activity in A^2 transferase.

In addition to A_2, there are other rare subgroups with weaker serological reactivity compared to A_1. These can be serologically differentiated and include A_3, A_x, A_{end}, and A_{el}. Similarly, B phenotypes with weaker than normal B activity are designated as B_3, B_x, B_m, B_{el}. These weak subgroups are of great importance because they allow us to characterize their specific glycosyltransferase by studying the mutations in these alleles. A_3 erythrocytes are characterized by a mixed-field pattern of agglutination with anti-A or anti-A,B reagents. B_3, the counterpart of A_3 in B, is similarly characterized by a mixed-field agglutination of erythrocytes with anti-B or anti-A,B reagents. Because roughly two thirds of the red cells are agglutinated, A_3 and B_3 are also called A_{60} and B_{60}, respectively [5].

Here we report the identification of mutations in the nucleotide sequences of the coding region in some cases of A^3 and B^3 alleles.

Materials and Methods

Blood Grouping

We have analyzed a total of 7 cases of weak subgroups; one A_1B_3, two A_3, two A_3B, two B_3. Blood groups were determined using immunological methods. One case (A_1B_3) was confirmed to posses a B^3 allele through the grouping of its family members.

PCR Amplification

Genomic DNA was prepared from blood specimens by Proteinase K-SDS method [6]. Reagents for PCR [7] reaction were purchased from Perkin-Elmer Cetus. The pairs of synthetic oligodeoxynucleotides used for PCR are as follows:
fy-121, CGGAATTCCATGTGACCGCACGCCT and
fy-122, CGGAATTCTCTACCCTCGGCCACC;
fy-101, CGGAATTCCCGTCCGCCTGCCTTGCAG and
fy-113, CGGAATTCACCTCTTGCACCGACCC;
and fy-112. CGGAATTCCGTTCTGCTAAAACCAAG
and fy-114, CCTGGTGTGCGTGGAC, or
fy-127, CGGAATTCCTGGTGTGCGTGGAC.
The oligodeoxynucleotide fy-127 is essentially identical to fy-114 except for the additional *Eco*RI site at the 5' end. All the *Eco*RI sites are artifical in order to facilitate the subsequent subcloning. The reaction mixture was denatured for 5 min at 95 °C followed by 45 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C in a DNA thermal cycler. DNA was extracted with phenol:chloroform:isoamylalcohol = 25:24:1 and precipitated with ethanol.

Subcloning and DNA Sequencing

After resuspension, DNA was digested with *Eco*RI, electrophoresed on an agarose gel, and the major fragments with expected size were excised and DNA was extracted with GeneClean kit (Bio 101). Gel-purified DNA was then ligated with either *Eco*RI-digested, dephosphorylated sequencing vector pT7T3U18 (Pharmacia-LKB) (fy-121-fy-122, fy-101-fy-113, and fy-112-fy-127), or the same vector double digested with *Eco*RI and *Sma*I (fy-112-fy-114). Ligated DNA was used to transform *XL1-blue* strain of *Escherichia coli*. Plasmid DNA was prepared from a multiple number of transformants, analyzed for the insert, denatured, and used for nucleotide sequencing by the Sanger method [8] with Sequenase (United States Biochemical).

Results

Figure 1 shows the schematic comparison of the nucleotide and the deduced amino acid sequences of each allele from 7 individuals showing phenotypes of weak subgroups, A_3 or B_3, with those of A^1, O, and B alleles.

Two pairs of oligo primers, fy-101 & fy-113 and fy-112 & fy-114/fy-127, were used to amplify the coding sequence in the last coding exon. The corresponding sequence for primer fy-101 resides in the intron preceding this last coding exon (the last base of fy-101 is located just adjacent to the splicing acceptor site), and that of fy-112 resides in the 3' untranslated region (the last base of fy-112 is located 53 bases away from the termination codon on the 3' side). Primers fy-113 and fy-114/fy-127 are homologous to the sequences in the last exon except for the artifical *Eco*RI sequences of fy-113 and fy-127, and the amplified fragments from these pairs of oligos overlap one another. The third pair of oligos was used to amplify the preceding exon. The corresponding sequences for fy-121 and fy-122 reside in the introns preceding and following this exon, respectively. The last base of fy-121 is located 16 bases away from the 5' side of the splicing acceptor site and that of fy-122 is located 11 bases away from the 3' side of the splicing donor site. Nucleotide sequencing of multiple (more than 10) clones of transformants, transformed with each PCR-amplified fragment, was performed to discriminate between the two alleles on the two homologous chromosomes as well as the occasional errors introduced by *Taq* DNA polymerase.

We were able to discriminate the two alleles on homologous chromosome pairs in 5 out of the 7 samples tested, based on differences in the nucleotide sequences. However, even with our extensive sequencing we were unable to discriminate the two homologously paired alleles in the remaining 2 cases (both with a phenotype of A_3) in the coding sequence of the last coding exon. More than twenty transformant clones showed an identical sequence suggesting that these A^3 alleles are identical to A^1 alleles in this region. Figure 1 shows this conclusion. There appears
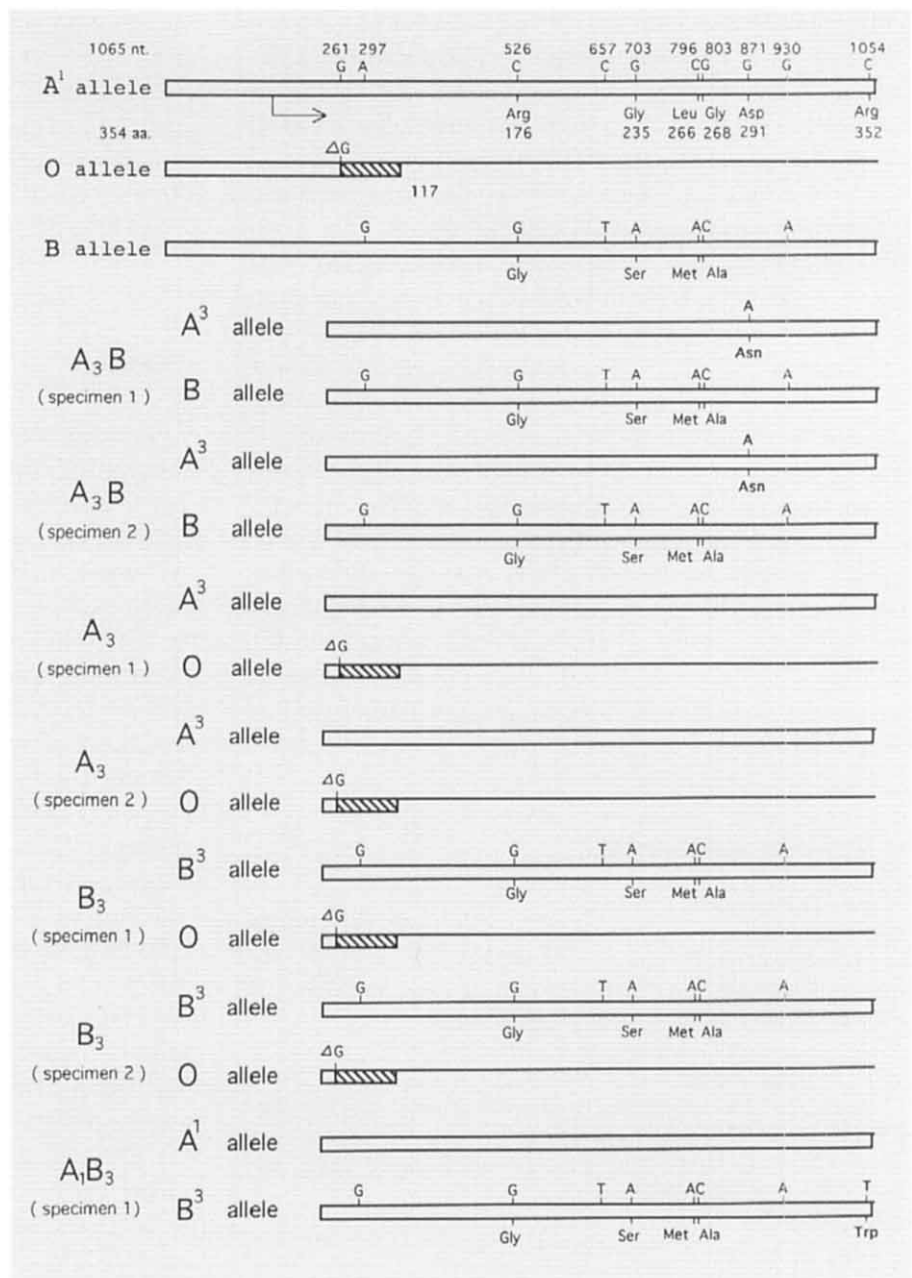
Fig. 1. Schematic comparison of the nucleotide and the deduced amino acid sequences of each allele from individuals showing weak subgroup phenotypes, A₃ or B₃, with those of A¹, O, and B alleles. The A residue and the methionine residue of the initiation codon of A¹ transferase are numbered 1 (because of the deletion of the three nucleotides in FY-59-5 clone, the numbering is different from that of FY-59-5 presented in reference [2]. Because only the last two coding exons were analyzed for A³ and B³ alleles, only the sequences in this region are compared and the differences are indicated. For each specimen, the two alleles which made up homologous chromosome pairs were discriminated from one another by differences in the nucleotide sequences. For the two A³O individuals, we were unable to discriminate between the two homologously paired alleles in the last coding exon. We assume these A³ alleles are identical to A¹ alleles in the last coding exon because no differences were found among more than twenty transformant clones. The figure shows this conclusion. The hatched rectangle in O alleles indicates the entirely different deduced amino acid sequence due to frame shifting caused by the single-base deletion. The arrow shows the N-terminal of the soluble form of A¹ transferase.

to be at least two kinds of A³ and B³ alleles. The A³ alleles from two A₃B individuals have a single-base substitution at nt. 871 (G → A) resulting in an amino acid substitution at aa. 291 (aspartic acid → asparagine) which discriminates these A³ alleles from A¹ alleles. The B³ allele from one A₁B₃ individual has a single-base substitution at nt. 1054 (C → T) resulting in an amino acid substitution at aa. 352 (arginine → tryptophan), which discriminates this B³ allele from B alleles.

Discussion

Heterogeneity among the single subgroups has been reported for A and B variants based on the analysis of transferase activity in the serum of these individuals. Cartron et al. [9] reported three types of A₃ phenotypes. One group consisted of A₃ phenotypes with a low level of A transferase activity in the serum, and the optimal pH of the transferase was approximately 7 (in 7 out of 11 cases). The second group consisted of A₃ phenotypes with no activity of A transferase (3 out of the 11). A high A trans-

Yamamoto/McNeill/Yamamoto/Hakomori/
Harris/Judd/Davenport

Molecular Genetics of A³ and B³ Alleles

ferase activity optimum at pH 6 was detected in the last of eleven A_3 samples. Nakamura et al. [10] reported an active A transferase which has characteristics (the level of activity, pH optimum, K_m, V_{max}, and heat stability) identical to those of A^1 transferase in the serum of A_3 phenotypes. These authors postulated that the weak A expression is due to the suppression of A transferase production only in hemopoietic tissues. B transferase activity in the serum of individuals with B_3 phenotype has been reported to vary from 10 to 100% of control level [11]. Due to the restricted availability of blood specimens, we have not yet performed any enzymological analyses.

Because of our incomplete knowledge of the exon-intron boundaries of ABO genes and the restricted availability of sample specimens, we focused our efforts on the identification of differences in the coding sequence of the last two coding exons. Although there may be more differences somewhere outside of these exons (including differences in the splicing pattern), 91% of the soluble form of A^1 transferase is contained in these two exons and extensive nucleotide and deduced amino acid sequence homologies with $\alpha 1 \rightarrow 3$ galactosyltransferase gene are observed only in this region [12] suggesting the importance of the last two coding exons for transferase activity.

We have identified nucleotide substitutions, which result in amino acid substitutions, in the last coding exon of some of A^3 and B^3 alleles. The fact that these differences were not found common to all the samples with the same weak subgroups indicates heterogeneity in each group.

There is a possibility that only those cases which identify differences in the nucleotide sequence are genuine weak subgroups and the rest are caused by changes in physical conditions due to illness, diet etc. A decrease of A and B antigens in patients with leukemia has been well documented [13].

The computational calculation using the IBI Pustell Sequence Analysis Program indicates the decrease of flexibility around these amino acid substitutions (aspartic acid → asparagine at aa. 291 in two A^3 alleles and arginine → tryptophan at aa. 352 in a B^3 allele) as well as the changes in the isoelectric point. The amino acid substitution at aa. 291 has also been calculated to disrupt the α-helix structure around this amino acid. These changes may be responsible for the weak activities of these alleles. Future studies by molecular biological approaches (construction of expression constructs with these nucleotide and resulting amino acid substitutions and expression study by DNA transfection) may clarify whether these changes are sufficient for explaining these weak phenotypes. When the complete exon-intron boundaries and promoter sequences are defined for ABO genes, mutations in other A^3 and B^3 alleles will be clearly identified.

References

1 Landsteiner K: Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. Zentralbl Bakteriol 1900;27:357–362.
2 Yamamoto F, Marken J, Tsuji T, White T, Clausen H, Hakomori S: Cloning and characterization of DNA complementary to human UDP-GalNAc: Fucα1→2Gal α1→3 GalNAc transferase (histo-blood group A transferase) mRNA. J Biol Chem 1990;265:1146–1151.
3 Yamamoto F, Clausen H, White T, Marken J, Hakomori S: Molecular genetic basis of the histo-blood group ABO system. Nature 1990; 345:229–233.
4 Yamamoto F, McNeill PD, Hakomori S: Human histo-blood group A² transferase coded by A² allele, one of the A subtypes, is characterized by a single base deletion in the coding sequence, which results in an additional domain at the carboxyl terminal. Biochem Biophys Res Commun 1992;187:366–374.

5 Issit PD: Applied Blood Group Serology, ed 3. Montgomery Scientific Publications, 1985.
6 Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1982.
7 Saiki RK, Scharf SJ, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N: Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985;230:1350–1354.
8 Sanger F, Miklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977;74:5463–5467.
9 Cartron JP, Badet J, Mulet C, Salmon CH: Study of the α-N-acetylgalactosaminyltransferase in sera and red cell membranes of human A subgroups. J Immunogenet 1978;5:107–116.

10 Nakamura I, Takizawa H, Nishino K: A₃ phenotype with A¹ gene-specified enzyme character in serum. Exp Clin Immunogenet 1989;6: 143–149.
11 Salmon C, Cartron JP, Ronger P: The Human Blood Groups. New York, Masson Publishing USA Inc, 1984.
12 Yamamoto F, Hakomori S: Sugar-nucleotide donor specificity of histo-blood group A and B transferases is based on amino acid substitutions. J Biol Chem 1990;265:19257–19262.
13 Race RR, Sanger R: Blood Groups in Man, ed 6. Oxford, Blackwell Scientific Publications, 1975.