REVIEW

The O^2 allele: questioning the phenotypic definition of an *ABO* allele

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There are three main alleles in the ABO blood group system, *A, B,* and *O*. The former two alleles encode glycosyltransferases resulting in the wild-type A and B phenotypes, whereas the latter allele does not encode a functional enzyme owing to a frameshift polymorphism in the majority of cases. Thus the group O phenotype is the absence of A or B sugars. More than 15 years ago the O^2 allele was described; this allele did not feature the usual crippling 261delG polymorphism, which up to that point was the hallmark of an allele encoding group O, but instead had several other nucleotide polymorphisms that reduced or eliminated the activity of its resulting protein. The classification of this type of allele as encoding group O has been called into question of late as some individuals with an O^2 allele appear to have a weak A phenotype. Others with the same allele do not demonstrate any A antigens on their RBCs but might be involved in reverse typing discrepancies. Even within the same pedigree these alleles do not necessarily produce a consistent phenotype. This paper will summarize the detailed biochemical and population-based evidence both for and against the O^2 allele's ability to create A antigens or the absence of anti-A in plasma. ***Immunohematology* 2008;24:138–147.**

Key words: ABO, O, O^2, allele, nondeletional

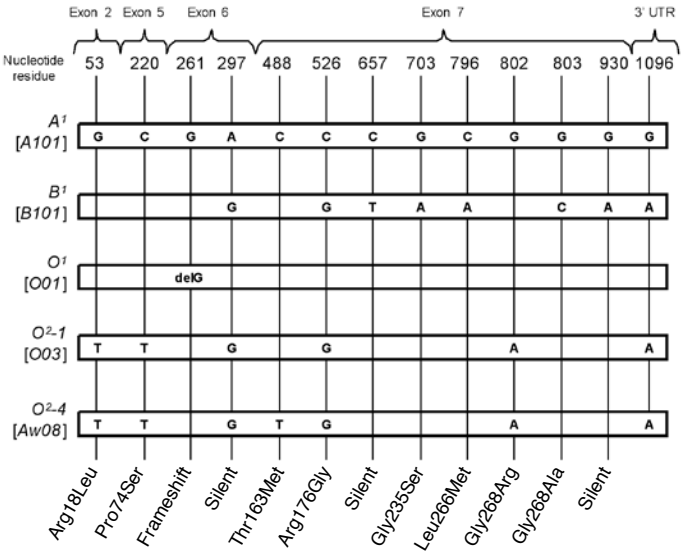

Fig. 1. Summary of exonic SNPs in the alleles described in this paper. The A^1 [*A101*] allele is the consensus allele to which all others are compared. Vertical bars through the schematic allele indicate a consensus nucleotide. UTR = untranslated region.

Introduction to the Concept of *ABO* Alleles

For a short period, things were simple in the nascent field of ABO molecular genetics. There were three known alleles of the *ABO* gene, A^1, *B,* and *O,* and they correlated well with the four major ABO phenotypes. It was known that all three alleles contained 1065 bases unequally divided among seven exons, and that *A* alleles consistently differed from *B* alleles at seven in-frame nucleotide residues (Fig. 1), of which four of these single-nucleotide polymorphisms (SNPs) caused amino acid substitutions in the enzyme (Fig. 2).[1–3] At that time it was also known that the *O* allele was one base shorter than the consensus A^1 allele; the deletion of a G nucleotide at residue 261 (261delG) introduced a frameshift mutation and a premature stop codon such that the protein was predicted to have only 117 of the usual 354 amino acids and completely lack an enzymatic center.[1] Lacking the glycosyltransferase A (GTA) ability to transfer the A-determining sugar *N*-acetylgalactosamine (GalNAc) or the glycosyltransferase B (GTB) activity of transferring the B-determining moiety galactose (Gal), an O phenotype clearly is essentially the absence of A or B antigens.

Thus in the early part of the 1990s, three *ABO* alleles produced four phenotypes. Those halcyon days were, however, the calm before the storm; shortly after the landmark description of the structure of the *ABO* gene by Yamamoto et al.,[1] the alleles underlying various A and B subtype phenotypes were reported.

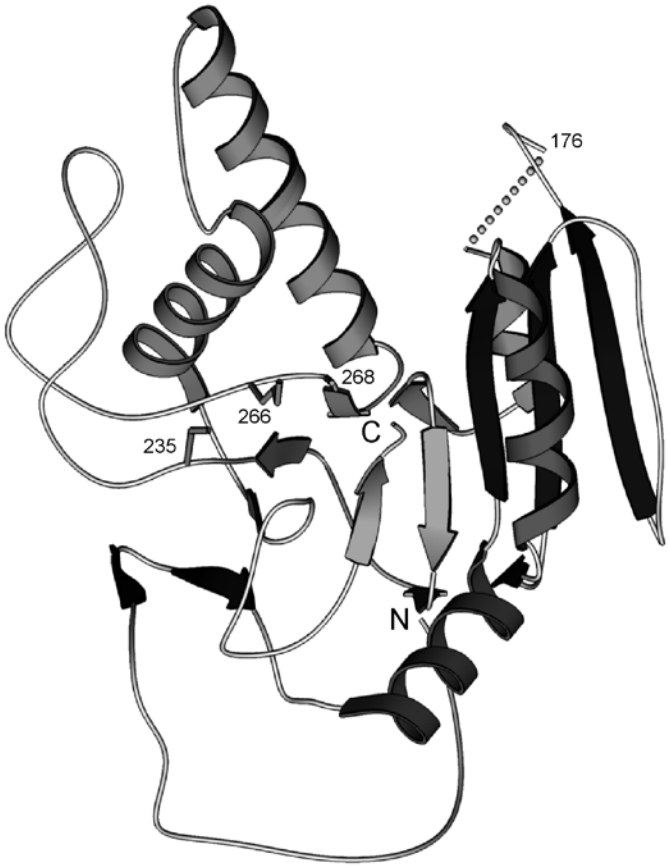

Fig. 2. Three-dimensional ribbon diagram of the ABO glycosyltransferase enzyme. The numbered amino acid residues indicate those which differentiate GTA from GTB while highlighting residue 268, which is critical in causing the dramatic change in enzymatic activity in the O^2 allele–encoded protein. Figure generously supplied by Drs. Stephen Evans and Monica Palcic.

It became clear that different SNPs could be manifest in a similar phenotype. This is not surprising given that A and B subtypes are normally first detected in the blood bank by using serologic techniques that, in a relatively crude manner, determine the amount and type of A and B antigen expressed on the RBCs. A specific subtype SNP is seldom betrayed by its phenotype; however, the same SNP can sometimes give rise to different phenotypes. Thus, almost 20 years after the cloning of the *ABO* gene, there are many known SNPs underlying A and B subtype phenotypes, and the process of *ABO* genotyping is further complicated when hybrid alleles are added to the mix. In summary, the genetics of the ABO blood group is no longer simple.

But what about group O, the null phenotype of the ABO system? Simply put, any allele that does not give rise to an A or B phenotype, no matter how weak, is de facto an *O* allele. Given the heterogeneity and number of SNPs underlying A and B subtypes, why should we not assume that *O* alleles have an even greater variety of SNPs eliminating enzyme activity? How fine a line is there between an enzyme with *greatly reduced* activity and one *completely devoid* of activity? And an even more intriguing question is whether the same enzyme can behave differently in different circumstances.

As initially described, the *O* allele was virtually identical to the consensus A^1 allele (now also referred to as *A101*) save for the 261delG SNP.[1] In time, other *O* alleles with the same 261delG SNP were described. The second most commonly encountered *O* allele, O^{1v} [*O02*], has 10 SNPs scattered throughout exons 3 through 7 including 261delG,[4] as well as numerous SNPs in its introns that discriminate it from *A101*.[5,6] Despite sharing the 261delG SNP, O^1 and O^{1v} are not at all related in evolutionary terms.[7,8] A variety of rare and common *O* alleles with and without 261delG (reviewed in Hosseini-Maaf et al.[9] and Chester and Olsson[10]) have been deposited in the Blood Group Antigen Gene Mutation Database (dbRBC),[11] which currently harbors 42 entries of alleles containing 261delG. *O* alleles that feature 261delG are broadly referred to as "deletional" *O* alleles owing to the nature of their crippling SNP, and they account for virtually all of the *O* alleles in all populations studied in which an O^2 allele was not identified. Lacking an active center, these deletional *O* alleles cannot give rise to functional enzymes, although it has been speculated that these truncated proteins might play a role as minor histocompatibility antigens.[12] Thus, when a deletional *O* allele is inherited with a consensus or subtype *A* or *B* allele, the RBC phenotype will be defined only by the allele in *trans* to the deletional *O* allele.

1. Do *O* Alleles with 802G>A Give Rise to GTA Activity? No.

As mentioned above, given the panoply of SNPs producing A and B subtypes, it should be somewhat surprising to find only one main genetic mechanism of eliminating A and B glycosyltransferase activity, namely 261delG. But group *O* alleles are not devoid of their own intrigue. Consider an interesting patient first described in 1993 by Yamamoto et al.[13] Their patient likely had a B_3 phenotype by modern subtype nomenclature (60% of this patient's RBCs were agglutinated with anti-B reagents; this represents mixed field agglutination); thus their genotype was

expected to be B^3/O as the authors assumed that a B^3/B^3 genotype was very rare.[13] However, on sequencing exons 6 and 7, the 261delG SNP was not found and in fact no SNPs were discovered that would have limited the translation of this new allele's transcript. Thus, for the first time, an allele without apparent enzymatic activity that lacked the 261delG SNP had been discovered! Other perturbations from the *A101* allele were present: 297A>G, 526C>G, and 802G>A.[13] Remarkably, the former two SNPs are present in a wild-type *B* allele, whereas the 802G>A SNP was novel (Fig. 1). 802G>A predicts a Gly268Arg substitution in the enzyme; amino acid residue 268 is one of the four residues that discriminate a wild-type GTA from GTB and, along with the amino acid at residue 266, it helps to confer the enzyme's donor sugar specificity. The 526C>G SNP predicts an Arg176Gly amino acid mutation, which again is one of the four GTA versus GTB discriminating amino acids, although the amino acid at residue 176 is more likely involved in enzyme turnover rate, not in conferring donor sugar specificity (reviewed in Yazer and Palcic[14]). The authors transfected HeLa cells with the cDNA of their newly discovered "nondeletional" *O* allele and could only detect background levels of A and B antigen expression by flow cytometry.[13]

One year later, Danish investigators Grunnet et al.[15] gave this new *O* allele a name. By PCR-RFLP analysis of 150 phenotypically group O blood donors and laboratory volunteers, they found 11 individuals who they described as having either an "*A* or a *B* allele without the (261delG) deletion."[15] Further analysis of this allele confirmed the finding by Yamamoto et al.[13] of a *B* allele–related 526G, and also found an *A* allele–related 703G (Fig. 1). This latter residue is another *A* versus *B* determining nucleotide. By sequencing exons 6 and 7, these authors also confirmed the presence of both the 297A>G and 802G>A SNPs that had already been described. It was now clear that this new allele was composed of both *A* and *B* allele sequences. The Danish investigators thus recognized that the nondeletional allele they were describing was identical to the one published a year earlier, and they named it O^2.[15] As all 11 of their donors with the O^2 allele were also heterozygous for the deletional O^1 allele, the authors calculated an O^2 gene frequency of 3.7 percent in their population. This frequency is within the range of O^2 allele frequencies later reported in several populations.[10] Grunnet et al. did not perform functional assays on the O^2 enzyme, nor did they employ adsorption–elution to detect whether very small quantities of A antigen were present on RBCs from their O^2 donor.[15]

The O^2 allele surfaced again a few years later in studies on A_{el}[16] and A_x subgroups.[17] Even though it was striking to find this infrequent *O* allele present in several of the rare pedigrees presented,[16,17] there were no data to suggest that O^2 was able to increase the amount of A antigen expression when present, a finding that was consistent with the literature at that time.

In 2000 another comprehensive study focused on both healthy blood donors and patients with gastric carcinoma.[18] The O^2 gene frequency was established to be 4.2 percent and 3.9 percent, respectively, but this study was important for several other reasons. It was the first to sequence all seven exons of the O^2 gene, and in so doing it revealed two SNPs in exons 2 and 5 that had not previously been detected in this allele. The 53G>T SNP is interesting because it represented a polymorphism specific to the O^2 allele; up to this point, the O^2 gene appeared to be a hybrid of *A* and *B* alleles without any novel sequences of its own except for 802G>A, but the new data suggested that this allele had undergone its own evolution (Fig. 1).[18] Later investigators would reveal more similarities between the O^2 and A^1 alleles in the upstream CCAAT-binding factor (CBF) enhancer region (both have only one 43-bp element as opposed to the other major alleles, which have four).[19] Analysis of the introns of the O^2 allele revealed that it shares some common sequences with other *O* alleles in introns 2, 4, and 5, and with *B* alleles in intron 6, while also demonstrating a few unique SNPs in introns 2 through 5.[5,6]

The authors of the previous studies on O^2 predicted that if a protein was to be translated from this gene, it should contain all 354 amino acids and it should thus cross-react with antibodies to both the wild-type GTA and GTB enzymes themselves. Indeed this latter postulate was shown to be the case; when Sf9 insect cells were transfected with the full-length O^2 transcript, staining with an antibody that binds to amino acid sequences common to wild-type GTA and GTB revealed a Golgi pattern of localization for the O_2 protein. This pattern of staining was observed in most normal and neoplastic tissues from O^1O^2 gastric cancer patients.[18] So whereas the O_2 protein was expressed in its expected location, its functionality remained dubious. Expression constructs of both

the full-length and soluble forms of the enzyme failed to show activity above background levels with either GalNAc or Gal.[18] Similar findings had been demonstrated in 1996 when Yamamoto and McNeill[20] produced expression constructs of an *A101* gene and mutated the amino acid at residue 268 with all 20 amino acids; when the construct featured Arg268, A antigen was not detected on the HeLa cells by flow cytometric analysis and this mutant construct failed to show GTA enzymatic activity. However, it should be noted that the other O^2-related SNPs were not present in this construct. When the normal and neoplastic gastric tissues from the O^1O^2 patients were stained for A antigens, only an equivocal amount of immunoreactivity was observed in a few specimens.[18]

Thus in 2000, the overwhelming weight of evidence was on the authors' side when they concluded[18,20] that this allele's designation as an *O* allele, despite its production of a full-length transcript whose protein localizes where functional GTA and GTB enzymes do, was valid as it did not appear to have enzymatic activity.[18]

Crystallographic structure of O_2 protein reveals mechanism for reduced or abolished enzymatic activity

More support for classifying O^2 as an *O* allele came in 2005 when Lee et al.[21] studied an *Escherichia coli*–optimized model of the soluble truncated form of the O_2 enzyme (lacking amino acids 1–53). In their construct, the O_2 enzyme had six orders of magnitude less ability to transfer GalNAc to the synthetic acceptor substance (H antigen) compared with the wild-type GTA enzyme. Incorporating all three amino acid mutations, the activity level (k_{cat}) of O_2 was 3.4×10^{-5} s^{-1} compared with the wild-type GTA activity of 17.5 s^{-1} with a GalNAc donor! The soluble form of O_2 also had a marked increase in the binding constant for the GalNAc donor substrate (K_B) with a smaller increase for the H antigen acceptor substrate (K_A). Moreover, they showed that the principal deactivating mutation was Gly268Arg (caused by the 802G>A SNP), as revealed in the enzyme's crystal structure; the bulky side chain of Arg268 causes steric blockade of the access of GalNAc to its binding site in the enzyme's active center, hence the observed increase in the K_B and its resulting inability to catalyze the formation of A antigen.[21] Given that in the clinical studies described above no A antigens were regularly detected on the RBCs or tissues of individuals with an O^2 gene, and compounded with these in vitro data, it looked like the meager k_{cat} of O_2 with a GalNAc donor was basically equal to zero.

Interestingly, the 802G>A SNP turned out not to be unique to the O^2 family of alleles. The dbRBC contains seven alleles that feature this SNP,[11] of which two are not specifically identified as group *O* alleles. These others include *Aw08* (see below),[5] and *B^w-18*.[22] This latter allele produced a B_x phenotype caused by an Ala268Thr amino acid mutation. Interestingly, the steric interference observed with the O_2-specific Arg268 does not appear to occur with Thr268. Nevertheless, the k_{cat} of the B_w-18 enzyme is reduced by more than 100-fold compared with a wild-type GTB with Gal donor, again indicating the importance of residue 268 for the enzyme's activity.[22]

2. Do *O* Alleles with 802G>A Give Rise to GTA Activity? Yes.

Given the results of all the investigations on the O^2 allele, it was a surprise when, in 2003, two blood donors who were homozygous for nondeletional *O* alleles featuring the 802G>A SNP (also referred to as *O03* alleles) with an RBC phenotype consistent with the A_{el} subgroup were described.[5] Both donors also had weak anti-A_1 and strong anti-B antibodies in their serum.[23] One of the two donors had another type of nondeletional *O* allele; in addition to the previously reported SNPs for O^2, this donor's allele also featured a 488C>T polymorphism, which predicts a Thr163Met change. As the authors believed that this new nondeletional allele had some intrinsic GTA activity, they named it *ABO*Aw08* (probably identical to $O^{2\text{-}4}$;[9] Fig. 1). It was hypothesized that Thr163 somehow allowed the enzyme to overcome the significant steric inhibition caused by Arg268, thus "reactivating" the enzyme and allowing it to create a very weak A phenotype.[5] The authors did not comment on the fact that although only one donor was heterozygous for *ABO*Aw08,* both donors had identical RBC phenotypes. Thus even the donor who was homozygous for two standard nondeletional *O* alleles (O^2) without the Thr163Met mutation was allegedly capable of producing what they described as an A_{el} phenotype. This speaks against the notion of a reactivating mutation, as does the subsequent finding of an apparently identical allele in a blood donor with a normal group O phenotype and strong anti-A and anti-A_1.[9]

Allelic enhancement might contribute to the O_2 enzyme's GTA activity

Allelic enhancement is a phenomenon that can lead to unexpected phenotypes, typically noted when a pedigree analysis is performed.[24] It has been reported in the setting of two full-length products from the *ABO* locus; typically one enzyme is mutated and would be expected to produce a weak A (or possibly B) phenotype, whereas the second ABO glycosyltransferase (GT) is fully functional. Under most circumstances, the normal ABO GT would then outcompete the weakened enzyme and can even result in the failure to detect any of the mutated enzyme's antigens.[25] Uncommonly though, greater than expected antigen production by the mutated enzyme can result when it is expressed along with a fully functional and full-length ABO GT. In the original report, A^x and *B* alleles were studied in a family, and a nearly 10-fold increase in the number of RBC A antigen sites was observed in the A^x/B family members compared with their A^x/O relatives. In addition, serum GTA activity was only detected in the A^x/B family members.[26] Because wild-type GTB is known to have some low-level intrinsic GTA activity, it was even more exciting when O^2 was implicated in allelic enhancement in a Polish family; the propositus demonstrated a weak A phenotype with an A^x-4/O^2-1 genotype, while her parents both had O phenotypes and were heterozygous for A^x-4 or O^2-1 alleles with common deletional *O* alleles in *trans*.[27] The biochemical basis of allelic enhancement is still unclear, although in an *E. coli*–optimized model of soluble O_2 proteins, dimerization appeared to occur by means of the enzyme's stem region.[21] Thus, there is a potential to produce more enzymatically favorable protein heterodimers leading to increased catalysis if both fully functional and less optimal ABO-GT are present in heterozygous individuals. A scenario supporting this speculation has recently been reported for other types of glycosyltransferases in which the enzymatic activity increased significantly after heterodimer formation.[28]

RBCs from blood donors with an O^2 allele appear to demonstrate A antigens

Seltsam et al.[29] followed up their initial report of nondeletional group *O* alleles with limited GTA activity with a larger survey of both blood donors with a group O forward type but variably reduced levels of anti-A and normal levels of anti-B on their reverse type, samples that were referred to their laboratory because of a suspected A subtype, and in vitro expression studies of both the O^2 (*O03*) allele and the *ABO*Aw08* allele. Some of the results were difficult to interpret, in particular some of the serologic findings among the 12 donors (and 4 family members) who were at least heterozygous for a nondeletional *O* allele. All but one had an uncomplicated group O forward type, although some demonstrated weakening of their serum anti-A_1, and some completely lacked anti-A. These reverse typing perturbations can be considered a surrogate marker for A antigen production, if not on the RBCs themselves then perhaps on some other tissue such that the immune system produces a muted antibody response to this antigen as it recognizes the low level of A antigen as a "self" antigen. In other individuals the level of anti-A as assessed by agglutination was normal. A antigens were not detectable on any of these RBCs by flow cytometry. Using both polyclonal anti-A and anti-A,B, A antigens were barely detectable on some of these RBCs using two different adsorption–elution techniques,[29] which is unusual because even in the weakest possible A subgroup, A_{el}, the eluate generally reacts very strongly (typically 3–4+). In this study, Seltsam et al.[29] did not report the strength of the eluates prepared by using their anti-A and anti-A,B reagents with other A subgroup cells as a positive control, nor did they specify the results of testing the "last wash" supernatant; thus it is unclear how to interpret the very weak (often unconventionally reported as 0.5+) agglutination they observed in their eluates. Furthermore, the pattern of eluate positivity is hard to interpret; some donors showed very weak reactivity using both eluate methods and both reagents, whereas others showed reactivity only with one reagent and one eluate method. The lack of consistent eluate reactivity patterns coupled with the generally weak agglutination makes drawing firm conclusions about the GTA activity of O_2 proteins difficult in this study.

In this study, one donor who was heterozygous for a nondeletional *O* allele apparently demonstrated macroscopically visible agglutination on forward typing using one particular monoclonal anti-A reagent (reported as 1+ agglutination using the Seraclone reagent from Biotest AG, Dreieich, Germany), yet the donor's eluate was completely negative with both reagents and both elution techniques![29] This individual also featured nearly normal agglutination

of anti-A_1 on reverse typing. It is difficult to reconcile these findings: there were sufficient A antigens on this individual's RBCs to cause macroscopically visible agglutination, yet anti-A could not be eluted from them, nor was any A antigen detectable by flow cytometric analysis. It must be considered that the apparent weak A activity noted in some O^2 donors and with some reagents may actually represent not the true GalNAc-based A antigen but some alternative A-like structure that cross-reacts with certain antisera and sometimes downregulates the anti-A response of the individual. For instance, it should be possible to assess whether the O_2 protein is slightly better at transferring a sugar other than GalNAc to the H precursor. Interestingly, minor reactivity with alternative carbohydrate substrates has been reported with wild-type GTA and GTB.[30]

The *Aw08* allele was once again detected in two donors who had *O03/Aw08* genotypes; the presence of the *Aw08* allele did not cause increased A antigen expression on their RBCs compared with those of donors with an *O03* allele.[29] In the in vitro component of their experiments, anti-A was elutable from HeLa cells transfected with the *O03* and *Aw08* genes, and eluates from both of these transfections demonstrated identical agglutination strengths.[29] It is possible that the transfection experiments do not necessarily reflect the activity of these alleles in vivo; the eluates from the RBCs of donors with nondeletional *O* alleles ranged from negative to weakly positive, and the strengths did not change significantly between eluates prepared from anti-A or anti-A,B reagents. In the transfection experiments, the agglutination strengths were generally much higher than those of the donors, and in all cases showed a full grade stronger agglutination with the anti-A,B reagent.[29] As in their donor studies, Seltsam et al.[29] did not report the results of the last wash supernatant for the transfection adsorption–elution experiments, nor was this technique originally intended to be used on HeLa cells.

In their analysis of 16 donors and family members with an O^2 allele, an interesting side note emerged. Although it is difficult to compare the eluate results among donors when the strength of agglutination was so weak, there did not appear to be a difference in the diminution of anti-A_1 or anti-A on reverse typing, nor an increase in strength of the eluates in donors who were homozygous for nondeletional *O* alleles compared with those who were heterozygous for a deletional *O* allele.[29] This appears to weigh against zygosity as a contributing factor in the (possible) production of A antigen.

Further evidence that homozygosity of nondeletional *O* alleles is not required to cause ABO discrepancies on automated typing instruments came from another German study of 2196 group O blood donors by Wagner et al.[31] In 45 of these samples their Olympus PK7200 could not determine the blood group because of irregularities with the anti-A reverse typing, and in 38 of those cases, the donors were at least heterozygous for an O^2 allele (1.7% of group O donors). Given that the expected frequency of the O^2 allele in the German population is approximately 1.6 percent,[29,32,33] it was expected that in total approximately 65 to 70 donors with at least one O^2 allele should have been discovered in this study. That only 38 such donors were identified suggests that the allele frequency in that specific donor population is lower than expected or, alternatively, it is possible that there are other as yet unidentified factors that regulate the activity of the O_2 protein such that in the approximately 30 donors who were not identified their O_2 protein lacked sufficient GTA activity to create an ABO discrepancy.

Curiously, two donors who were heterozygous for the O^2 allele produced normal levels of anti-A, yet they were enumerated among those samples in which the instrument could not interpret the ABO type because of diminished titers of anti-A.[31] In this study too, zygosity did not seem to influence the diminution of anti-A level; in the 10 donors who were devoid of anti-A, all were heterozygous for the *O03* allele along with a deletional *O* allele. Three donors were homozygous for nondeletional *O* alleles, and they demonstrated weak (but not absent) anti-A on the reverse type, again indicating that zygosity is not likely affecting these results, and also that the Thr163Met mutation in the Aw08 enzyme does not confer on it extra GTA activity compared with the O03 (O_2) protein.[31] Unfortunately, adsorption–elution studies were not performed (Franz Wagner, personal communication) on the RBCs from the donors with an O^2 allele to try to determine whether A antigens were present or the diminution of anti-A on reverse typing had another cause. Even supplying the levels of anti-B would have indicted whether the diminution in antibody levels was restricted to anti-A (as the authors suggest), or it was related to a global reduction in antibody titers.

Although the *O²* allele was present in the majority of donors with reduced titers of anti-A, overall the results of this study were not clear-cut; there were seven donors who were homozygous for deletional *O* alleles who also demonstrated weak anti-A on reverse typing.[31] Surely another explanation for the diminished reverse typing must be sought in these donors as deletional *O* alleles cannot produce A antigens. Conversely some donors with *O03* alleles did not demonstrate any diminution of their plasma anti-A on reverse typing, whereas one *O03* heterozygous donor demonstrated diminution of both anti-A and anti-B on repeat testing.[31] The diminution of anti-B is not explicable by any intrinsic GTA activity of the O_2 protein.

In total, these authors have interpreted their results to indicate that the members of the *O²* family of alleles have intrinsic GTA activity. As Lee et al.[21] reported, the in vitro activity of the soluble form of the O_2 enzyme was 3.4×10^{-5} s^{-1}; some of the aforementioned results suggest that this number, although very small, is not quite equal to zero. Still, in the absence of unusual confounders it can be predicted that an A phenotype will be produced if a consensus *A* gene is present and a B phenotype will be produced if a wild-type *B* gene is present. However, the RBC phenotype cannot be predicted with a high degree of certainty when a nondeletional *O* allele is present. This is perhaps even more evident in the presence of nondeletional *O* alleles other than *O²*.[9,34]

The weak A phenotype created by the O^2 *allele is highly variable*

The variability of the *O²*-associated phenotype was highlighted in a recent paper analyzing 19 *O²* heterozygous samples from patients and donors from around the world.[9] Three samples produced the expected normal O phenotype on both the forward and reverse typing, 10 samples had diminished or absent anti-A in the setting of an otherwise normal O phenotype, and in the remaining 6 samples some degree of A antigen expression was detected, generally by adsorption–elution. A variety of *O²* family alleles (including a few new family members, i.e., *O²* alleles with one or two additional missense mutations) were detected among these 19 heterozygous donors, including *Aw08,* which was also found in a donor with a completely normal O phenotype as mentioned above.[9]

An interesting family study also serves to highlight the variability of A antigen synthesis in donors with an *O²* allele. The male propositus had an *O^{1v}/O²-2* genotype and an A_{el} RBC phenotype. His father, who genotyped as *O¹/O²-2,* had a completely normal O phenotype including normal-strength anti-A! Thus the same allele within the same family can behave differently.[9]

This may be the mechanism for the GTA activity associated with the presence of O^2

Hosseini-Maaf et al.[9] offered an interesting explanation for how an *O²* allele might demonstrate GTA activity when it is inherited with a common deletional *O* allele; terming it "autologous chimerism," the authors postulate that if recombination or gene conversion occurs between these two *O* alleles such that the 261delG of the deletional allele is replaced with the otherwise consensus DNA in this area from the nondeletional *O* allele, then the mRNA from the (formerly) deletional *O* allele will lack the premature stop codon and translation can proceed normally. This hybrid transcript would not feature the crippling 802G>A SNP characteristic of the *O²* family of alleles, and thus a functional hybrid *A* allele could be produced. As this could be a somatic mutation occurring randomly during mitosis it would not be transmitted from generation to generation. This randomness would explain why the son with the *O^{1v}/O²-2* genotype produced an apparent A_{el} phenotype, whereas his father with basically the same genotype did not. It should be noted that it still remains to be shown experimentally whether this appealing hypothesis is valid or not. The fact that other nondeletional *O* alleles carrying early (exon 2) or late (exon 6 or 7) nonsense mutations resulting in premature stop codons behave similarly to *O²* in that they can produce different phenotypic variants of A expression or no A at all favors a general explanation that can be applicable to any combination of a deletional and a nondeletional *O* allele.[9,34]

However, autologous chimerism, the potential process of creating a *trans-cis* hybrid and in this case a functional *A* allele, would not explain the suspected GTA activity in those rare individuals homozygous for nondeletional *O* alleles (*O²* is the only example so far) because identical alleles cannot overcome each other's problematic polymorphisms. Finally, it will be interesting to see whether a future *O* allele based on a *B* allele backbone results in a similar phenomenon with variable expression of the B antigen.

3. Do Group O Donors with an 802G>A O^2 Allele Produce A Antigens? Maybe not.

By the end of 2005, several types of studies had begun to describe the enigma of the O^2 allele: (1) studies based on samples referred to a reference laboratory because of an ABO discrepancy or suspicion of a weak A subtype phenotype,[9,29] (2) studies based on blood donors whose predonation samples created ABO typing discrepancies,[29,31] (3) studies of group O blood donors whose predonation samples did not feature ABO discrepancies,[29] and (4) in vitro studies of the expression and activity of the O^2 allele.[13,18,20,21,29]

As illustrated above, these studies came to different conclusions because of variable results concerning the activity and consequences of the O^2 allele and the encoded O_2 enzyme. In some cases the overall conclusion was that the O^2 allele was not enzymatically functional, whereas in other studies, there did appear to be both direct and indirect evidence for its weak enzymatic activity.

Yazer et al.[35] took a different approach to the O^2 allele when they studied its incidence and properties in labeled group O units that were available for transfusion from their blood banks in Pittsburgh, Pennsylvania, and Lund, Sweden. The genotypes of these group O donors were not known at the time of their donation. These investigators assayed a total of 779 group O units for the presence of an O^2 allele and found 40 donors (5.1%, allele frequency 2.6%) who were heterozygous for an O^2 allele. None of these units had caused ABO discrepancies either on automated ABO typing instruments used for routine donor typing or when the ABO type was performed manually after these units were identified. Unlike in the previous study of blood donors with an O^2 allele,[29] when three donors from Pittsburgh had adsorption–elution performed on their RBCs using a polyclonal anti-A reagent, anti-A was not recoverable in the eluate. The A antigen was not detected when some of these donor RBCs were subjected to a very sensitive flow cytometry assay capable of detecting the minute quantity of naturally occurring A antigen on B cells,[36] nor when the plasma from the O^2 donors was used to upload A antigens onto group O RBCs. In fact, the activity of the plasma-borne O_2 enzyme from these donors was not above the water blank baseline. However, although A antigens could not be directly demonstrated on these RBCs, on average the titer of anti-A_1 and in particular anti-A was clearly diminished compared with that from control O^1/O^1 sera.[35] Unfortunately, titers of other antibodies were not performed to exclude a global reduction in immunoglobulin titer, but again this appears unlikely to coincide with O^2 carrier status. Thus, as described earlier, the presence of A antigens on other tissues cannot be excluded.

The authors of several earlier studies that had shown some weak GTA activity of the O_2 protein speculated that despite the low level of A antigens expressed on these RBCs, it should be safe to transfuse them to group O and B donors who would be expected to have naturally occurring anti-A in their plasma.[29,31] Yazer et al.[35] provided some confirmation of this hypothesis by performing a retrospective analysis on 19 of the O^2 units that were transfused to recipients with naturally occurring anti-A. There were no reported immediate hemolytic events, and in six recipients for whom more detailed information was available, all six had the expected increase in their posttransfusion hemoglobin level. The biochemical markers of hemolysis were not ordered on any of the recipients after transfusion, which also indicates that the attending clinicians did not suspect hemolysis after transfusion of these O^2 units.[35]

In a small prospective study, so far only reported in abstract form, the investigators monitored the anti-A levels in group O recipients after transfusion of RBC units from group O donors positive for an O^2 allele (i.e., *O03* and *Aw08*).[37] They found no changes in these levels when pretransfusion and posttransfusion samples were compared. This further supports the notion that O^2 units do not pose a risk of hemolysis by introducing the A antigen into group O recipients.

Conclusions

Whether the O_2 protein has consistent GTA activity remains to be seen, and the factors that regulate any intrinsic GTA activity have not been elucidated. At most, the O^2-encoded protein might be able to produce a very weak A phenotype, although in many cases evidence for the presence of A antigens on the RBCs is indirect, through reduced anti-A_1 and anti-A levels on reverse typing. In other situations individuals with an O^2 allele do not seem to demonstrate even a weak A phenotype, nor weakened anti-A or -A_1 in plasma. In the meantime, the ABO system continues to serve as a wonderful model system for polymorphic carbohydrate biology, and the study

of the O_2 protein has shed some more light on the fascinating biology of the ABO glycosyltransferases and their underlying loci.

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