Variable Hemagglutination Reactions with *Ulex europaeus* Lectin and Group O Erythrocytes

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ABSTRACT

Group O red blood cells (RBCs) express the highest amount of H antigen of any other blood group and are therefore expected to demonstrate strong reactivity when tested with *Ulex europaeus* anti-H lectin, unless the RBCs are from individuals possessing the Bombay or para-Bombay phenotypes. We report several examples of group O RBCs not belonging to the aforementioned groups that consistently produced weak reactions with quality-controlled *Ulex europaeus* lectin.

ABBREVIATIONS: AHG - antihuman globulin, CH and RS - specimen identifiers, EDTA - ethylenediamenetetraacetic acid, MLS - medical laboratory science, PCR - polymerase chain reaction, RBC - red blood cell, QC - quality control.

INDEX TERMS: H antigen, hemagglutination, blood banking, *Ulex*, fucose, lectins.

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INTRODUCTION

The H antigen (CD173) is an integral part of the membranes of RBCs, endothelial cells, platelets, and epithelial cells. The antigen is formed by the action of the high-incidence gene *FUT1* located at 19q13.33, which elicits the production of alpha-2-L-fucosyltransferase to synthesize L-fucose to type-2 precursor chains on the RBC membrane, and acts in concert with the closely-linked *FUT2* (secretor) gene to synthesize L-fucose to type-1 precursor chains in secretions. The H antigen serves as the precursor to both the A and B antigens and provides a scaffold for additional fucose residues that comprise the Le^a and Le^b antigens. Deficiencies in expression of the H antigen have been observed in the Bombay and para-Bombay phenotypes. H-antigen expression on the RBC can be detected through hemagglutination techniques using human-source allo-anti–H antibodies. However, due to the rarity of the phenotypes capable of producing these allo-anti–H antibodies in the general population, anti-H lectins can be employed. Lectins are nonimmune proteins or glycoproteins derived from plant or animal sources that are capable of producing hemagglutination reactions similar to RBC antibody-antigen reactions.

Because the *FUT1* gene is present in over 99.99% of the population, the H antigen is expressed on the RBCs and various tissues of most individuals. The autosomal recessive Bombay phenotype (O_h), first reported by Bhende et al,[1] arises from mutations in both *FUT1* and *FUT2*, which fail to produce the functional fucosyltransferases necessary to produce H antigens on RBCs and in secreted substances. The Bombay (*h/h*) genotype is ethnically restricted to subcontinental India.[2]

The para-Bombay phenotypes can exhibit total or partial H deficiency. A silenced *FUT1* gene with an active *FUT2* gene can lead to total H deficiency. Partial H deficiency ($H+^w$) can arise from a mutated *FUT1* gene with or without an active *FUT2* gene, in which case the mutated *FUT1* gene produces low levels of alpha-L-fucosyltransferase and causes inefficient synthesis of L-fucose to the precursor substances, leading to weak H-antigen expression on the RBC.[5]

Over 25 *FUT1* silencing or weakening mutations have been reported in various populations, although these mutations appear to be sporadic and not ethnically restricted as with the Bombay phenotype.[4,5] Lack of H-antigen expression does not appear to compromise the function, integrity, or survival of the RBC. An increased risk of disease or infection caused by the lack of this antigen has not been reported.[6] A rare leukocyte adhesion deficiency, LADII or CDGII, is associated with H deficiency; however, this is caused by changes in the GDP-fucose transporter gene (*SLC35C1*); the lack of H expression is a byproduct of the gene change and is not a cause of the defect in leukocyte adhesion. Individuals who lack a functional *FUT2* (secretor) gene are termed *nonsecretors* and cannot generate soluble H antigen in secretions, though the *FUT1* gene may be functionally normal. Lack of soluble H antigen in mucosal secretions is associated with resistance to certain strains of Norovirus.[7]

A specific mutation, *FUT1* 349C>T, is responsible for H-antigen deficiency observed in White populations in the Cilaos area of Reunion Island.[3] This mutation produces weak H-antigen expression on the RBC, and homologous titers of anti-H antibodies that react with all RBCs except O_h Bombay cells have been found in the sera of these individuals.[3]

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The allo-anti–H antibodies found in the sera of O_h Bombay individuals may cause acute intravascular hemolytic transfusion reactions. The anti-H antibodies present in the para-Bombay phenotypes can be reactive at body temperature but are usually weaker than the antibodies found in the Bombay phenotype. A weakly reactive, clinically benign H-like antibody (anti-IH) can be detected at low temperatures in the sera of most individuals with normal H-antigen expression. Anti-H antibodies have also been detected in the serum of oxen, goats, chickens, eels, cats, dogs, pigs, sheep, and rabbits.[8] However, antibodies from these sources have been difficult or impractical to procure.

Use of Lectins for H-Antigen Detection

The first lectin to demonstrate hemagglutinating properties was prepared in 1888 by Stillmark from the seeds of the castor-oil plant *Ricinus cummunis*.[9] Lectins specific to the H antigen were first extracted from the seeds of flowering legumes *Cytisus sessilifolius*, *Laburnum alpinum*, and *Lotus tetragonolobus* by Renkonen in 1948.[10] Then in 1952, Cazal and Lalaurie discovered the anti-H specificity of lectins in 3 species of gorse, thorny evergreen shrubs native to western Europe and northwest Africa.[11] Gorse, genus *Ulex*, also belongs to the legume family *Fabaceae*.

Lectins with H-antigen specificity can also be derived from the European eel *Anguilla anguilla*, a species that has sharply declined since the 1970s and is now critically endangered; from the ornamental flowering spindle tree *Euonymus europaeus*, a plant that also serves as a source of lectin specific to the B antigen; from the roots of spiny restharrow *Ononis spinosa*, a European plant that was historically used in the processing of bulat steel in medieval Russia; and also from the fruit pulp of *Clerodendrum viscosum,*[8] a tropical legume commonly used in Ayurveda and Unani traditional medicine.[12] However, the hemagglutinating properties of H-specific lectins derived from various plant sources are variable.[13-15] Extracts from various species of fungi including *Pleurocybells porriagens*, *Naematoloma sublateritium*, and *Pholiota squarrosa* have also produced anti-H reactivity.[9] Today, commercially available anti-H lectin is most often derived from the seeds of the common gorse *Ulex europaeus*.

Group A_1B and A_1 RBCs are typically not agglutinated or only weakly agglutinated by *Ulex europaeus* lectin because most of the H antigens on these cells have been converted to A_1, A, and/or B antigens and the relatively few existing, unconverted H-antigen sites on these cells are not exposed. Because group A_2 and B cells have fewer converted H antigens than A_1B and A_1 cells, 1+ to 2+ reactivity is typically observed when these cells are tested.[16] Group O cells express the highest amount of H antigen and will, therefore, typically demonstrate the strongest reactivity with anti-H lectin.[16]

During a routine laboratory classroom experiment within a medical laboratory science (MLS) program, it was noted that a specimen of group O RBCs (specimen CH) consistently produced reactions with expired, but acceptable, quality-controlled (QC) Ulex europaeus lectin that were much weaker than expected. To conserve money, expired reagents that pass QC parameters may be used to provide educational experiences for MLS students. A separate specimen from CH was drawn and tested with an in-dated and QC lot of Ulex europaeus lectin, and it produced the similarly weak results. This prompted our investigation into the underlying cause of the unexpected reactivity, in addition to running a comparison of the outdated and in-dated reagents. In this study, we report several examples of group O RBCs not belonging to the Bombay or para-Bombay phenotypes that demonstrated unexpectedly weak reactivity with QC Ulex europaeus lectin.

MATERIALS AND METHODS

Whole-blood samples were collected via venipuncture into EDTA tubes and refrigerated at 1-6° C for less than 72 hours prior to testing. ABO, Rh, H, and Lewis antigen testing was performed according to the standard operating procedures published in the manufacturer's reagent package inserts, using the traditional tube agglutination technique aided by a lighted hemagglutinin viewer. The antibody screen and cold panel were performed according to the standard operating procedures, using low ionic strength solution enhancement media and polyspecific antihuman globulin (AHG). The anti-H lectin (Immucor Inc) used in this study was a commercially prepared phytohemagglutinin of *Ulex europaeus* seeds for the detection of H substance.

The red cells were washed once in normal saline prior to preparing the cell suspension for testing. Blood group genotyping of the *ABO*, *FUT1*, and *FUT2* genes was performed by Grifols Diagnostics (San Marcos, TX). The genotyping methodology used specific primers located in flanking intron regions of the blood group genes to amplify relevant exons by polymerase chain reaction (PCR). The template used genomic DNA extracted from whole blood collected using EDTA. PCR-amplified exons subjected to bidirectional DNA sequence analysis used standard Sanger dideoxy chemistry. Seqscape software (ABI) was used to analyze the sequence data by comparing the obtained sequence to a reference sequence from the National Center for Biotechnology Information.

An additional sample from student RS was collected into an EDTA tube and phenotyping for Lewis and H antigens was performed with QC reagents for confirmation. Care was taken to ensure the blood was collected, stored, and tested according to the procedure published in the package insert, and tested by the same individual (with the one exception of RS performing Lewis and H testing

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on a second specimen) on the same day and with the same centrifuge, in order to reduce variability in test tube reading technique, specimen storage, and centrifugation. To further investigate the potency of the lectins, an additional 30 EDTA specimens from de-identified blood donors were tested with both the expired and in-dated lots of anti-H lectin. Lewis phenotyping was performed on all group O blood donor specimens to investigate whether secretor or nonsecretor status correlated with the strength of anti-H lectin reactivity. All specimens that initially demonstrated 2+ or weaker reactivity were repeated and confirmed to rule out technologist error or over-shaking.

The initial lectin typing experiment was performed for educational purposes only and as part of a scheduled immunohematology student laboratory activity. An outdated lot of commercially prepared anti-H lectin was used as part of a cost-saving strategy for the university's MLS program. The specimens tested include those submitted by MLS students after a routine ABO blood grouping activity, and also those that had been salvaged from a local blood center, which were not drawn for the purposes of the study. In-dated, commercially prepared lectin (also from Immucor Inc) was then used for confirmation testing and comparison. The study was approved by the Institutional Review Board.

RESULTS

Of the 38 Group O specimens tested, 9 produced reaction strengths of 2+ or weaker. Unusual agglutination in the reverse blood grouping by tube technique was initially seen (Table 1) with the sample from student CH. This was followed by microscopic investigation and confirmation of rouleaux formation. The saline replacement technique was employed to confirm the reverse type reactions were due to true agglutination and not pseudoagglutination caused by rouleaux. The patient's initial reactivity with antibody screening cells and the autocontrol displayed an unusual appearance at the 37 °C phase (Table 2). Rouleaux formation was also confirmed under microscopic examination but was not visible after 3 wash cycles with normal saline and addition of polyspecific AHG. Rouleaux formation can be caused by excess plasma proteins that were most likely sufficiently removed in this patient's test by the wash steps in the antibody screen. A cold panel with normal saline was performed on the sample from CH (Table 2) to investigate the presence of clinically benign anti-H or anti-IH antibodies, and the panel was negative will all cells tested at the immediate spin, room temperature, and 16 °C phases.

The unexpectedly weak (2+) reactivity with expired anti-H lectin and the CH specimen prompted repeated testing to rule out technologist error, and the repeated results were the same. A new specimen from CH was recollected into an in-dated EDTA tube and retested with a different lot (in-dated) of anti-H lectin that passed quality control, and the result was the same as with the expired lot (2+). Because weak H reactivity could be the result of the neutralizing effect of soluble H antigen present in the blood samples of secretors, Lewis antigen testing was performed (Table 1). The Lewis phenotype in an adult Le(a+b-) is indicative of nonsecretor (*se/se*) status, which was later confirmed by genotyping.

To further investigate the weak lectin reactivity, DNA sequencing of the *ABO*, *FUT1*, and *FUT2* genes was performed on a separately collected specimen from CH (Grifols Diagnostics, San Marcos TX). The ABO genotype results (Table 4) were reported as *ABO*O67, ABO*O03*. *ABO*O67* is a known allele of *ABO*O.01*, and *ABO*O03* is named *ABO*O.02.01* in International Society of Blood Transfusion nomenclature. Thus, CH's genotype is *O1/O2*, and the predicted phenotype is O. The *FUT1* gene was detected (Table 5), and no polymorphisms were detected in Exon 4. All reported Bombay and para-Bombay phenotypes are associated with polymorphisms in Exon 4.[17] The *FUT2* genotype results were as follows: *FUT2*171G, 216T, 428A, 739A, 960G*, (*se/se)* which results in the nonsecretor predicted phenotype, which is concordant with the Lewis type Le(a+b-).

To evaluate the potency of the lectins used, blood from a cohort of MLS students ($n = 12$) and donor blood samples ($n = 30$) were tested with the same reagents including both the expired and in-dated lots of lectins (Table 3). Out of the total group ($N = 42$), 38 were group O, 2 were group AB, one was group A, and one was group B. During the initial phase of testing, it was noted that an additional group O student (RS) also demonstrated similar unexpectedly weak reactivity with the lectins. RS submitted a second specimen several weeks later and performed the Lewis and H-antigen typing with the same vials. The results obtained with the second specimen were identical to the initial results recorded in Table 3.

DISCUSSION

It was expected that all of the Group O specimens tested would produce agglutination reactions of 3+ or stronger when tested with the in-dated and QC anti-H lectin. However, 9 of the specimens tested produced reactions that were weaker than expected. According to the package insert of the anti-H lectin, weaker than normal reactions may be seen with bacterial contamination of the specimen or reagent, aged blood specimens collected in EDTA stored longer than 7 days, inappropriate storage outside of 1 °C to 10 °C, too-heavy cell suspension, improper incubation time or temperature, improper calibration of the centrifuge, vigorous shaking of the test tube during examination, and other deviations from the recommended procedure.

Although both the in-dated and expired lots of anti-H lectin passed the quality control parameters and the strength of reactivity with both lots was the same, the

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expired antisera nearly consistently reacted one reaction strength lower with each RBC specimen tested. Nevertheless, it is of interest to note that the lectin that expired nearly 2 years prior to testing was still capable of producing robust reactions with the majority of the group O cells tested. According to the manufacturer's package insert, it is usually variation in reaction strength that carries the most significance in testing with the lectin, and comparison with control tests may be important in the interpretation of the test results.

The cause of the unexpectedly weak reactivity with the CH, RS, and 4 donor specimens has not been identified. Secretors of soluble H substances can exhibit weaker reactivity with the lectin if the soluble H antigens in the specimen neutralize the lectin and attenuate its ability to bind H antigen on the RBCs[18]; however, based upon Lewis phenotyping results, CH and 4 of the weakly reactive blood donors are (*se/se*) nonsecretors (Table 3). The rouleaux noted in the CH specimen could be a result of hyperproteinemia, which may have interacted with the lectin during testing. However, all RBC specimens tested were washed one time and resuspended to an approximate 2% to 4% cell suspension in saline prior to testing per the manufacturer's protocol.

According to a study in which H-antigen sites of native human O erythrocytes were converted *in situ* into blood-group-A determinants by transferring labelled N-acetylgalactosamine residues to the H antigens using human group A transferase, the number of H-antigen sites per group O RBCs is estimated to be 1.7-1.9 x 10^6.[18,19] The expression of H antigens on the RBCs of a neonate is considerably less than that of an adult, with only approximately 0.325×10^6 H-antigen sites expressed on the RBC of a group O neonate.[18] However, this study did not investigate variations in the development or expression of H antigen among group O individuals of different age groups, and the estimates published were based upon averages. While the ages of the blood donors in our study were not documented, none of the blood donor specimens included in our study could be from an individual under the age of 16 years. Our observations may indicate either the variability in the ability of certain lots of *Ulex europaeus* lectin to agglutinate H-positive RBCs from group O individuals, or the variability of H-antigen expression among group O individuals.

Reactions of 2+ or weaker were observed in 9 of the 38 (23.7%) group O specimens tested in this study. Lewis phenotyping was performed on all group O donor blood specimens as well as on the specimens submitted by RS and CH ($n = 30$). Of these, 18 samples were secretors, 9 were nonsecretors, and 3 were unable to be determined because the Le(a-b-) phenotype is the result of *le/le* genes and could be either a secretor or nonsecretor. Secretor status did not correlate with the strength of lectin reactivity observed in this study (Table 3).

CONCLUSION

Anti-H is expected to produce reactions with RBCs of various blood groups in the following order: O > A_2 > A_2B > B > A_1 > A_1B.[18] It was, therefore, unusual to observe the ability of the A_2 cells used for QC to consistently produce strong (3+) reactions with both the in-dated and expired lots of lectin, and yet several group O specimens tested produced 2+ or weaker reactions (Table 3). It is known that different lots of lectin can produce inconsistent agglutination reactions as the titer of the lectin produced from *Ulex europaeus* seed extraction is usually low and is subject to batch variation.[20] However, it is unexpected to encounter this variability within the same vial and same lot. Human-source allo-anti–H antibodies are difficult to procure as they are only produced by the rare phenotypes lacking H antigens previously discussed. Murine monoclonal anti-H antibodies have been produced as an H-antigen typing reagent,[20] but this specialty reagent is not commercially available or offered by any of the major manufacturers of blood typing antisera. The limitations of this study include our inability to compare the hemagglutination reactions produced by our lectins against human and murine monoclonal antigen-antibody reactions, and the inability to quantify the number of H-antigen sites or characterize their placement or expression on the RBCs from our sample pool. Our observations suggest there may be quantitative and/or qualitative differences in H-antigen expression among group O individuals not belonging to the Bombay or para-Bombay phenotypes or, alternatively, the natural variability of the lectin's hemagglutinating properties with different expressions of H antigen among these individuals.

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