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New and unusual *O* alleles at the *ABO* locus are implicated in unexpected blood group phenotypes

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BACKGROUND: In the ABO blood group system mutations in the *A* gene may lead to weak A subgroups owing to a dysfunctional 3- α -*N*-acetylgalactosaminyltransferase. **STUDY DESIGN AND METHODS:** Blood and DNA were investigated to correlate weak A phenotypes with genotype, and an overrepresentation of the infrequent *O*² allele was observed. Consequently, 57 available *O*² alleles were examined in detail.

RESULTS: Two new *O*² alleles were identified having mutations resulting in Gly229Asp with or without Arg217Cys. A recently described *O*² variant (488C>T; Thr163Met) was also found. Surprisingly, both the original and the variant *O*² alleles were associated with either *O* or A_{weak} phenotypes. Three novel *O* alleles surfaced in six other samples with suspected A subgroups. These were A¹-like alleles having nonsense mutations causing premature truncation at codons 56, 107, or 181. A second example of the rare *O*³ allele was also identified. A newly described *O*¹ allele having 768C>A was found to be the third most frequent *O* allele among Swedish donors. Of the five novel *O* alleles, three were incorrectly interpreted as A¹ following routine *ABO* genotyping.

CONCLUSION: Apparent *O* alleles lacking 261delG may cause weak A expression on red blood cells and/or inhibit anti-A production. A hypothesis that exchange of genetic material between principally dissimilar *O* alleles during mitosis ("autologous chimerism") restores glycosyltransferase activity in some cells would explain this interesting phenomenon.

The ABO blood group gene contains seven exons spanning 19.5 kb^{2,3} (see also GenBank Accession No. AC000397 and AF134412-40) and codes for glycosyltransferases that synthesize blood group A and/or B active structures, the correlation of which is crucial for transfusion and transplantation outcome. Since the cloning of DNA complementary to mRNA transcribed from the blood group ABO locus in 1990,⁴ various *O* alleles coding for nonfunctional glycosyltransferases have been described by several research groups (for review, see Chester and Olsson⁵).

ABBREVIATIONS: ASP(s) = allele-specific primer(s); nt = nucleotide.

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Part of this work has been published in abstract form (M.L. Olsson et al., *Vox Sang* 2002;83(Suppl 2):117-8).¹

The following GenBank accession numbers were obtained for the novel *O* alleles presented in this paper: AY611640 (*O*²⁻²), AY611641 (*O*²⁻³), AY611638 (*O*⁴), AY611639 (*O*⁵), and AY611637 (*O*⁶).

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The first blood group *O* allele described was identical to the consensus *A* allele except for a nucleotide (nt) deletion, 261delG, in exon 6 resulting in a frameshift that alters the protein sequence after amino acid 88.⁶ A stop codon halts translation after amino acid 117, and the resulting protein is enzymatically inactive. Another common *O* allele has the same inactivating deletion (261delG) as the original *O* allele (*O*¹-1 [O01]), but in addition has nine point mutations spread throughout exons 3 to 7⁸ and an additional 13 mutations in intron 6⁹ (as well as several further mutations in introns 2-5^{10,11} and intron 1 [GenBank Accession numbers AC000397 and AL158826]). We refer to this allele as *O*^{1variant} (*O*^{1v}-1) [O02]⁸ since the inactivating mutation was the same as in the original *O*¹ allele.

ABO allele nomenclature poses significant problems that are still under consideration by the International Society of Blood Transfusion (ISBT). It is useful and convenient to classify *O* alleles according to the cause of their inactivity. *O*² is inactivated by a different mutation, as are several alleles found subsequently. Minor variants within these subclasses are designated by additional numbers, for example *O*¹-2, *O*^{1v}-3, and so forth (see also Chester and Olsson).^{3,5} The allele designations given in square brackets the first time the allele is mentioned in the text refer to the entry in the Blood Group Antigen Gene Mutation Database (<http://www.bioc.aecom.yu.edu/bgm/abo.htm>).

Additional polymorphisms associated with blood group *O* have been found up to approximately 4300 bp from *ABO* exon 7,¹² some of which correlated with *O*¹ and *O*^{1v} alleles.¹³ The majority of *O* alleles are *O*¹-1 or *O*^{1v}-1⁵, although several variations on these themes exist, which usually arise from single-point mutations compared to *O*¹-1,¹⁴⁻¹⁹ or *O*^{1v}-1,^{13,14,16-18} but also includes various hybrids of *O*¹-1 and *O*^{1v}-1.^{17,19,20} Since *O*¹ and *O*^{1v} alleles are inactivated by the same 261delG mutation all hybrids of these alleles also contain this inactivating mutation.

Hybrids formed by interaction of *O*¹ or *O*^{1v} alleles with *A* or *B* alleles are interesting; they can give rise to obvious *O* alleles if nt 261 is donated by the *O*¹ or *O*^{1v} allele,^{17,20-22} but if nt 261 is consensus (*A* or *B*) and combined with an *O*^{1v} allele, then there are various consequences depending on the crossover point.^{9,10,19,20,23,24} Hybrid alleles apparently exhibit a whole range of serologic activity.^{1,9,11,19,23}

Other *O* alleles not due to 261delG also exist that are caused by other inactivating mutations along the reading frame. The first allele described of this type (*O*²-1 [O03])^{25,26} has a critical mutation (802G>A) causing an amino acid change (Gly268Arg) that prevents the enzyme from utilizing the nucleotide sugar donor.²⁷ This *O* allele comprises approximately 2 to 5 percent of *O* alleles in Caucasian persons but seems to be absent, or at least very rare, in other populations (see Chester and Olsson⁵).

A few other rare *O* alleles have subsequently been found in single samples, such as *O*³ [O08] that has both the common *A*²-allele deletion at nt 1059 to 1061 and the *A*^{el}

insertion in the string of Gs at nt 798 to 804;²⁸ **O*301 [O14] and **O*302 [O15]¹⁹ that have an 893C>T (Ala298Val) or a 927C>A (Tyr309stop) mutation, respectively. **O*301 and **O*302 was the terminology originally used by the authors. Most commonly used genotyping methods rely on the presence or absence of 261delG in determining whether an *O* allele is present, so inactive alleles lacking this mutation complicate most genotype screening methods.²⁹

This study was undertaken to identify and characterize a number of novel *O* alleles found in samples that were referred to Lund, Sweden, for genomic *ABO* typing because of suspected *A* subgroups following discrepant serologic blood grouping results. Our findings expand the current understanding of *ABO* subgroups, the molecular background of which has been associated so far with alterations in *A* or *B* alleles, either by point mutations or by recombinant hybrid formation, which results in *A/B* glycosyltransferases known or presumed to have suboptimal capabilities to synthesize the *A* or *B* antigens on red blood cells (RBCs) and other cells. Many of the novel alleles constitute a risk of error in all *ABO* genotyping methods used to date.

MATERIALS AND METHODS

Sample collection, blood group serology, and DNA preparation

Blood was collected and DNA was prepared with standard laboratory procedures. Samples from 35 individuals referred to Lund for genomic analysis of the *ABO* locus were investigated thoroughly because the phenotypes and genotypes could not be correlated by routine methods.

Blood samples from 456 apparently healthy blood group *O* donors were collected at the Blood Center, University Hospital, Lund, and 46 blood group *O* samples were obtained from Swiss blood donors (Swiss Blood Transfusion Service, Bern, Switzerland). Randomly chosen samples from 172 donors of mixed ethnic and/or geographic origin with other blood groups than *O* were also utilized for screening purposes. Additionally, 64 random blood samples from Jordanian blood donors (The National Blood Center, Amman, Jordan) were examined.

ABO serology was performed with commercially available monoclonal, polyclonal, and lectin anti-*A*, anti-*B*, anti-*A*₁, anti-*A*,*B*, and anti-*H* reagents according to the routines of the laboratories from which the sample(s) originated. If the RBC condition allowed serologic investigation on samples referred to Lund, this was performed as previously described.³⁰

ABO genotyping and DNA sequencing

Oligonucleotide primers (Table 1) used for genotyping, sequencing, and screening were synthesized by DNA Technology ApS (Aarhus, Denmark). A duplex polymerase

TABLE 1. New oligonucleotide primers and primer combinations used in this study*

Primer name†	Sequence (5'→3')	Location	Primer combination	Control primers
ABO-53T-F	AACCAAAATGCCACGCACTTCT	Exon 2	mo-31R	mo-57b-F/mo-46R
ABO-88insG-F	TTCCTAATAATGCTTGTCTTGGG	Exon 2	mo-31R	JK-F3/JK-R3
ABO-106G-R‡	TAGACTTCTGGGGCTTAGGAC	Exon 3	mo-21s	
ABO-220T-F	CAAGGATGGTCTACCCCACT	Exon 5	ABO-297B-R	mo-21F/mo-31R
ABO-261G-R	GGAGCCAGCCAAGGGGTC	Exon 6	ABO-220T-F	mo-21F/mo-31R
ABO-297B-R	GTTGAGGATGTCGATGTTGAAC	Exon 6	ABO-220T-F	mo-21F/mo-31R
ABO-322stop-R	GGTGTTCTGGAGCCTGAACCTA	Exon 7	mo-21F	JK-F3/JK-R3
ABO-488T-R	TGCCGACCGGTCCCCAGCA	Exon 7	ABO-261F-C	JK-F3/JK-R3
ABO-542 A-R	GCGCATGGACACGTCCTGCT	Exon 7	ABO-261F-C	JK-F3/JK-R3
ABO-649T-R	CGCCACGTGGTCCGCA	Exon 7	ABO-261F-C	mo-21F/mo-31R
ABO-689 A-R	CCGGGGTGCAGGGTGT	Exon 7	ABO-261F-C	mo-21F/mo-31R
ABO-768C-R	AATCGCCCTCGTCCTTA/GGGG	Exon 7	mo-101s	mo-21F/mo-31R
ABO-768 A-R	AAATCGCCCTCGTCCTTA/GGGT	Exon 7	mo-101s or ABO-261F-C	mo-21F/mo-31R
mo-75r‡	TCCATGTCCACGTCCACGCAC	Exon 7	mo-101 s or ABO-261F-C	mo-21F/mo-31R
ABO-i2-126AA-R	CAGAGCTGGACGACGCAATT	Intron 2	ABO-53T-F	JK-F3/JK-R3
mo-57b-F	GAGGCGCATGTGGGTGGCAC	Intron 5	Control primer	With mo-46R

* Primers mo-46R, mo-21F, mo-31R, JK-F3, JK-R3, ABO-261F-C, and mo-101s have been described previously.²⁴

† All primers except mo-75r and mo-57b-F were designed to detect the polymorphisms indicated in their names.

‡ The primer was used to amplify general and/or specific alleles for sequencing.

chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method³¹ was used for the initial *ABO* genotyping. A method linking mutations in exons 6 and 7 by PCR with allele-specific primers (ASP) across intron 6 was used when exclusion of recombinant hybrid alleles was required.^{24,32}

The enhancer region (CBF/NF-Y)³³ and exons 1 to 7²⁴ were analyzed as described previously. Briefly, primers located in introns approximately 50 bp from the exons were used to amplify exons 1 to 7. Exons 2 and 3 and the intervening intron were amplified in one fragment. To determine on which allele the novel mutations were located, ASPs (Table 1) were used to amplify allele-specific fragments subsequently used for sequencing to verify *cis* coupling of polymorphisms. A cycle sequencing kit (Big Dye Terminator cycle sequencing kit, Applied Biosystems, Foster City, CA) and genetic analyzer (ABI Prism 310, Applied Biosystems) were used for direct DNA sequencing according to the manufacturer's instructions.

Screening for O-related mutations by PCR-ASP

For each reaction, 5 pmol of each primer (Table 1) and 2 pmol of each of the two internal control primers were mixed with 100 ng of genomic DNA, 2 nmol of each dNTP, 0.5 U AmpliTaq Gold (Perkin Elmer/Roche Molecular Systems, Branchburg, NJ), and glycerol and cresol red at final concentrations of 5 and 0.01 percent, respectively, in the buffer supplied. The final reaction volume was 10 μ L.

Primers used for PCR-ASP to detect the nine mutations (53G>T, 88insG, 220C>T, 322C>T, 488C>T, 542G>A, 649C>T, 689G>A, 768C>A) are shown in Table 1. Thermocycling was performed in a PCR system (GenAmp 2400, Perkin Elmer Cetus, Norwalk, CT). For detection of the 53G>T and 88insG mutations the following cycling

conditions were used: initial denaturation at 96°C for 10 minutes followed by 10 cycles at 94°C for 30 seconds, 65°C for 2 minutes, and then 25 cycles at 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 2 minutes, and a final extension at 72°C for 5 minutes. For the other mutations the cycling conditions were initial denaturation at 96°C for 10 minutes was followed by 32 to 35 cycles at 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 minute, and a final extension 72°C for 2 minutes.

RESULTS

Analysis of blood and/or DNA samples submitted for *ABO* genotyping to the Nordic Reference Laboratory for Genomic Blood Group Typing at the Blood Center in Lund indicated the presence of novel *O* alleles and an apparent overrepresentation of *O*² alleles, compared to the low frequency expected according to population studies.⁵

Presence of the *O*² allele and its variants in blood samples with abnormal phenotypes

No fewer than 28 *O*² alleles were detected in the 35 referred samples, the majority of which was associated with samples involved in ABO blood grouping discrepancies (see later). To identify more *O*² alleles for comparison, blood samples from 456 Swedish blood group O donors were screened for the presence of non-261delG-mutated *O* alleles.³¹ Twenty-seven of these were heterozygous for the *O*² allele (approx. 3% of *O* alleles), which is consistent with the expected frequency in Caucasian persons. Also included were two other blood donors (one group A and one B) known to be heterozygous for an *O*² allele in the study. In total, 57 *O*² alleles were examined. Homozygosity for *O*² was not detected in any sample.

A flow chart summarizes the findings obtained with the referred samples (Fig. 1). Nineteen of the 28 referred blood samples shown to be heterozygous for the O^2 allele also contained an O^1 or O^{1v} allele and could thus be ABO phenotyped without interference from A or B alleles. Three of these 19 samples were unequivocally phenotyped as group O, lacking A and B antigens on the RBCs and containing anti-A and anti-B of normal strength in the plasma. The remaining 16 samples were examined in more detail. Serologically, the blood group was unclear, with seven of these having group O RBCs but lacking plasma anti-A; three had weak plasma anti-A; three samples were suspected to be A_{el} based on weak or absent anti-A and positive adsorption-elution test results at least once with anti-A and/or anti-A,B; and the remaining three were classified as A_{weak} based on either weak RBC agglutination with anti-A,B and/or

adsorption-elution tests combined with weakened or absent plasma anti-A activity.

DNA sequencing of four O^2 alleles selected at random from the 16 discrepant samples described above showed expected O^2 sequences from exons 1 to 7 and the enhancer region in two cases. The third case was a Danish sample lacking plasma anti-A, which showed the expected O^2 sequence except for an additional polymorphism (488C>T; Thr163Met. O^2 -4 in Fig. 2 and Table 2) that was very recently reported¹¹ in a single A_{weak} sample. By PCR-ASP screening (Fig. 3C) we found this mutation in the O^2 allele of a serologically anomalous Swiss sample and in one serologically normal Swedish group O blood donor but not in any other allele (Table 3).

The fourth case was the proband in an Israeli family who was studied further, together with his parents. His mother was group A_1 (A^1O^{1v}) and his father was O (O^1O^2).

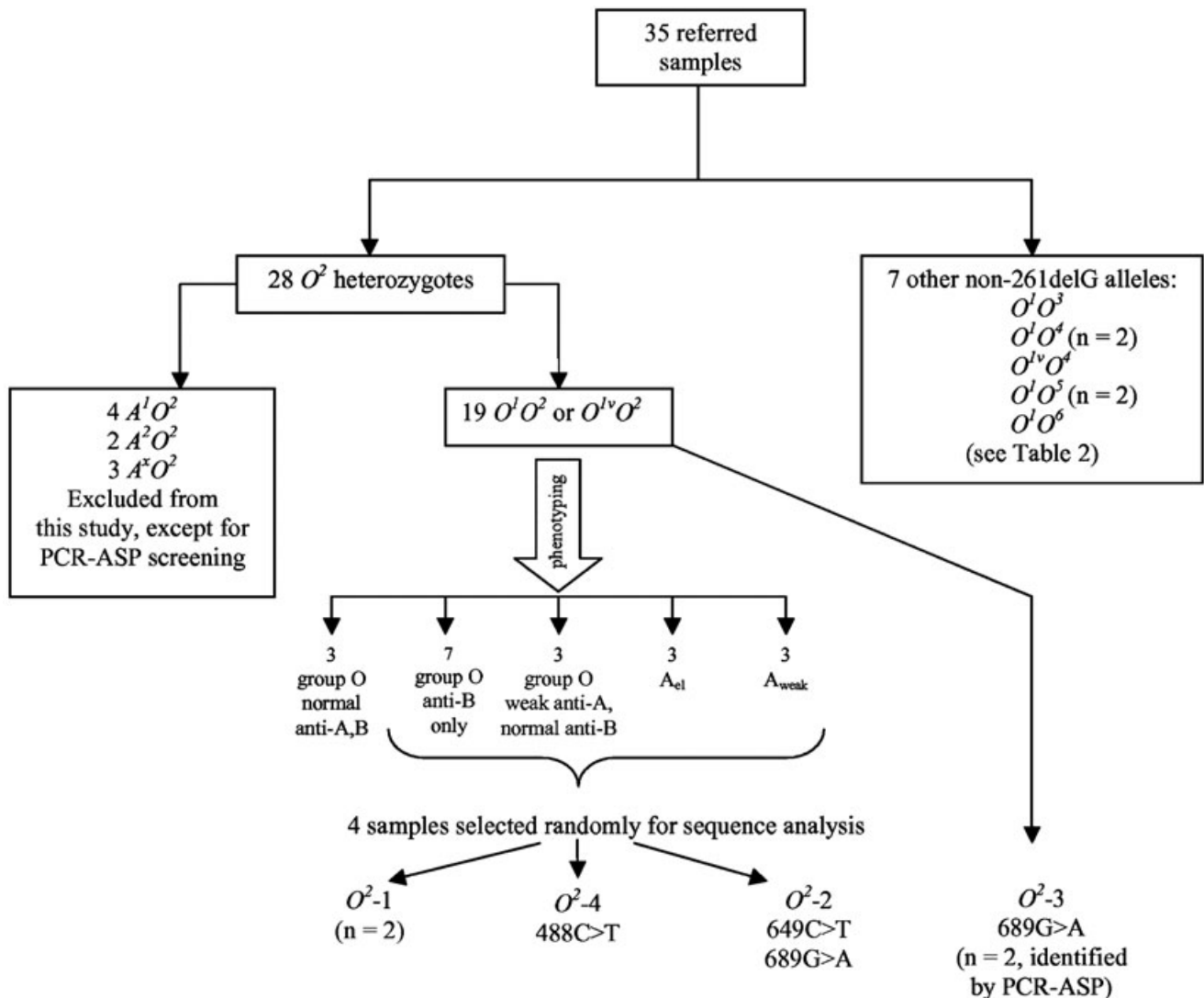


Fig. 1. Flow chart summarizing the results obtained in the 35 referred samples.

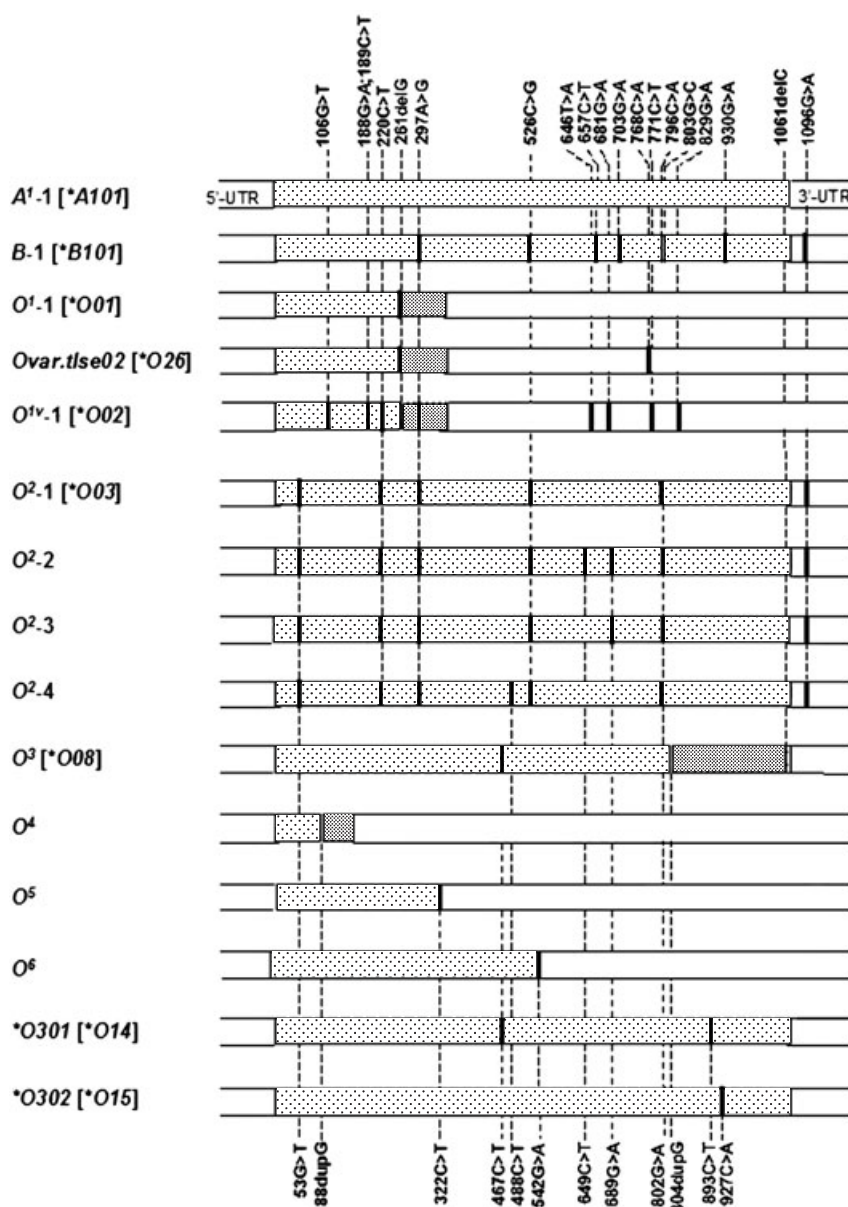


Fig. 2. Common ABO alleles and known O alleles caused by mutations other than 261delG. The common A^1 , B , O^1 , and O^{1v} alleles are shown for reference. Only changes from the consensus A^1 [*A101] sequence are shown (thick vertical lines). The reading frame is shown as light shaded bars. The dark cross-hatched bars indicate a reading frameshift. UTR = untranslated region.

The son lacked anti-A in plasma and had weak expression of A antigen on his RBCs according to adsorption-elution experiments with anti-A. His genotype was $O^{1v}O^2$ and subsequent direct sequencing of all seven exons indicated a normal O^{1v} allele and an O^2 allele containing all the expected O^2 -specific polymorphisms plus two new ones, 649C>T and 689G>A, resulting in Arg217Cys and Gly229Asp, respectively (O^2 -2 in Fig. 2). The father's O^2 allele was identical to that of his son but in contrast he had a typical blood group O phenotype (Table 2). All available

O^2 alleles were screened for mutations at these two positions (Figs. 3D and 3E) and 11 O^2 alleles were found to have 689G>A but all lacked 649C>T (O^2 -3 in Fig. 2). Thus, no further allele containing both mutations was detected, indicating that the 649C>T polymorphism is a rare occurrence, although 689G>A occurs in 17 percent of O^2 alleles. Neither of these mutations was present in non- O^2 alleles (Table 3).

Screening for previously known polymorphisms in blood samples containing O^2 alleles

All 57 O^2 alleles differed from the A^1 consensus sequence in exons 6 and 7 at nt 297, 526, 802, and 1096 as previously described.^{25,26,31} Like the exonic nt 297 and nt 526, nt 1096 located in the 3'-untranslated region is a useful B/O^2 -specific polymorphic position used in ABO genotyping methods. The more recently described polymorphisms in earlier exons at nt 53 and nt 220 found in six O^2 -containing Portuguese blood samples tested by Amado and coworkers³⁴ were confirmed here by PCR-ASP (Figs. 3A and 3B) to be a general feature of the O^2 allele, as shown by their presence in all 57 alleles (Table 3 and Fig. 2). The 53G>T polymorphism is the second mutation exclusive to the O^2 exons, whereas 220C>T is also present in O^{1v} and its variants.^{8,10} In addition to screening for exon polymorphisms, it was also confirmed that all 57 O^2 alleles had only one copy of the 43-bp minisatellite repeat enhancer region as expected from previous work.³³

Other unusual O alleles in blood samples with abnormal phenotypes

Not all O alleles lacking 261delG are O^2 , although alleles that are not O^1 , O^{1v} , or O^2 are very rare or at least difficult to detect with current screening methods. When examining samples referred to Lund because of suspected weak A expression, four such alleles were encountered.

A Swedish blood donor had a weakly reacting anti- A_1 in plasma but no detectable anti-A. No A antigen could be demonstrated on his RBCs, however. The presence of the A^{e1} -characteristic insertion at nt 804 and the A^2 -characteristic polymorphisms at nt 467 and nt 1061 indicated the

TABLE 2. Summary of the serologic results obtained in samples heterozygous for 261delG and an unusual O allele

Allele involved	Donors	Number	RBC reaction			Plasma reaction*		
			Anti-A	Anti-A,B	Adsorption-elution	A ₁ cells	A ₂ cells	Genotypes
O ² -1	Multinational	19	—	—	ND	++++	++++	O ¹ O ² , O ^{1v} O ²
O ² -1	Multinational	13	— to +	— to +	Positive†	— to +++	— to +	O ¹ O ² , O ^{1v} O ²
O ² -2	Israeli (father)	1	—	—	ND	++++	++++	O ¹ O ²
O ² -2	Israeli (son)	1	—	—	Positive	—	—	O ^{1v} O ²
O ² -3	Multinational	9	—	—	ND	++++	++++	O ¹ O ² , O ^{1v} O ²
O ² -4	Swedish	1	—	—	ND	++++	++++	O ¹ O ²
O ² -4	Danish	1	—	—	ND	—	—	O ^{1v} O ²
O ² -4	Swiss	1	mf‡	mf	ND	—	—	O ¹ O ²
O ³	Swedish	1	—	—	Negative	++ to +++	—	O ¹ O ³
O ⁴	American	1	—	—	Positive	+ to ++	—	O ^{1v} O ⁴
O ⁴	American	1	—	—	ND	—	—	O ¹ O ⁴
O ⁴	Austrian	1	—	—	Positive	+	—	O ¹ O ⁴
O ⁵	Austrian§	2	—	—	ND	+	—	O ¹ O ⁵
O ⁶	Swiss	1	—	—	ND	+ to ++	—	O ¹ O ⁶

* All samples had strong anti-B, reacting ++++ in plasma.

† Not tested on all samples.

‡ mf = mixed field agglutination.

§ Father and daughter.

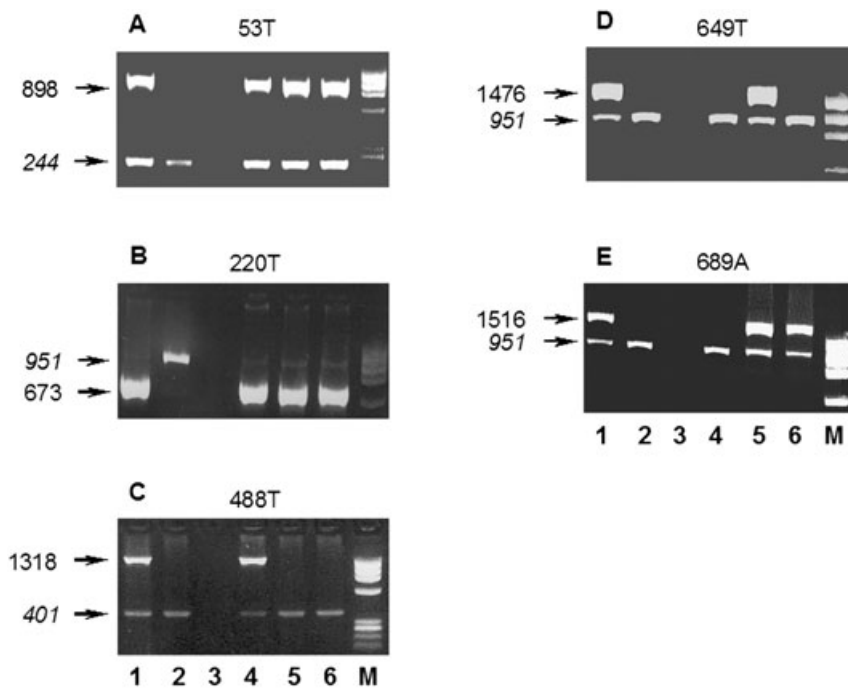


Fig. 3. Gel electrophoretograms from PCR-ASP screening for newly described polymorphisms in the O² allele. (A) 53G>T. Amplification with primer pair ABO-53T-F/mo-31R. (B) 220C>T. Amplification with primer pair ABO-220T-F/ABO-297B-R. (C) 488C>T. Amplification with primer pair ABO-488T-R/ABO-261F-C. (D) 649C>T. Amplification with primer pair ABO-649T-R/ABO-261F-C. (E) 689G>A. Amplification with primer pair ABO-689A-R/ABO-261F-C. Lane 1, positive control; Lane 2, negative control; Lane 3, contamination control (H₂O replaced DNA sample); Lane 4, sample containing the O²-4 allele; Lane 5, sample containing the O²-2 allele; Lane 6, sample containing the O²-3 allele; M, molecular size marker, ϕ X174F DNA/HaeIII (Life Technologies, Gaithersburg, MD). The numbers (italicized for the control bands) on the left of each gel indicate the size (bp) of the indicated amplified DNA fragments.

presence of an O³ allele.²⁸ No additional polymorphisms were detected after sequence analysis of the other exons. The other allele was O¹.

Two blood donors, from the US and Austria, had weak RBC A activity detectable only by absorption-elution and plasma antibodies that reacted weakly with A₁ cells but not at all with A₂ cells. Initial screening and ABO genotyping²⁴ indicated the presence of a consensus (A¹) allele associated with either an O¹ or O^{1v} allele in both cases (Table 2). Another American donor whose RBCs were O but whose plasma did not react with group A RBCs showed similar results. Because this genotype was inconsistent with the phenotypes, the full coding region (exons 1-7) and two proposed regulatory motifs of the ABO gene were sequenced for each sample. This revealed a G insertion at nt 88 in an otherwise normal consensus (A¹) allele (Fig. 4a) resulting in a frameshift and a premature stop at codon 56. We provisionally named this new allele O⁴ (Fig. 2). No further examples of this polymorphism were found after subsequent screening of common ABO alleles (Table 3).

Two other Austrian blood samples (father and daughter) with RBCs that phenotyped as O, but whose plasma contained a weak anti-A₁ and lacked anti-A, were screened (an A¹O¹ genotype

TABLE 3. Screening for some polymorphisms in the ABO gene by PCR-ASP

Allele	Mutation																	
	nt 53		nt 88		nt 220		nt 322		nt 488		nt 542		nt 649		nt 689		nt 768	
	G	T	G	insG	C	T	C	T	C	T	G	A	C	T	G	A	C	A
A ¹ [A101]	50	0	57	0	44	0	66	0	37	0	47	0	52	0	52	0	36	0
A ² [A201]	18	0	27	0	15	0	24	0	14	0	16	0	18	0	18	0	17	0
B [B101]	12	0	18	0	12	0	23	0	32	0	32	0	27	0	27	0	11	0
O ¹ -1 [O01] + O ^{iv} -1 [O02]*	105	0	137	0	†	†	86	0	41	0	62	6‡	36	0	36	0	291	27
O ² [O03]	0	57	57	0	0	57	57	0	54	2§	57	0	55	0§	44	11§	12	0
Total	185	57	296	0	71	57	256	0	178	2	214	6	188	0	177	11	367	27

* These alleles had the deletion at nt 261 and were hence either Oⁱ or O^{iv}. No distinction was made here.

† Not tested. All Oⁱ alleles examined so far have nt 220C and all O^{iv} alleles have nt 220T.

‡ The 542G>A mutation was only found in the O^{iv} allele, as expected.

§ The index case(s) are excluded here. Three of the individuals having O²-4 alleles were related.

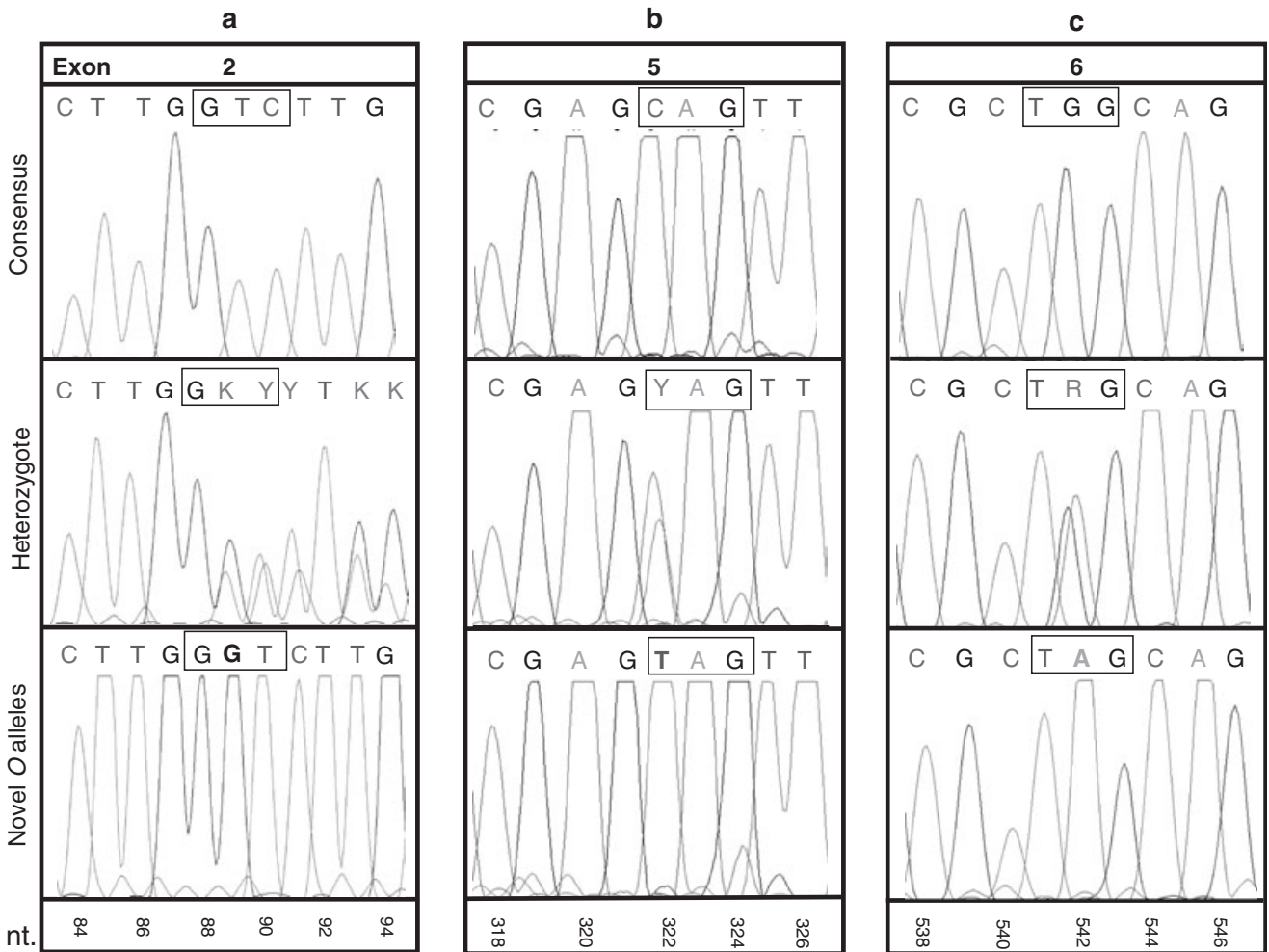


Fig. 4. DNA sequencing chromatograms showing the sequence surrounding the three new polymorphic sites. The top panel in A through C shows the consensus sequence from an A¹ allele and the bottom panel shows the sequence of the new O alleles. (a) nt 84 to 94 in exon 2 showing the 88-insG polymorphism (Oⁱ); (b) nt 318 to 326 in exon 6 showing the nt 322C>T polymorphism (O⁵); and (c) nt 538 to 546 in exon 7 showing the nt 542G>A polymorphism (O⁶). The chromatograms from heterozygous samples are shown in the middle panels.

was indicated) and subsequently sequenced (see Fig. 4b). In both cases a single mutation (322C>T) in the consensus (A^1) allele created an immediate stop codon that would lead to truncation of the enzyme protein in the putative stem region after amino acid 107. This new allele was named O^5 (Table 2 and Fig. 2). Screening failed to show any further examples of this mutation (Table 3).

Finally, a blood sample from a Swiss blood donor, also phenotyped as O, but whose plasma contained a weak anti- A_1 and lacked anti-A (see Table 2) was genotyped as A^1O^1 on PCR-RFLP and PCR-ASP screening. Subsequent direct sequencing (Fig. 4c) showed a polymorphism (542G>A) previously found only in some O^{1v} alleles (O^{1v} -4 [O11]).^{13,16} In the Swiss sample examined here, this mutation is present in an otherwise normal A^1 allele (named O^6 in Fig. 2) and results in an immediate stop codon after amino acid 180. Screening of ABO alleles (Table 3) unearthed six more with the 542G>A change, but these were all shown to be in the O^{1v} allele (O^{1v} -4) mentioned previously.

Unexpected high frequency of an O allele involved in an ABO genotyping discrepancy

A discrepancy between phenotype and apparent genotype after genomic typing of DNA from an Australian blood group O donor was not conclusive with a PCR-sequence-specific oligonucleotide method at the referring center.³⁵ Further ABO genotyping analysis (duplex PCR-RFLP and PCR-ASP) in Lund indicated the O^1O^1 genotype but DNA sequencing indicated homozygosity for a silent mutation (768C>A).

The same mutation was found in 27 O [O^1 or O^{1v}] alleles among Swedish blood donors with PCR-ASP. This corresponds to a frequency of approximately 9 percent of all O alleles. The total absence of this mutation among the Swedish donors in alleles lacking the 261delG was demonstrated and it was also shown that the 768C>A polymorphism was exclusively on the O^1 allele (Table 3). Also examined were blood samples from Jordanian (n = 64, including 82 O alleles) and Swiss (n = 54, including 100 O alleles) blood donors and found three O^1 alleles with 768C>A in each of these populations, suggesting a widespread distribution of this polymorphism, albeit at lower frequencies than in Sweden.

DISCUSSION

In this study a number of novel null (O) alleles are described in the ABO blood group system, the clinically most important antigen system for transfusion and organ transplantation. Although the overwhelming majority of O alleles are caused by the deletion at nt 261, other O alleles can be expected, and indeed are found, having point mutations leading to amino acid changes or early

termination during the synthesis of the enzyme protein. Three of the novel O alleles described here result in an early translation stop and the expected protein would lack the catalytic domain of the enzyme and hence be totally inactive. These rare alleles when associated with either of the common, inactive O^1 or O^{1v} alleles would be expected to give rise to the O phenotype. Each of these alleles surfaced owing to uncharacteristic serologic reactions, however (Table 2). Had the new allele been associated with a common A allele then no serologic abnormalities would have been found.

A disproportionate number of samples submitted to us for genotyping attributed to suspected weak A subgroups contain only O alleles, one of which is frequently O^2 . The O^2 allele, although not common, occurs frequently enough to be observed regularly in samples sent for analysis. It has some interesting features, appearing to contain alternating A- and B-like sequences³³ interspersed with some intronic O^1 and O^{1v} characteristics.^{10,11} The recently described³⁴ mutations in exon 2 (53G>T) and exon 5 (220C>T) were shown here to be ubiquitous in this allele. This indicates that these latter mutations are a basic property of O^2 alleles and not restricted to a Portuguese population. The 220C>T polymorphism is interesting because it is the only mutation found so far in exon 5 and previously only in O^{1v} alleles.^{6,8}

The 488C>T mutation (in O^{2-4} ; Table 2) initially detected in an anomalous Swiss sample was also found on screening not only in an anomalous Danish sample but also in one of the serologically normal Swedish O samples. This indicates that the phenotypic anomaly should not be due to the presence of an O^{2-4} allele because the Swiss and Swedish samples had apparently identical ABO genotypes. This polymorphism was recently described in a German blood donor genotyped as O^2O^2 but phenotyped as A_{weak} .¹¹ The allele was consequently named $Aw08$, but it is identical in all exons and the introns examined (introns 2-6)⁹⁻¹¹ to the O^{2-4} allele described here. Our data argue against the idea that 488C>T would result in an amino acid substitution with the capacity to restore some enzymatic function.

The majority of O individuals have one of the genotypes O^1O^1 , O^1O^{1v} , or $O^{1v}O^{1v}$ and have the classical phenotype with RBCs unreactive against anti-A and anti-B and with plasma reacting strongly with A_1 , A_2 , and B RBCs. The frameshift in exon 6 of the ABO gene causes expression of a truncated protein incapable of glycosyltransferase activity. The A and B antigens cannot be produced by other enzymes coded in the human genome. The O^2 allele was shown to be completely inactivated by the mutation 802G>A changing an amino acid at the enzyme's active site.^{27,34} It is therefore remarkable that in the Israeli family studied here having the novel O^{2-2} allele the father has a normal O phenotype whereas the son has the same allele but shows the A_{weak} phenotype (Table 2). That this rare

allele is not involved in the discrepancy seems to be too great a coincidence.

The new alleles O^4 , O^5 , and O^6 described here either have a frameshift (O^4) resulting in early truncation or point mutations leading to an immediate and early stop codon (O^5 , O^6) leaving only 8, 31, or 52 percent of the correct coding region intact, respectively. By comparison, the totally inactive O^1/O^{1v} and O^3 alleles retain 25 and 76 percent, respectively. In all cases these alleles are expected to be totally incapable of producing an active enzyme and, hence, when present with another inactive allele (usually O^1 or O^{1v}) should produce a normal O phenotype. This is clearly not the case (Table 2).

One could postulate that an alternative translational start codon³⁶ could exist that initiates the formation of an N-terminally truncated protein (at least in allele O^4 , after nt 88). This protein would lack the transmembrane region, however, and its ability to function correctly is questionable. This explanation is inadequate with O^5 and O^6 where the nonsense mutations are much closer to the enzymatically active C-terminal domain.

If alternative splicing can occur in a way that eliminates the exon containing the lethal mutation while retaining the reading frame, then some enzyme activity may be restored. This would not help allele O^4 where the reading frame would not be retained if exon 2 (containing 88dupG) was eliminated. The mutation in allele O^5 is in exon 6, which could be omitted with retention of the reading frame. Joining exon 5 directly to exon 7, however, just happens to create a stop codon. In the Austrian family the father and daughter have the same phenotypic anomaly (lack of plasma anti-A). Both have a hitherto unique O allele. It would appear too coincidental if this novel allele was not involved in the abnormal phenotype. It is difficult to accept, however, that this allele and its predicted product could generate A activity and therefore seek another explanation. Finally, allele O^6 has a mutation creating an immediate stop codon in exon 7, which contains the enzymes catalytic site. It is therefore highly unlikely that alternative splicing can regenerate any enzyme activity in this allele.

A *Chi* sequence (5'-GCTGGCGG-3'), originally described in bacteria, has been shown to influence the recombination frequency in *Escherichia coli*.^{37,38} We and others have published several examples of inheritable, recombinant *ABO* alleles (generated during meiosis).^{9,17,19-24} The duplicated *Chi* sequences in the 3'-end of intron 6 in the *ABO* gene may also increase the probability of recombination in somatic cells (during mitosis). Based on the data set presented here, it is hypothesized that gene conversion or recombination events in somatic cell clones in or outside the marrow may result in dual cell populations within the same individual. If O alleles with principally different defects (e.g., 261delG and 802G>A) are present, there is a possibility to exchange genetic material between

the alleles so that one functional A-like allele is created, either by recombination or by gene conversion (Fig. 5). We suggest that this phenomenon could be called "autologous chimerism" and would explain and be responsible for inhibition of the natural production of anti-A. It is appealing to consider that this idea would account for the correlation of unusual *ABO* phenotypes with all the non-261G-deleted O genotypes encountered here. One could also speculate that the random formation of mitotic hybrids explains why the same genotype can give rise to different phenotypes, as seen here within families as well as when comparing unrelated blood donors. This "random hit" model may also be a general explanation why A_{weak} as well as "O without plasma anti-A" phenotypes arise. If recombination occurs in erythropoietic cells (or even in cells producing glycolipids that may be passively adsorbed to RBCs at a later stage), then weak A expression may result. If other cells are affected, then weak A may not be seen in blood grouping tests but could well inhibit anti-A production. It is probable that A

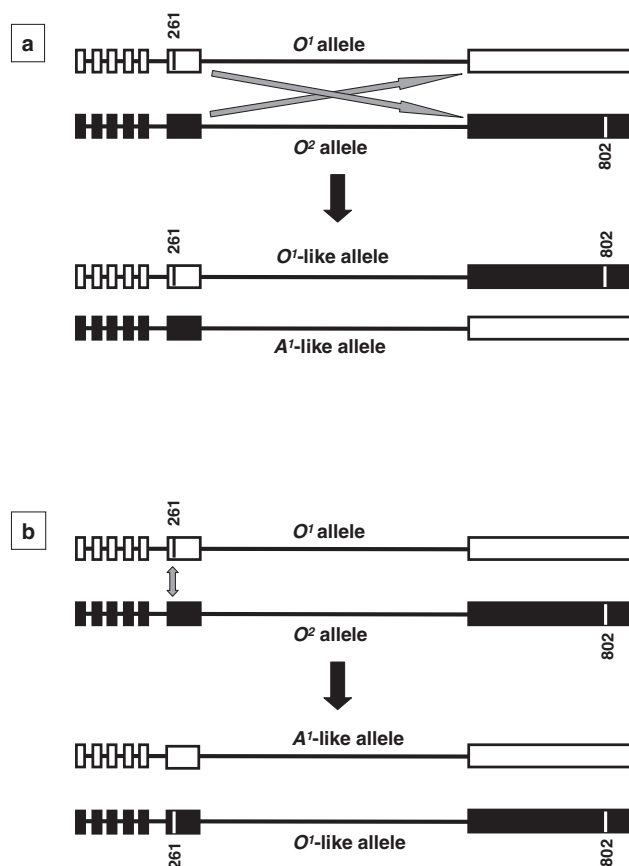


Fig. 5. Recombination or gene conversion events. Possible creation of enzymatically active gene products from inactive alleles by (a) recombination and (b) gene conversion. The thin black arrows show the positions of the *Chi* and *Chi*-like sequences.

antigens must appear early in life so that an immunologic response is not stimulated. It can also be discussed if this event must occur in a "stem cell-like" cell with self-renewal capacity.

We have, however, also seen donors and patients homozygous for 261delG who lack both A expression on RBCs and anti-A in plasma (unpublished data), so other mechanisms obviously exist. Assuming that an O^2O^2 sample with suspected weak A expression¹¹ was correctly typed (and not pseudo-homozygous owing to either loss of heterozygosity or amplification of only one allele), then this genotype may not fit in our explanatory model.

At present, only inexplicable group A RBC activity or lack of plasma anti-A has been described, but not the equivalent abnormal B expression. This could merely be due to the higher A frequency in the populations studied rather than an inherent property of the A alleles.

An O^1 allele (*Ovar.tlse02* [O26] in Fig. 2) having an additional mutation (768C>A) was recently described in 3 of 110 samples (220 O alleles of which 126 were allele $O01$, indicating a frequency of 2%-3% in $O01$ alleles) from Basques from Navarre/Navarra (France/Spain),¹⁸ but was absent from Berber (Morocco), Akan (Ivory Coast), and Amerindian persons (Ecuador/Bolivia). In Swiss and Jordanian blood donors, the frequency has been found to be similar to that found in Basque persons. Our findings that the same allele is present in 27 of 318 O^1/O^{1v} alleles in a Swedish population (Swedish $O^1:O^{1v}$ ratio 55:40⁵) indicates a much higher frequency (15%) in O^1 alleles and makes this the third most common O allele among Swedish blood donors. The calculated frequencies are shown in Table 4, based on results reported here and previously.^{5,13} As far as is currently known other O alleles are rare.

It is disconcerting that three O alleles (87-88insG, 322C>T, and 542G>A) were incorrectly interpreted as A^1 with our ABO genotyping methods (and presumably would be in all currently published methods). Furthermore, the 768C>A polymorphism was brought to our attention because of detection failure with a published method³⁵ that includes a probe aimed at detecting the O^{1v} -

specific 771C>T polymorphism and where the 768C>A polymorphism prevents its binding.

Unusual O alleles lacking the common 261delG or the O^2 -characteristic 802G>A mutation represent a serious risk for erroneous interpretation in genomic blood grouping. The inability to detect a mutation known or expected to cause a total inactivation of the glycosyltransferase responsible for the production of an A or B antigen does not necessarily mean that the enzyme is inactive and that no such antigen exists.

It is becoming apparent that genotype-phenotype correlation in the ABO system is more complex than previously imagined. In the past, apparently identical alleles have been classified as both O and A_{weak} depending on the ensuing phenotype.^{9,11,19,23} The discrepancies have usually been reconciled by suggesting that the difference is marginal and that different laboratories reach different phenotypic conclusions. This is possible in certain instances, but highly unlikely on the scale now becoming apparent. Alternatively, it has been suggested that gene sequences outside the regions examined could explain the differences. This is also becoming a less likely explanation as larger and larger segments of the ABO alleles are being sequenced. Most enigmatic is the presence of A activity in individuals homozygous for alleles that are totally inactive in the vast majority of the population. It is also highly unlikely that another independent gene is capable of initiating the biosynthesis of the human blood group A antigen. If this were the case, this rare occurrence would surely be more prevalent.

In conclusion, our data appear to challenge the usual way of looking at blood group inheritance and also complicates allele terminology further. In a predictable manner, O alleles are supposed to give rise to O phenotypes and A alleles to A or possibly A_{weak} phenotypes. This is obviously no longer the case.

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TABLE 4. The O allele frequency among Swedish blood donors*

Allele	Nucleotide position: Consensus (A^1) sequence:	220 C	261 G	297 A	542 G	689 G	768 C	802 G	Total O (%)
O^{1-1} [O01]			delG						47
O^{1v-1} [O02]		T	delG	G					35
<i>Ovar.tlse02</i> [O26]			delG				A		8
O^{1v-4} [O11]		T	delG	G	A				5
O^{2-1} [O03]		T		G				A	4
O^{2-4}		T		G		A		A	1

* Only differences from consensus are shown. Additional mutations in the reading frame are 53G>T specific for O^2 and 106G>T, 188G>A, 189C>T, 646T>A, 681G>A, 771C>T, and 829G>A specific for O^{1v} .

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