AQP3 is a water and glycerol channel present on human erythrocytes and in various tissues. By protein and molecular biology analysis, two unrelated probands who developed alloantibodies to the high frequency antigen GIL were found to be AQP3-deficient. The defect is caused by homozygous mutation affecting the 5' donor splice site of intron 5 of the AQP3 gene. This mutation causes the skipping of exon 5 and generates a frameshift and premature stop codon. Functional studies by 90° light scattering using a stopped-flow spectrometer revealed the absence of facilitated glycerol transport across red cell membranes from the probands, but the water and urea transports were normal. Expression studies into COS-7 cells followed by flow cytometry analysis showed that only cells transfected with AQP3 cDNA strongly reacted with anti-GIL antibodies. These findings represent the first reported cases of AQP3 deficiency in humans and provide the molecular basis of a new blood group system, GIL, encoded by the AQP3 protein.

Integral membrane proteins that facilitate the transport of water or and solutes belong to the major intrinsic protein (MIP) family and are involved in many physiological processes and the pathophysiology of several clinical disorders (1–4). They are divided into three subgroups according to their sequences and function properties: (i) the aquaporins (AQP), permeable only to water; (ii) the glycerol facilitators, permeable only to glycerol (not present in mammals); and (iii) the aquaglyceroporins, which present a mixed selectivity. AQP1, the archetype of the MIP family selectively permeable to water (5), is present in various tissues including red blood cell (RBC) membranes from which it was first purified (6).

The high glycerol permeability of human RBCs is due to aquaglyceroporin AQP3 (7, 8), which is moderately permeable to urea (9–11). AQP3 is present in rat RBCs but absent from mouse RBCs (8). It is encoded by a single-copy gene composed of six exons distributed over 6 kilobases of DNA located on human chromosome 9p13 (12). The predicted protein of 292 residues is organized into six bilayer-spanning domains with the NH2- and COOH termini located intracellularly (13). On human RBCs, AQP3 presumably is glycosylated on all subunits at Asn-141, the putative N-glycosylation site located in loop “c” at the external face of the cell membrane (14).

So far, AQP1 and AQP3 are the only two known proteins of the aquaporin family identified in human RBCs. AQP1 expresses Colton blood group antigens (15, 16), the question was raised as to whether AQP3 might also be encoded by a blood group gene. We reasoned that if AQP3 carries a blood group specificity, the corresponding antigen should be of high frequency because AQP3 is a common protein of human RBCs. Accordingly, we performed an immunostaining analysis of human RBCs proteins from patients who had developed alloantibodies against high frequency antigens that caused delayed or severe hemolytic transfusion reactions. This approach led us to identify the first examples of AQP3null individuals and to define a new blood group system called GIL.

**EXPERIMENTAL PROCEDURES**

**Blood Samples**—Proband 1 is a white French woman born in 1925. She had 10 pregnancies before 1979 when an antibody against a high frequency antigen (GIL) reacting with all human RBCs except her own was identified following a hemolytic reaction that occurred during orthopedic surgery (17). Her RBCs were genotyped O, GIL-negative. Blood samples from proband 1 and her family were collected after informed consent at the Centre National de Référence pour les Groupes Sanguins (CNRGS, Paris, France). Proband 2 is an unrelated white American woman born in 1956. She had no previous history of blood transfusion. In 1979, the RBCs from her first child had a weakly positive direct antiglobulin test, but there was no clinical sign of hemolytic disease of the newborn (17). Her serum contains an anti-GIL antibody and she was phenotyped A, GIL-negative. Serum and DNA from proband 2 came from the frozen collection of the Bristol Institute for Transfusion Sciences (Bristol, UK).

**Reagents**—Modifying enzymes came from New England Biolabs (Hertfordshire, UK). Expand High Fidelity PCR, Titan One Tube RT-PCR systems, and N-glycosidase F (PNGase-F, 50 units/mg) were from Roche Molecular Biochemicals. Nucleotide primers were purchased from Genset (Paris, France). For primer designation, nucleotide (nt) position +1 was taken as the first nucleotide of the initiation codon of the human AQP3 cDNA clone (NCBI accession NM_004925). All reagents and peptide came from Sigma. AEBSF protease inhibitor was from Interchim (Montluçon, France). Rabbit polyclonal sera against human AQP1 (total protein) and the COOH-terminal region of rat AQP3 (residues 263–292) were characterized previously (7, 18).

**Immunoblot Analysis**—RBC membrane proteins were prepared by
hypotonic lysis in the presence of 0.5 mM AEBSF as described (19). After SDS-polyacrylamide gel electrophoresis, immunoblotting was performed with rabbit anti-hAQP3 (1:4000) or anti-rAQP3 (1:500) antisera as described (8). N-glycosidase F treatment of RBC membrane proteins was performed overnight at 37 °C according to manufacturer’s instructions.

**Amplification by Reverse Transcription Coupled to Polymerase Chain Reaction**—Total RNA extracted by the acid-phenol-guanidinium method (20) was used for the first PCR in the Titan One Tube reverse transcription polymerase chain reaction (RT-PCR) system between primers SP1 (nt −52 to −36) and ASP1 (nt 932–913) according to the manufacturer’s instructions with annealing temperature of 62 °C and elongation time of 45 s. The second PCR was performed with one fifthth of the first reaction in the same condition, except that the fourth step of the first cycle was omitted and the elongation temperature was 72 °C, using primers SP2 (nt −23 to −7) and ASP2 (nt 907–889) and Expand High Fidelity system. The PCR products were subcloned and sequenced on both strands using the Big Dye Terminator Cycle Sequencing ready Reaction Kit and analyzed on an ABI-Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Splice Site and Restriction Analysis**—Direct PCR amplification was carried out on genomic DNA between primers SP3 (5′-aaccagctgtaggccttc-3′, nucleotides −171 to −152 upstream exon 4) and ASP3 (nucleotides 807–788 in exon 6) using the Expand High Fidelity system according to manufacturer’s instructions with annealing temperature of 62 °C and elongation time of 1 min. PCR products were subcloned and sequenced as above. The AQP3(Δ5) mutation was detected by PCR restriction fragment-length polymorphism (PCR-RFLP). The 1027-bp PCR product was digested with 5 units of PmlI endonuclease and analyzed on a 1% (w/v) agarose gel. Each PCR reaction was performed in a total volume of 50 μl containing 100 ng of genomic DNA extracted from cells using a Wizard Genomic DNA purification kit from Promega (Madison, WI).

**Glycerol Transport by Stopped-flow Analysis**—Glycerol influx was induced by mixing a fresh or thawed RBC suspension (1.5% hematocrit) with an equal volume of a hyperosmotic solution of glycerol to produce a 100 mosmol/kg H2O inwardly directed osmotic glycerol gradient. The kinetics of RBC volume changes were followed by 90° light scattering using a stopped-flow spectrophotometer Biologic SFM3 instrument (Clax, France) as described (7). Glycerol efflux was induced by equilibrating the RBC suspension in 200 mosmol/kg H2O glycerol for 1 h prior to analysis (to insure that RBCs would be loaded with glycerol even if a glycerol proteic pathway were absent) and mixing them with a 200 mosmol/kg H2O mannitol solution in isoosmotic conditions to produce a 100 mosmol/kg H2O outwardly directed glycerol gradient. Inhibition studies were performed by incubating RBCs for 10 min with 0.1 mM CuCl2, and in some cases, 1 mM Gly-Gly-His peptide was added to the samples to induce the glycerol uptake or efflux rate constants (k, in s⁻¹).

**Cell Culture, Transfection, and Flow Cytometry**—Transient expression of AQP3 in COS-7 cells obtained from American Type Culture Collection (Manassas, VA) was performed by DEAE-dextran transfection with the full-length hAQP3 cDNA (kindly provided by Dr. Ishihashi) subcloned into the pcDNA3 expression vector (Invitrogen, Leek, The Netherlands) as described (21). Four days after transfection, 2.5 × 10⁵ cells resuspended in LiSS/glycine buffer (NaCl 30 mM, glycine 237 mM, NaH2PO4 1.3 mM, NaHPO4 1.2 mM, pH 7.2) supplemented with 1% (w/v) bovine serum albumin and goat serum (1:20) were incubated at 37 °C with all-trans-retinol (1 μM in LiSS/glycine/bovine serum albumin buffer). After 20 min, the cells were washed and stained with phycoerythrin-conjugated F(ab)2 fragments of goat anti-human IgG (1:40). Flow cytometry analysis (FACScanibur, BD Biosciences) was performed as before (20). Occasionally, the cells were fixed first in HCHO 1% (v/v) for 15 min at room temperature and permeobilized with 1% (w/v) N-Octylglucoside before hybridization with anti-rAQP3 and fluorochrome labeling with a second antibody.

**RESULTS**

**Identification of AQP3Null Probands and Characterization of the AQP3 Gene Mutation**—RBCs from 24 individuals who developed alloantibodies directed against unidentified high frequency antigens were analyzed by immunoblotting with the anti-rAQP3 antibody, which cross-reacts with human AQP3 (hAQP3) protein (7). RBC membranes from one of these individuals, proband 1, lacked the deglycosylated (25 kDa) and glycosylated (45 kDa) AQP3 protein forms visualized in control RBCs (Fig. 1A). Proband 1 exhibited the rare phenotype called GIL-negative, and her serum contained an alloanti-GIL (17). RBCs from a second GIL-negative individual (proband 2) were also found AQP3-deficient (Fig. 1A). As control, all tested RBCs showed a normal expression of AQP1 protein (not shown).

To determine the molecular basis of AQP3 deficiency, AQP3 transcripts from proband 1 and a control were amplified by RT-PCR (712 bp). The same transition was found in the alternative splicing of exon 5, was identified in proband 1 (Fig. 1B). As control, all tested RBCs showed a normal expression of AQP3 protein (not shown). The AQP3Δ5 transcript would potentially encode a truncated polypeptide of 281 amino acid residues (versus 292) with a new COOH-terminal region generated by a frameshift and premature termination. The predicted AQP3(Δ5) polypeptide would lack amino acids 165–237 (encoded by exon 5) overlapping the fourth and fifth transmembrane domains and the “e” extracellular loop of the hAQP3 protein (see Fig. 2B).

To confirm this finding and screen the AQP3Δ5 silent allele in the proband 1 family and in random blood donors, and because the g to a substitution correlated with a PmlI restriction site polymorphism, we were able to develop a PCR-RFLP genotyping assay (Fig. 2A). PCR-RFLP analysis indicated that the 1027-bp product amplified from AQP3-positive individuals was cleaved into fragments of 841 and 186 bp (Fig. 2A and 3, top panel). In contrast, the PCR product amplified from proband 1 (Fig. 3) and also from proband 2 (not shown), carrying the AQP3Δ5 mutation, remained uncut, demonstrating the homozygosity of this mutation in both individuals. Examination of the proband 1 family for the AQP3Δ5 mutation indicated that her sister (I.2) and 10 children (II.1 to II.10) were heterozygous, as a combination of digested fragments of 1027,
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Fig. 2. Identification of a splice site mutation in AQP3*Δ15. A, partial schematic representation of the AQP3 gene. The introns (I) and exons (E) around the mutation are shown as well as the SP3 and ASP3 primer pair used to amplify the 1027-bp PCR products. Left, sequence electropherograms of the 3′-intron exon 5 boundaries showing that the invariant g nucleotide of the donor splice site of intron 5 in the normal AQP3 allele (top) is substituted by an a in the AQP3*Δ15 null allele (bottom), thus destroying the PmlI restriction site overlapping the 3′-intron exon 5 junction. Right, PCR-RFLP genotyping of the AQP3*Δ5 mutation by PmlI endonuclease digestion. B, sequence alignment of normal AQP3 polypeptide (upper sequence in bold) with the AQP3*Δ5 spliceform (lower sequence) deposited to the GenBankTM/EBI Data Bank (accession no. AJ493597). The missing amino acids encoded by exon 5 are indicated by dots, and the new reading frame is underlined. The limits of coding exons, membrane-spanning regions, and putative N-glycosylation site (Aan-141) are indicated by arrowheads, gray boxes, and an asterisk, respectively.

Fig. 3. AQP3 genotyping and immunoblot analysis. Family tree of proband 1. Proband I.1 is indicated by an arrow. The top panel shows the PCR-RFLP genotyping performed on family members. The two lower panels show the same membrane of RBC membrane proteins first immunostained with the anti-aAQP3 serum and then washed and immunostained with the anti-hAQP1 serum. Glycosylated forms are indicated by glyAQP. The asterisk represents a nonspecific band.

from the glycerol influx with a concomitant water flow (Fig. 4B, control). Conversely, only a very low decrease in light intensity was detected with RBCs from probands, indicating that the glycerol permeability due to the facilitated pathway was absent. Rate constants (k) of the apparent cell volume swelling due to the glycerol uptake were 0.127 ± 0.033, 0.003 ± 0.001, and 0.001 s⁻¹ at 22 °C for control, proband 1 and proband 2 RBCs, respectively. Interestingly, the glycerol permeability of two heterozygotes (I.2 and II.1) was approximately half that of the controls with rate constants of 0.051 and 0.044 s⁻¹, respectively (not shown), in accordance with the reduced AQP3 expression protein detected on an immunoblot (Fig. 3). Additionally, the glycerol transport of control RBCs incubated with CuCl₂, an inhibitor of the glycerol-facilitated pathway (22), was drastically inhibited (Fig. 4A, control + Cu²⁺) and was equivalent to proband RBC glycerol transport. However, when the copper chelator peptide Gly-Gly-His was added after incubation with CuCl₂, the glycerol transport across control RBCs recovered (Fig. 4A, control + Cu²⁺ + His). The measurements performed at different temperatures allowed us to calculate the activation energy (Ea) of the glycerol transport (Fig. 4B, inset), which were 3.6 and 12.1 kcal/mol for control and proband 1 RBCs, respectively, thus excluding another glycerol channel in human RBCs.

Glycerol efflux was followed in isoosmotic conditions (RBCs loaded with glycerol, generating an outwardly directed glycerol gradient) with control and proband 1 RBCs. The efflux of glycerol induces a shrinkage of control RBCs illustrated by an increase of the scattered light intensity (Fig. 4C). In contrast, the scattered light intensity remained unchanged with proband 1 RBCs and with control RBCs incubated first with copper; the glycerol transport recovered in the latter sample after the addition of histidine peptide (Fig. 4C). Control experiments further indicated that AQP1-mediated water transport and
hUT-B1-mediated urea transport of proband RBCs were normal (not shown).

**AQP3 Carries the Blood Group GIL Specificity**—Since both AQP3-deficient probands were GIL-negative and had alloantibodies to the GIL antigen in their serum (17), flow cytometry analysis was performed to confirm that proband sera reacted with control RBCs (group O, GIL-positive) but not with proband 1 RBCs (O, GIL-negative), as shown in Fig. 5. As expected, proband sera reacted strongly with control RBCs even at low dilution and when the RBCs were trypsin- or papain-treated (not shown), whereas no increase of the fluorescence was seen with enzyme-treated proband 1 RBCs, which lacked the AQP3 protein. Thus, the development of antibodies in proband sera after transfusion or pregnancy, as well as the absence of AQP3 in their RBC membranes seen by immunoblot, strongly suggested that both events were related.

To provide the formal proof that AQP3 carried the blood group GIL specificity, transient expression of AQP3 protein in COS-7 cells was performed by cloning AQP3 cDNA into the human cytomegalovirus promoter-controlled expression vector, pcDNA3. Flow cytometry analysis of AQP3 transfectants was performed with rabbit anti-rAQP3 serum and anti-GIL antibodies. The anti-rAQP3 reacted with the AQP3 transfectants but not with parental COS-7 cells, whereas neither type of cells reacted with a pre-immune rabbit serum (not shown). Moreover, immunoblotting with the anti-rAQP3 showed that the glycosylated (45 kDa) and deglycosylated (25 kDa) forms of the AQP3 protein were present in AQP3 transfectants but not in parental COS-7 cells (Fig. 6, left). Further studies with human sera from both probands reacted with AQP3-COS-7 but not with parental COS-7 cells, whereas neither type of cells reacted with a pre-immune rabbit serum (not shown). In contrast, a human serum sample devoid of anti-GIL antibody used as control reacted neither with AQP3 COS-7 nor with COS-7 parental cells. These findings indicate that the pcDNA3-AQP3 construct directed the synthesis of a AQP3 cell surface protein recognized by sera containing alloanti-GIL antibodies developed by GIL-negative probands 1 and 2 who were genotyped AQP3null in these studies.

**DISCUSSION**

This is the first report of human AQP3 deficiency; it was discovered by probing, with a rabbit serum against the AQP3 protein, RBCs from two unrelated patients who developed alloantibodies against high frequency blood group antigens following transfusion or pregnancy. The two probands belong to the rare blood group phenotype called GIL-negative, found among five cases published previously (17). Although both probands, who exhibited the same molecular genetic basis, were apparently unrelated, it could not be determined whether they
might have a common ancestor. Transcript sequencing and genomic analysis indicated that the lack of AQP3 protein was caused by a single nucleotide substitution at the conserved 5' donor (gt→at) (where italics represent the location of mutated nucleotides) splice site of intron 5 of the AQP3 gene, which resulted in exon 5 skipping, frameshifts, and premature termination of translation. PCR-RFLP genotyping further indicated that the probands were homozygous for the AQP3(Δ5) mutation, whereas the sister and 10 children of proband 1 were heterozygous. In addition, there was a clear gene dosage effect, whereas the sister and 10 children of proband 1 were heterozygous. In addition, there was a clear gene dosage effect, whereas the sister and 10 children of proband 1 were heterozygous.

These results suggest that the AQP3(Δ5) isoform is either not functional or not expressed at the RBC membrane. The potential reduction of water and urea movements in proband RBCs could not be estimated because AQP1 and hUT-B1 were normally present and responsible for at least 90% of the water and urea movements across RBCs, respectively. Residual mercury-sensitive water permeability detected in Coltonnull RBCs, which lack AQP1, was previously accredited to AQP3 (7). The high activation energy of glycerol permeability found in AQP3null cells suggests the absence of another glycerol channel. However, normal mice RBCs are deficient in AQP3 expression but, surprisingly, showed a phloretin-sensitive glycerol permeability as deduced from analysis of in-and-out facilitated glycerol fluxes.

Another important result of these studies is the conclusive demonstration that AQP3 carries blood group GIL specificity: (i) AQP3null individuals belong to the GIL-negative phenotype; (ii) the rabbit anti-AQP3 antibody reacts on immunoblots with GlyAQP3 and AQP3 components present in GIL-positive but not GIL-negative RBCs; (iii) the glycerol permeability of GIL-negative RBCs is severely reduced; (iv) allanti-GIL in probands' sera reacted by flow cytometry with AQP3-positive but not AQP3null RBCs; (v) AQP3-transfected cells reacted with anti-GIL present in the serum of GIL-negative individuals. Altogether, these findings provide the necessary information to define the molecular basis of a new blood group system, GIL, encoding the aquaglyceroprotein AQP3. Accordingly, it can be deduced that the GIL locus is located on the short arm of chromosome 9p13 and is the second blood group on this chromosome with the ABO locus located on the long arm at 9q34.

As AQP3 protein has a wide tissue distribution in the epithelial cells of kidney, airways, and skin and in immature dendritic cells, suggesting a role in water reabsorption, mucosal secretions, allergic diseases, and cell volume regulation (24–27), it is surprising that the AQP3null individuals identified here, or the reported GIL-negative donors (17), did not suffer any obvious clinical syndromes. Moreover, AQP3, which efficiently transport glycerol, might be implicated in energy metabolism (28). The absence of clinical disorders in normal life conditions has also been observed in Coltonnull patients who are deficient for AQP1 (29, 30). It is only under stress conditions that a defective urinary concentrating ability (31) and a decrease in pulmonary vascular permeability (32) have been detected in Coltonnull individuals. Thus, detailed clinical studies of AQP3null individuals, like those conducted with AQP1null individuals, should provide useful information on the biological role of the AQP3 protein. Interestingly, investigations of AQP3null mice revealed a marked polyuria, urine hypoosmolarity, and partial response to V2 agonists representative of a nephrogenic diabetes insipidus (23, 33). A hypotriglyceridemia was also detected in AQP3null mice. Additionally, AQP3 might be involved in skin hydration, as a defect of stratum corneum hydration has been described in these animals (27).

An important benefit that emerges from these studies is the identification of AQP3null mutation in humans and the characterization of the novel blood group system GIL. These findings should stimulate future investigations in RBCs and various organs to probe the physiological role of this aquaporin and to understand the significance of its channel selectivity.

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REFERENCES