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Molecular Basis for Lewis α(1,3/1,4)-Fucosyltransferase Gene Deficiency (FUT3) Found in Lewis-negative Indonesian Pedigrees*

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The Le[a] and Le[b] human blood group antigens are synthesized in tissues producing exocrine secretions; they also circulate in plasma, where they are adsorbed by erythrocytes. They are synthesized by two fucosyltransferases, encoded by *Lewis* (FUT3) and *secretor* (FUT2) loci. This genetic model has been challenged because some erythrocyte Lewis-negative individuals express Lewis antigens in saliva. To define the molecular basis of this apparent discrepancy, we sequenced FUT3 in Lewis-negative individuals. We identified two single base pair changes. One, termed L1, yields a Leu-20 → Arg substitution in the enzyme's transmembrane domain. When expressed in COS-7 cells, enzyme substrate affinities are essentially identical to those of wild type. However, the mutant enzyme is found at substantially reduced levels in transfected cells. This suggests that the L1 mutation may alter the Golgi membrane anchoring of the enzyme. It was found alone in double dose in 10 of 30 erythrocyte Lewis-negative individuals, nine of whom express Lewis antigens in saliva. Therefore, L1 can account for erythrocyte/saliva-discrepant Lewis typing results. The L2 mutation creates an Ile-356 → Lys change in the enzyme's catalytic domain and inactivates the enzyme. It was found in double dose in 18 of 19 individuals bearing the double erythrocyte and salivary Lewis deficiency and can account for this phenotype.

In 1946, the first anti-Le[a] antibody,[1] agglutinating erythrocytes of about 20% of the population, was described (1). Two years later, an anti-Le[b] antibody agglutinating erythrocytes of the majority of Le[a]-negative individuals was characterized (2). Since then, three red cell phenotypes have been identified in Caucasians: Le(a+b−) 20%, Le(a−b+) 70%, and Le(a−b−) 10% (3). These red cell phenotypes are not secondary to two *a* and *b* alleles at a single *Lewis* locus as was originally thought, but are the result of epistatic interactions of the products of two loci, FUT2 or salivary secretor of ABH (*Se,se*) and FUT3 or Lewis (*Le,le*), encoding the secretor α(1,2)-fucosyltransferase and the Lewis α(1,3/1,4)-fucosyltransferase, respectively, in tissues producing exocrine secretions.

The genetic control of Lewis antigens expression is complex, because FUT2 and FUT3 each encode a different fucosyltransferase, and the final oligosaccharide products are the result of the competitive interactions of these enzymes on the same oligosaccharide acceptors of type 1 Galβ1→3GlcNAcβ1→R and type 2 Galβ1→4GlcNAcβ1→R.

The Le[a] epitope, Galβ1→3(Fucα1→4)GlcNAcβ1→R, results from the transfer of fucose in α1→4 linkage onto type 1 by the Lewis enzyme. This terminal product cannot be further glycosylated and is the main oligosaccharide antigen found in Lewis-positive ABH nonsecretor individuals. It is responsible for the Le[a] phenotype in saliva and for the Le(a+b−) phenotype on erythrocytes.

The Le[b] epitope, Fucα1→2Galβ1→3(Fucα1→4)GlcNAcβ-1→R, is the product of the transfer of two fucoses onto the same type 1 precursor, one in α1→2 linkage onto the terminal galactose transferred by the secretor enzyme and the other in α1→4 linkage onto the subterminal *N*-acetylglucosamine transferred by the Lewis enzyme. It is also a terminal product and the main epitope found in Lewis-positive, salivary ABH secretor individuals. It is responsible for the Le[b] phenotype in saliva and for the Le(a−b+) phenotype on erythrocytes.

In Lewis-positive, salivary ABH secretor Caucasians, the secretor enzyme is highly active and transforms most of type 1 into H type 1, which is in turn transformed into Le[b] by the Lewis enzyme, giving the Le(a−b+) phenotype on erythrocytes. Very few precursor chains are left to make Le[a], some of which can be detected in saliva, but usually not on erythrocytes. However, a fourth erythrocyte phenotype Le(a+b+) has been found in Lewis-positive, salivary ABH secretors, among Australian aborigines (4), Japanese (5), Chinese (6), and Polynesians (7); it is due to the existence of a secretor weak enzyme (Se^w) leaving a larger proportion of unsubstituted precursor chains which can be transformed into Le[a] by the Lewis enzyme (7).

The cDNA encoding the Lewis α(1,3/1,4)-fucosyltransferase has been cloned (Fuc-TIII; Ref. 8) and registered in the Genome Data Base as FUT3. The sequences of FUT3 alleles of Lewis-negative individuals in Sweden and Japan have revealed the existence of at least three point mutations that lead to a loss of

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[1] The abbreviations used are: Le[a], Galβ1→3(Fucα1→4)GlcNAc; Le[x], Galβ1→4(Fucα1→3)GlcNAc; α(1,3/1,4)-fucosyltransferase, GDP-fucose:β-D-*N*-acetylglucosaminide 3/4-α-L-fucosyltransferase; H type 1, Fucα1→2Galβ1→3GlcNAc; H type 2, Fucα1→2Galβ1→4GlcNAc; sialyl-Le[a], NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAc; sialyl-Le[x], NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAc; PCR, polymerase chain reaction; bp, base pair(s); RFLP, restriction fragment length polymorphism; ASO, allele-specific oligonucleotide; *H* gene, FUT1; *Se* gene, FUT2; *Lewis* gene or Fuc-TIII, FUT3; Fuc-TIV, FUT4; Fuc-TV, FUT5; Fuc-TVI, FUT6.

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enzyme activity in transiently transfected cells. The first, Thr-105 → Met, was found to be homozygous in 5 of 18 Swedish blood donors of erythrocyte Le(a−b−) phenotype (9). A second mutation, Gly-170 → Ser, was reported to be homozygous in 4 of 8 Lewis-negative individuals by two Japanese teams (10, 11). Finally, a third missense mutation, Asp-336 → Ala, was found on a heterozygous girl, who had inherited the Gly-170 → Ser mutation from her father and the Asp-336 → Ala mutation from her mother (11). These last two teams also reported another point missense mutation in the transmembrane region of the enzyme, changing Leu-20 → Arg, but they detected Lewis enzyme activity in COS cells transfected with this FUT3 allele and concluded that this mutation was irrelevant for enzyme activity (10, 11).

The Lewis α(1,3/1,4)-fucosyltransferase enzyme is expressed in epithelial cells producing exocrine secretions and its oligosaccharide final products (Le^a, Le^b, Le^x, or Le^y) are shed into secretions, mainly as glycoproteins and into plasma, mainly as glycolipids, but there is no detectable Lewis enzyme activity in plasma, irrespective of the Lewis phenotype of the individual. In contrast, large amounts of plasma α(1,3)-fucosyltransferase encoded by the FUT6 gene (Fuc-TVI; Ref. 12) are found in plasma.

In a large series of plasma α(1,3)-fucosyltransferase-deficient individuals in Indonesia, we found that this enzyme is inactivated by two single base pair mutations in the coding region of FUT6. A missense mutation yields Glu-247 → Lys and a nonsense mutation yields Tyr-315 → stop, which truncates the COOH terminus of the enzyme by 45 amino acids. Each mutation, when present in double dose, produce a plasma α(1,3)-fucosyltransferase-deficient phenotype. Furthermore, the mutation Glu-247 → Lys in double dose cosegregated with the plasma enzyme defect in the five Indonesian pedigrees tested (13).

The genes of three human α(1,3)-fucosyltransferases (FUT3, FUT5 also known as Fuc-TV (14), and FUT6) share 85–90% identity (12), they are expressed in different tissues and have different acceptor specificity patterns. FUT3 and FUT6 are closely linked, within 13 kilobases of each other on the short arm of chromosome 19 (15). Typing of 198 Indonesian random blood donors has shown that 17 of 18 plasma α(1,3)-fucosyltransferase-deficient individuals are also Lewis-negative on red cells. These data are compatible with FUT3 and FUT6 being in linkage disequilibrium (13).

We have now studied the five original plasma α(1,3)-fucosyltransferase-deficient families (13), plus three new Indonesian families ascertained by a propositus with the FUT6 defect, for both FUT6 and FUT3 mutations and corresponding phenotypes. The molecular basis of this FUT3 defect is shown here to be a consequence of two point mutations in the coding region of FUT3. One, termed L1, is identical to the Leu-20 → Arg mutation reported above (10, 11). The other, termed L2, is a new missense mutation, in the catalytic domain of the protein. We show here that the L1 mutation in double dose produces weak Lewis enzyme activity characterized by erythrocyte Le(a−b−) phenotype with secretion of Lewis antigens in saliva; the L2 mutation, in double dose, inactivates the enzyme giving the Lewis-negative phenotype on both erythrocytes and saliva.

EXPERIMENTAL PROCEDURES

Plasma α(1,3)-Fucosyltransferase-deficient Pedigrees—Eight deficient plasma α(1,3)-fucosyltransferase blood donors from Jakarta were selected for this study. Family trees were drawn giving a serial numeric order to each individual, starting from the oldest members. Missing numbers in the pedigrees indicate that the individuals were not available or that they refused to participate in the study. Pedigrees were validated by segregation of HLA haplotypes using serological and RFLP analysis by the Tissue Typing Department of the Red Cross Blood Transfusion Service (Sydney, Australia). Transformation by Epstein-Barr virus was made on frozen buffy coats (13), and the whole genomic DNA was extracted (16) and dissolved at 700 μg/ml in Tris-EDTA, pH 7.5 (20 mM Tris, 5 mM EDTA).

Erythrocyte Phenotypes—Routine red cell agglutination tests were performed on fresh blood samples with polyclonal anti-A, anti-B (CSL Ltd., Melbourne, Australia), lectins anti-H *Ulex europaeus* (Vector, Burlingame, CA) and anti-A1 *Dolichos biflorus* (Gamma Biologicals, Houston, TX) and six anti-Le^a and anti-Le^b including the monoclonal antibodies from the Second International Workshop on Monoclonal Antibodies Against Human Red Blood Cells (Lund, 1990) (17) and goat polyclonal antibodies (Ortho Diagnostics, Raritan, NJ).

ABH and Lewis Salivary Phenotypes—Salivas from all family members were collected, boiled for 10 min within 2 h of collection, and centrifuged, and the supernatants were kept frozen until tested. Tests were performed by inhibition of the corresponding erythrocyte agglutination reactions with *U. europaeus*, anti-A, anti-B, anti-Le^a, and anti-Le^b.

Molecular Cloning of FUT3 Alleles and Corresponding FUT3 Expression Vectors from a Lewis-deficient Individual—PCR was used to amplify the coding and 3′ flanking regions of the FUT3 gene of the B4 individual, who is plasma α(1,3)-fucosyltransferase-deficient and Lewis-negative on erythrocytes and saliva. Genomic DNA (200 ng) was amplified in a DNA Thermal Cycler (Perkin-Elmer), using a Gene-Amp PCR kit (Perkin-Elmer) and 0.5 μM of each primer. The PCR program included a 5-min, 94 °C initial denaturation step, followed by 30 cycles consisting of 1.5 min at 94 °C and 3.5 min at 72 °C. The sense primer of FUT3 anneals to nucleotides 1–31, beginning immediately with the ATG codon. The sequence of this primer contains additional nucleotides (lowercase) at its 5′ end including an *Eco*RI restriction site (underlined): 5′-gcgcgaattcATGGATCCCCTGGGTGCAGCCAAGCCACAAT-3′. The antisense primer is complementary of nucleotides 1125–1096, and the extraneous nucleotides (lowercase) at the 5′ end contain an *Xba*I restriction site (underlined): 5′-gcgctctagaGGCAGATGAGGTTCCCGGCAGCCCAGGCAC-3′ (sequence numbering according to Ref. 8).

The PCR product was digested with *Eco*RI and *Xba*I, and the 1125-bp fragment was cloned between the *Eco*RI and *Xba*I restriction sites of the mammalian expression vector pCDNAI (Clontech). Twelve representative plasmids were selected and both strands of their inserts were sequenced in their entirety, by dideoxy chain termination, using T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.). We first used oligonucleotides corresponding to flanking plasmid sequences, and subsequently we used primers corresponding to internal sequences of the wild type FUT3 gene (8).

The wild type pFUT3 vector, containing a sequence of 1125 bp, was prepared by amplifying the wild type FUT3 cDNA (8). To diminish the risk of PCR errors, 50 ng of cloned wild type cDNA were used as template for 20 cycles of PCR with the same program. After amplification, the fragment was digested with *Eco*RI and *Xba*I and cloned in pCDNAI, in the same way as the mutated clones. Inserts of two representative clones of pFUT3 were sequenced to confirm the absence of PCR-induced mutations. One plasmid containing mutations L1 and L2 (see "Results"), but otherwise identical to a wild type pFUT3 allele, was designated pL1,L2. Constructions of other mutant FUT3 expression vectors were accomplished via restriction fragment interchange. Vector pL1, containing only the L1 mutation, was constructed by replacing the wild type *Afl*III restriction fragment encompassing this sequence position in pFUT3, with the corresponding *Afl*III fragment isolated from the plasmid pL1,L2. The *Afl*III restriction enzyme cuts at position 1532 in the plasmid vector, upstream of the cytomegalovirus promotor and at position 656 in the coding region of the gene, after the L1 mutation. Vector pL2 containing only the L2 mutation was prepared by insertion of the wild type *Afl*III restriction fragment into the *Afl*III-digested pL1,L2 vector. The structure of each of these constructs was confirmed by restriction endonuclease digestion, by DNA sequencing across the restriction sites used for cloning, and by DNA sequencing across each polymorphic nucleotide position (Fig. 1).

Detection of the Single Base Differences Giving L1 and L2 Alleles of the FUT3 Gene, Using Allele-specific Oligonucleotides (ASO)—The FUT3 alleles of individuals from the eight pedigrees, the wild type pFUT3 construct, and the constructs containing the L1 and/or L2 mutated alleles were amplified by PCR with the same primers used for cloning FUT3. Hybridization with wild type and mutated ASO probes (Table I) was performed as described (13).

Transfection of COS-7 Cells—Cells were transfected with DEAE-dextran (18). An expression vector, containing the coding region of the bacterial chloramphenicol acetyltransferase (pCDM7-CAT) (19), was simultaneously transfected to allow normalization for transfection efficiency. Transfected cells were harvested after a 72-h expression period (12).

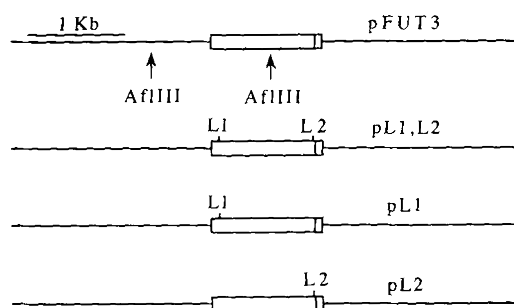

FIG. 1. **Mutated FUT3 allele expression vectors.** The FUT3 gene is represented by *open rectangles* representing its coding region (*large rectangle*) and 3′-untranslated region (*small rectangle* to the *right*). The *line* represents adjacent pCDNAI vector sequences. The relative positions of the mutations within the wild type sequence are indicated *above* the coding region of each vector, whose designation is given at *right*. Vectors with one or two mutations were constructed with restriction fragment interchange procedures using *Afl*III restriction sites as detailed in "Experimental Procedures." The relative positions of the *Afl*III sites within the vector sequence or within the FUT3 coding region are indicated by the *arrows*.

Flow Cytometry Analysis—Transfected COS-7 cells were labeled with different monoclonal antibodies: affinity-purified anti-H, anti-Le^a, and anti-Le^b (Chembiomed, Alberta Research Council, Edmonton, Canada; Ref. 17); anti-Le^x (SSEA1, D. Solter, Wistar Institute, Philadelphia, PA); anti-sialyl-Le^a (19.9 Centocor, Malvern, PA); and anti-sialyl-Le^x (CSLEX1, UCLA Tissue Typing Laboratory, Los Angeles, CA; Ref. 20). After incubation with the first antibody, the cells were washed and stained with fluorescin isothiocyanate-conjugated goat anti-mouse immunoglobulins and analyzed with a Becton Dickinson FACScan (12).

α(1,3)- and α(1,4)-Fucosyltransferase Enzyme Activities of Transfected COS-7 Cells with Different Acceptors—Fucosyltransferase assays were performed with GDP-[^{14}C]fucose (Amersham Corp., 300 mCi/mmol) and Galβ1→4GlcNAc, Galβ1→4Glc, Galβ1→3GlcNAc, Fucα1→2Galβ1→4Glc, and NeuAcα2→3Galβ1→4GlcNAc (Oxford Glycosystems, Oxford, United Kingdom) as described previously (12).

Plasma α(1,3)-Fucosyltransferase—Enzyme activity was measured with H type 2 acceptor, synthesized as a 8-methoxycarbonyloctyl glycoside, Fucα1→2Galβ1→4GlcNAcβ1→$(CH_2)_8COOCH_3$ (Chembiomed, Alberta Research Council, Edmonton, Canada) by the Sep-Pak C_{18} product isolation procedure (21).

Kinetics of α(1,3)- and α(1,4)-Fucosyltransferase Enzyme Activities—Kinetics were measured in 1% Triton X-100 extracts from transfected COS-7 cells using GDP-fucose and the 8-methoxycarbonyloctyl glycosides H type 2 and H type 1, Fucα1→2Galβ1→3GlcNAcβ1→$(CH_2)_8$-$COOCH_3$ (Chembiomed). Enzyme assays were performed with amounts of protein corresponding to initial velocity and giving similar fucose transfer for each cell extract (21).

Detection of Specific mRNA Expression—Total RNA was isolated from transfected COS-7 cells by the guanidium isothiocyanate-CsCl procedure (22). For RNA PCR analysis, 5 μg of total RNA were treated with 15 units of DNase I (Promega) for 15 min. The synthesis of the first cDNA strand was then performed on a final volume of 20 μl with the oligo$(dT)_{12-18}$ primer and a cDNA synthesis kit (RPN 2275, Amersham Corp.). The reaction mixtures were incubated for 2 h at 37 °C. The double-stranded cDNA was synthesized by PCR using 38 cycles of the above described program. The Gene-Amp PCR kit with 5-μl aliquots of each reverse transcriptase mixture and 0.5 μM of each of the sense primer used for cloning FUT3 alleles and the antisense primer 5′-CAGCCAGCCGTAGGGCGTGAAGATGTCGGA-3′, corresponding to the complement of nucleotides 600–571. The amplified 600-bp fragments were analyzed in 1.6% NuSieve agarose gel. For RNA dot-blot hybridization, 20 μg of total RNA were denatured in 1 mM glyoxal (23) and then immobilized onto nylon membrane (Hybond-N, Amersham). The dot-blot was prehybridized at 42 °C in the presence of 50% formamide and hybridized with [α-^{32}P]dCTP-labeled pFUT3 probe (Megaprime kit from Amersham) for 24 h at 42 °C, with 40% formamide and 10% dextran sulfate. The blot was rinsed once in 2 × SSC, 0.5% SDS, washed twice in the same buffer for 15 min at room temperature, and washed twice in 0.1 × SSC, 0.1% SDS at 65 °C for 20 min.

RESULTS

Missense Mutations within the FUT3 Alleles of a Lewis-negative Individual—DNA from an erythrocyte Le(a−b−), salivary Lewis-negative individual (B4) was used for cloning the FUT3-deficient allele. An *Xba*I-*Eco*RI PCR fragment of 1125 bp was amplified from genomic DNA with FUT3-specific primers. The inserts of 12 recombinant plasmids were sequenced and found to differ from the wild type FUT3 allele (8) by single base changes at two positions in the coding region (Fig. 2).

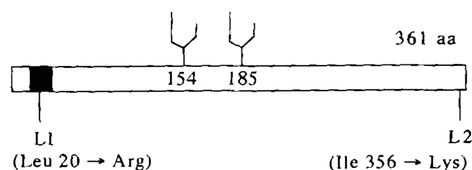

FIG. 2. **Location of L1 and L2 mutations and predicted glycosylation sites within the Lewis α(1,3/1,4)-fucosyltransferase.** The Lewis enzyme is depicted as a *rectangle*; its NH_2 transmembrane segment, containing the L1 mutation, is represented by the *small gray rectangle*. Positions and predicted amino acid sequence changes for each mutation are indicated *below* the scheme. Predicted *N*-linked glycosylation sites are indicated by the symbol ⋎ and the *residue number* for the corresponding asparagine.

The L1 mutation is a T → G substitution at position 59. This sequence alteration yields a leucine → arginine substitution (Leu-20 → Arg) in the transmembrane domain of the protein. The 12 clones sequenced contained this mutation, suggesting that the B4 individual is homozygous L1/L1.

The L2 mutation is a T → A substitution at position 1067. This sequence alteration changes an isoleucine codon to a lysine codon (Ile-356 → Lys) 6 amino acids before the COOH terminus of the protein. Eleven of 12 clones contained the L2 mutation, suggesting that L2 may also be homozygous in this individual. Homozygosity was further ascertained by the ASO technique. Only the two L1 and L2 mutant ASO probes (Table I) hybridized with genomic DNA, from the B4 individual, amplified with the same primers used for cloning FUT3, whereas neither of the two wild type ASO probes hybridized, confirming that this person is homozygous for both mutations.

Correlation of Lewis Genotypes with the Phenotypic Expression of Lewis Antigens on Erythrocytes and Saliva—The same ASO probes were used to ascertain the L1 and L2 genotypes of all family members typed for Lewis antigens on erythrocytes and saliva (Table II).

It was predicted that inactivating mutations in the coding region of FUT3, responsible for lack of expression of the fucosyltransferase products, should be homozygous. Therefore, the individual results of ASO typing were sorted for the presence of homozygous L1/L1 or L2/L2 genotypes (Table II). Four groups were defined: I, two individuals were homozygous L2/L2 only and did not express Lewis antigens, either on erythrocytes or saliva; II, 16 individuals were homozygous L2/L2 and L1/L1, and all of them also had Lewis-negative phenotypes on erythrocytes and saliva; III, 10 individuals were only homozygous L1/L1 and were also Lewis-negative on erythrocytes, but nine of them expressed Lewis antigens in saliva and one (A6) was negative on both erythrocytes and saliva; IV, 25 individuals had neither L1 nor L2 mutations in double dose, and they all expressed Lewis antigens in saliva. Only two individuals from this group typed as erythrocyte Le(a−b−). One expressed A and Le^a in saliva (G9), suggesting that he may be a weak secretor (Se^w) (7); the other expressed H and Le^b in saliva (C4), and it is not clear why he had erythrocyte Le(a−b−) phenotype (Table II).

All the homozygous L2/L2 (groups I and II) were plasma α(1,3)-fucosyltransferase-deficient (<300 dpm), while only 3 of 35 individuals (9%) without the L2 mutation in double dose were plasma α(1,3)-fucosyltransferase-deficient (H11 from group III and J1 and J3 from group IV, Table II).

A significant correlation was also observed between the presence of L2 in double dose and incidence of natural anti-Lewis

TABLE I
Allele-specific oligonucleotides (ASO) used to determine L1 and L2 genotypes of all members of the eight Indonesian families
Positions 59 (L1) and 1067 (L2) are underlined.

Mutation	Wild type	Mutated allele
L1	GCC GCA CTG CTA TTT	GCC GCA CGG CTA TTT
L2	CGC AGC ATA GCG GCT	CGC AGC AAA GCG GCT

antibodies (anti-Le^a or anti-Le^b) in plasma. Seven of 18 homozygous L2/L2 (groups I and II) had anti-Lewis antibodies in plasma (39%), while only one of 35 individuals (3%) without this mutation in double dose (groups III and IV) had weak anti-Le^b activity (G9, Table II). It is interesting to note that none of the individuals in group III had anti-Lewis antibodies in plasma, in spite of the fact that they all type as erythrocyte Le(a−b−), confirming that they are genetically Lewis-positive, as suggested by the presence of Lewis antigens in saliva in 9 of 10 individuals from this group III.

Segregation of L1 and L2 Mutations—The α(1,3)-fucosyltransferase was measured in plasma, but the Lewis α(1,3/1,4)-fucosyltransferase enzyme activity was only deduced from the presence of Le^a and/or Le^b antigens in saliva, because the Lewis enzyme in the available saliva samples was heat inactivated during preparation.

Cosegregation of lack of expression of Lewis antigens in saliva with the L2 mutation in double dose was confirmed in all families, with the exception of the A6 individual, who was Lewis-negative on both saliva and erythrocytes, but was heterozygous at the L2 position (Table II and Fig. 3). This individual was homozygous L1 and expressed very low levels of plasma α(1,3)-fucosyltransferase (440 dpm). The Lewis-negative phenotypes of this A6 individual could be secondary to incomplete maturation of expression of Lewis antigens, because he was only 5 years old when the samples were collected (24). However, in this particular family, it is possible that other mutations occur in the FUT3 gene, which may result in low enzyme activity, as has been proposed for the closely linked FUT6 gene product (13).

Segregation of the L1 mutation in double dose with the erythrocyte Le(a−b−) phenotype and salivary Lewis-positive phenotype is illustrated in family B, where B2 and B3 individuals are homozygous L1 and both have the erythrocyte Le(a−b−) phenotype with salivary Lewis-positive phenotype. The remaining members of this family are either heterozygous L1 and Lewis-positive on erythrocytes (B1, B5, B7, B8, and B9) or double homozygous L1 and L2 and Lewis-negative on both erythrocytes and saliva (B4, B10, and B11) (Table II and Fig. 3).

All individuals with the double homozygous L1 and L2 genotype had the same salivary and erythrocyte Lewis-negative phenotypes in the eight families. The inactivating effect of the L2 mutation in double dose was dominant over the effect of L1, as illustrated in family H, where all members were homozygous L1/L1, and the six members heterozygous L2/* expressed Lewis antigens in saliva (H2, H3, H6, H9, H11, and H15), while the six members homozygous L2/L2 were all negative for Lewis antigens in saliva (H1, H5, H7, H12, H14, and H16) (Table II and Fig. 3).

The plasma α(1,3)-fucosyltransferase-negative phenotype cosegregated with the Lewis-negative phenotype and homozygous L2/L2 genotype in most cases. However, there were three exceptions, J1, J3, and H11, who were plasma α(1,3)-fucosyltransferase-deficient but expressed Lewis antigens on erythrocytes and saliva and were heterozygous L2, suggesting a crossing over between FUT3 and FUT6 loci in the H family and another in the ancestry of J1 (Fig. 3).

Lewis Enzyme Activity of COS-7 Cells Transiently Transfected with FUT3—To determine the mechanisms by which L1 and L2 mutations yield the Lewis-deficient phenotypes, three series of experiments were performed: (i) flow cytometry analysis of COS-7 cells transfected with FUT3 expression vectors and stained with monoclonal antibodies specific for cell surface oligosaccharide antigens synthesized by the Lewis enzyme, (ii) measurement of the specific activity of the recombinant fucosyltransferases in these cells toward different low molecular weight oligosaccharide acceptor substrates, and (iii) determination of the apparent Michaelis constants of these recombinant enzymes for GDP-fucose, H type 1 and H type 2 substrates.

COS-7 cells were transfected with expression vectors encoding wild type FUT3 (pFUT3), or mutant forms of FUT3 containing the L1 substitution (pL1), the L2 substitution (pL2), or both changes (pL1,L2). After a 3-day expression period, these cells, or cells transfected with a negative control vector (pCDNAI), were stained with specific antibodies and subjected to flow cytometry analysis (Fig. 4). Cells transfected with the wild type FUT3 vector (pFUT3) express all four Lewis antigens, whereas cells transfected with the negative control vector (pCDNAI) do not express these antigens. Cells transfected with vectors containing the L2 mutation alone (pL2) or L2 in conjunction with the L1 mutation (pL1,L2) do not express detectable levels of the four Lewis antigens. Similarly, we found no detectable α(1,3)- or α(1,4)-fucosyltransferase activity in extracts prepared from cells transfected with pL2 or with pL1,L2 (Table III).

The specific FUT3 mRNA alleles were detected in all the transfected cells by reverse transcriptase-PCR and by hybridization with the FUT3 probe, confirming that pL2- and pL1,L2-transfected cells contained the corresponding FUT3 alleles, but the mutation L2 inactivates the cognate enzyme.

By contrast, the pL1 vector determines expression of the Le^a and sialyl-Le^a antigens on transfected COS-7 cells, although the fraction of cells that express these determinants is lower than the fraction of antigen-positive pFUT3-transfected cells. However, the Le^x and sialyl-Le^x antigens are expressed on pL1-transfected cells at levels barely above background. This observation is at odds with enzyme activity assays done on extracts prepared from pL1-transfected cells (Table III). These results indicate that the L1 mutant enzyme is, in fact, capable of utilizing type 2 acceptor substrates *in vitro*, although apparently not efficiently within the cell.

We explored this issue further by comparing the kinetic properties of the wild type FUT3 enzyme and the mutant L1 enzyme (Table IV). We found that both enzymes maintain similar apparent Michaelis constants for H type 1, H type 2, and GDP-fucose. However, the specific activity (and V_{max}) of the fucosyltransferase found in pL1-transfected cells was consistently 2–3-fold lower than the activity in pFUT3 transfectants (Tables III and IV).

Taken together, these results suggest that the L1 mutant fucosyltransferase exhibits a catalytic activity that is similar to that for the wild type enzyme, at least with respect to its affinity (K_m) toward three different substrates. However, the L1 mutation seems to decrease the overall specific activity of the enzyme. The wild type Lewis α(1,3/1,4)-fucosyltransferase is less active on type 2 acceptor substrates; it is therefore not surprising that diminution in expression efficiency in transfected cells is associated with a greater loss in expression of cell surface type 2 antigens than type 1 antigens.

Since the L1 mutation is localized to the transmembrane segment of this enzyme, its decreased activity might be a consequence of inappropriate membrane insertion. To test this hypothesis, total enzyme activity of the culture medium of pL1-transfected cells was tested and was found to contain 2.3 times more activity than the corresponding cells, whereas the difference was only of 1.5 times for wild type pFUT3-transfected

TABLE II

ABO and Lewis phenotypes and Lewis genotypes of all family members (Fig. 3)

Erythrocyte phenotypes were obtained by direct hemagglutination, and saliva phenotypes were obtained by inhibition of hemagglutination of the anti-A, -B, -H, -Le^a, and -Le^b reagents. Lewis genotypes at the 59 (L1) and 1067 (L2) positions were determined by ASO tests (* indicates wild type). The presence of natural anti-Le^a and anti-Le^b antibodies and α(1,3)-fucosyltransferase activity expressed as dpm of transferred [^{14}C]fucose (α-3-FT) were determined in plasma. After sorting for L2 and L1 homozygous genotypes, four groups could be distinguished: I, homozygous L2/L2; II, homozygous L2/L2 and L1/L1; III, homozygous L1/L1; IV, all others. Individuals within each group were sorted by increasing amount of plasma α(1,3)-fucosyltransferase activity (FUT6).

Group	Identity		Erythrocytes		Saliva					Genotype Lewis		Plasma	
	Family	No.	ABO	Le(a,b)	A	B	H	Le^a	Le^b	L1	L2	Antibody	α-3-FT
I	E	3	A1B	(−, −)	A	B	H	−	−	*/*	2/2	Le^b	108
	E	5	B	(−, −)		B	H	−	−	*/1	2/2		122
II	H	12	B	(−, −)		B	H	−	−	1/1	2/2	Le^b, Le^a	26
	H	7	B	(−, −)		B	H	−	−	1/1	2/2		43
	H	14	B	(−, −)		B	H	−	−	1/1	2/2	Le^b, Le^a	55
	H	1	O	(−, −)			H	−	−	1/1	2/2		84
	H	5	B	(−, −)		B	H	−	−	1/1	2/2		87
	D	1	A1B	(−, −)	A	B	H	−	−	1/1	2/2	Le^b	109
	B	10	O	(−, −)			H	−	−	1/1	2/2	Le^a	120
	D	2	A1	(−, −)	A		H	−	−	1/1	2/2		121
	J	4	A1	(−, −)	A		H	−	−	1/1	2/2		124
	H	16	O	(−, −)			H	−	−	1/1	2/2		128
	C	1	O	(−, −)			H	−	−	1/1	2/2		130
	A	3	O	(−, −)			H	−	−	1/1	2/2	Le^a	132
	B	4	A1	(−, −)	A		H	−	−	1/1	2/2		172
	B	11	A1	(−, −)	A		H	−	−	1/1	2/2	Le^b, Le^a	178
	G	4	A1B	(−, −)				−	−	1/1	2/2		198
	G	5	A1B	(−, −)				−	−	1/1	2/2		232
III	H	11	B	(−, −)		B	H	+	+	1/1	*/2		94
	A	6	A1	(−, −)	A		H	−	−	1/1	*/2		440
	H	9	B	(−, −)		B	H	+	+	1/1	*/2		1430
	E	4	O	(−, −)			H	+	+	1/1	*/2		1810
	H	15	B	(−, −)		B	H	+	+	1/1	*/2		2030
	H	6	O	(−, −)			H	+	+	1/1	*/2		2790
	B	3	O	(−, −)			H	−	+	1/1	*/2		3490
	B	2	A1	(−, −)	A		H	−	+	1/1	*/2		4300
	H	3	B	(−, −)		B	H	+	+	1/1	*/2		4720
	H	2	B	(−, −)		B	H	+	−	1/1	*/2		15160
IV	J	3	A1	(−, +)	A		H	+	+	*/1	*/2		52
	J	1	A1	(−, +)	A		H	+	+	*/1	*/2		69
	A	4	O	(+, +)			H	+	+	*/1	*/2		576
	A	1	O	(−, +)			H	+	+	*/1	*/2		910
	A	2	A1	(−, +)	A		H	+	+	*/1	*/*		1600
	J	2	A1	(−, +)	A		H	+	+	*/1	*/2		1665
	B	9	A1	(+, +)	A		H	+	+	*/1	*/2		2480
	E	1	A1	(+, +)	A		H	+	+	*/*	*/2		2830
	G	9	A1	(−, −)	A			+	−	*/1	*/2	Le^b	2860
	B	8	A1	(−, +)	A		H	+	+	*/1	*/2		2960
	B	5	O	(−, +)			H	+	+	*/1	*/2		3150
	A	5	O	(+, +)			H	+	+	*/1	*/*		3230
	B	1	A2	(−, +)	A		H	+	+	*/1	*/2		3500
	C	6	O	(−, +)			H	+	+	*/1	*/2		3530
	C	5	O	(+, +)			H	+	+	*/1	*/2		4050
	C	3	A1	(−, +)	A		H	+	+	*/1	*/2		4060
	C	4	O	(−, −)			H	−	+	*/1	*/2		4080
	G	8	A1	(−, +)	A		H	+	+	*/1	*/2		4080
	B	7	A1	(−, +)	A		H	+	+	*/1	*/*		4280
	C	2	A1	(−, +)	A		H	+	+	*/*	*/*		5290
	G	3	A1	(+, −)				+	+	*/*	*/*		6400
	G	7	B	(+, −)			H	+	+	*/1	*/2		10970
	G	2	A1b	(−, +)	A	B	H	+	+	*/1	*/2		11260
	G	1	O	(−, +)			H	+	+	*/*	*/*		13260
	G	6	O	(+, +)			H	+	+	*/*	*/*		15660

cells. This result favors the proposed hypothesis but does not account for all the difference between pL1 and pFUT3. New experiments have to be performed in carefully controlled cell viability conditions, with polarized epithelial cells, since some transfected COS-7 cells dye and can liberate their enzyme content in the culture medium.

DISCUSSION

The original genetic model of Lewis antigens resulting from epistatic interactions of the *Le-le* and *Se-se* loci considered as an all or none tissue expression of two fucosyltransferases, encoded by FUT2 and FUT3 loci (25) has recently been challenged, because Lewis enzyme activity and Le^a or Le^b antigens were detected in saliva of 3 of 6 individuals with the erythrocyte Le(a−b−) phenotype, who were called nongenuine Le(a−b−) individuals for this reason (26).

The occurrence of Lewis antigens in saliva of individuals with Le(a−b−) erythrocyte phenotype can now be seen to be due to the mutation L1. This mutation in double dose was found (without the homozygous L2 mutation) in 10 of 30 erythrocyte

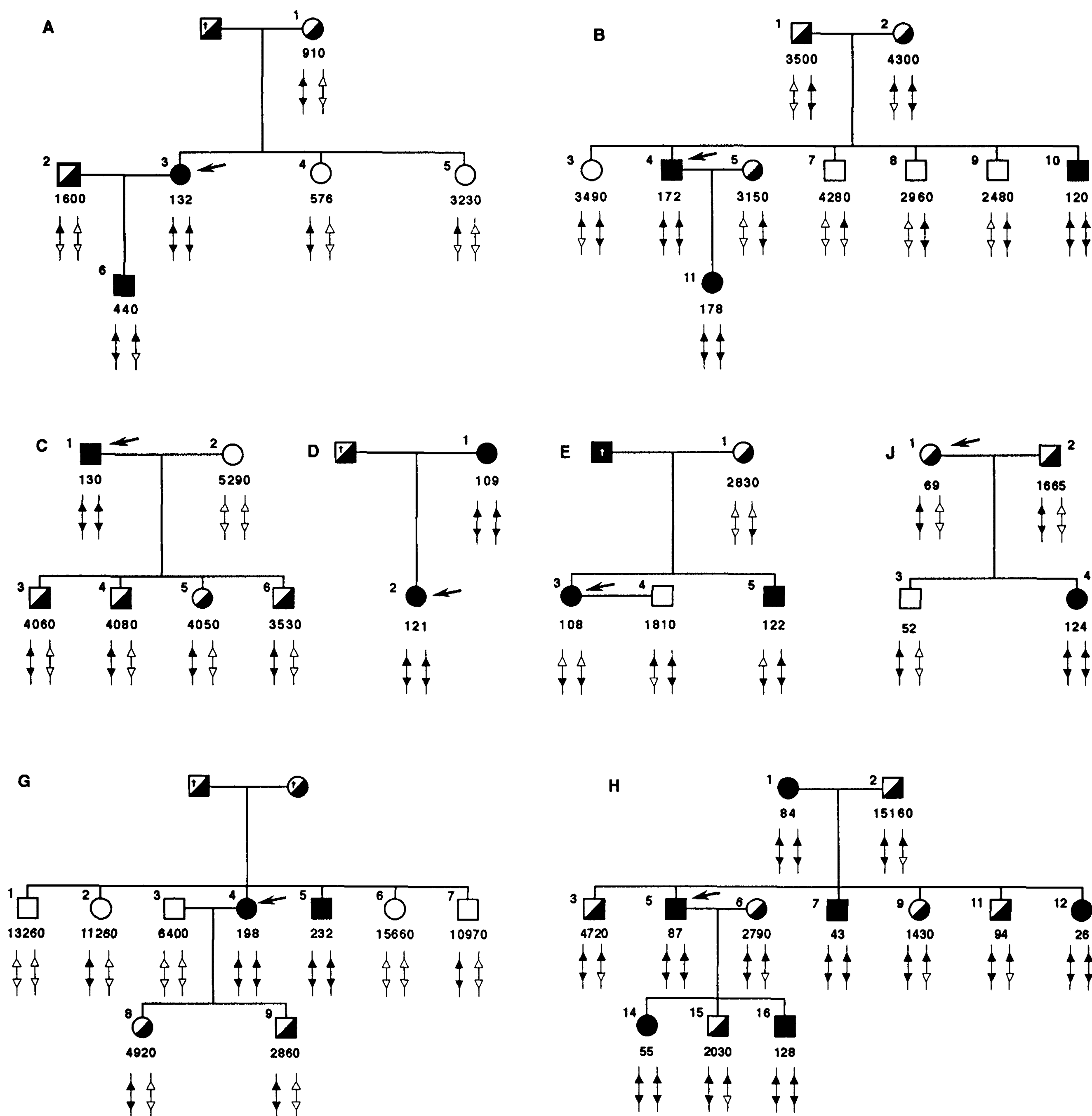

FIG. 3. **Segregation of FUT3 alleles in eight Indonesian families.** *Solid symbols* for males (■) and females (●) correspond to salivary Lewis-negative phenotype. The *half-black symbols* (◪, ◑) indicate Lewis-positive individuals, assumed to be Lewis heterozygous from the structure of the family tree. *Open symbols* (□, ○) correspond to Lewis-positive phenotypes, which can be either homozygous or heterozygous. A cross (†) inside symbols indicates that the person died before the study. The plasma α(1,3)-fucosyltransferase activity of each individual is expressed below the symbol, as disintegrations/minute of fucose incorporated onto H type 2. The L1 (▲, △) and L2 (▼, ▽) polymorphic alleles are displayed under enzyme activity numbers. Mutated alleles are represented by *solid triangles* and wild type alleles by *open triangles*.

Lewis-negative individuals, and 9 of them expressed Lewis antigens in saliva. Therefore, this kind of mutation in the transmembrane domain is probably responsible for the Le^w phenotype, previously described in individuals typing as Le(a–b–) on erythrocytes and Lewis-positive in saliva (27).

The reduction in expression of Le^a and Le^b antigens on red cells, induced by the L1 mutation, might be related to its location in the putative transmembrane domain. The change of a neutral leucine for a charged arginine in this hydrophobic domain can impair the proper anchoring of the enzyme in the Golgi membrane. The expected consequences of such an impediment would be a decreased amount of enzyme resident in the Golgi, which synthesizes the Lewis glycolipid epitopes shed into the plasma compartment. Enzyme kinetics favor this hypothesis since similar apparent K_m values were found in cell extracts of COS-7 cells transfected with wild type pFUT3 and pL1 constructs, but the pL1-transfected COS-7 cells had consistently lower V_{max} values, suggesting a 2–3-fold reduction of intracellular enzyme in the case of L1 mutant.

In addition, the so-called genuine Lewis-negative individuals are not completely negative, since they express small amounts of Lewis epitopes in other tissues (26, 28). Indeed, trace amounts of glycolipids bearing the pentasaccharide Le^a and the hexasaccharide Le^b were identified in genuine Lewis-negative

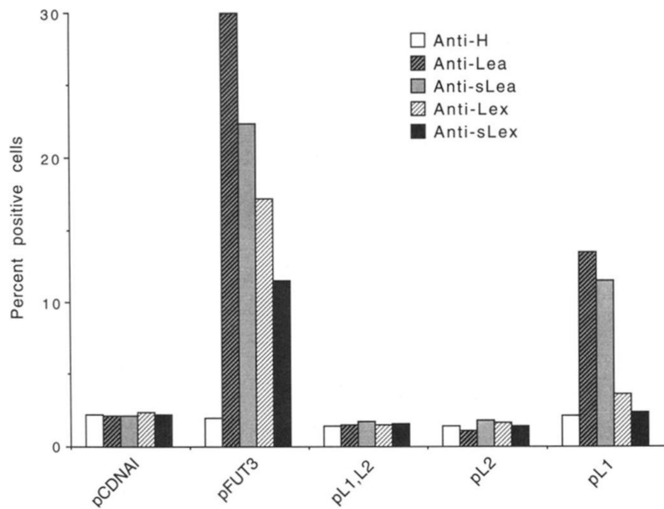

FIG. 4. **Flow cytometry analysis of COS-7 cells transfected with FUT3 expression vectors.** COS-7 cells were transfected and subjected to flow cytometry analysis as described under "Experimental Procedures," using vectors indicated below the histograms. Cells were stained with monoclonal antibodies specific for Le^a (*Lea*), sialyl-Le^a (*sLea*), Le^x (*Lex*), and sialyl-Le^x (*sLex*) determinants, or with negative control antibodies as anti-H and anti-Le^b (not shown). Data are normalized for transfection efficiencies, as determined by an internal control chloramphenicol acetyltransferase assay.

TABLE III
Enzyme activity in pmol/mg/h on different oligosaccharide acceptors of extracts of transfected COS-7 cells with FUT3 constructs, performed as described under "Experimental Procedures" (12)

Lacto-*N*-bioseI, Galβ1→3GlcNAc; 2′-fucosyllactose, Fucα1→2Galβ1→4Glc; lactose, Galβ1→4Glc; LacNAc, Galβ1→4GcNAc; sialyl-LacNAc, NeuAcα2→3Galβ1→4GlcNAc.

Constructs	Lacto-*N*-bioseI	2′-Fucosyllactose	Lactose	LacNAc	Sialyl-LacNAc
pFUT3	10,880	5020	2380	2214	1990
pL1	3995	1655	820	722	620
pL2	<1	<1	<1	<1	<1
pL1,L2	<1	<1	<1	<1	<1
pcDNA1	<1	<1	<1	<1	<1
Relative activity of pL1	37%	33%	34%	33%	31%

TABLE IV
Comparison of the kinetics of the Lewis enzyme produced by cell extracts of COS-7 cells transfected with wild type pFUT3 or pL1 constructions

H type 1, Fucα1→2Galβ1→3GlcNAcβ-$(CH_2)_8$-$COOCH_3$.
H type 2, Fucα1→2Galβ1→4GlcNAcβ-$(CH_2)_8$-$COOCH_3$.

Acceptors	Wild type pFUT3		pL1		Relative V_{max} of pL1
	Apparent K_m	V_{max}	Apparent K_m	V_{max}	
	µM	µM/h/mg	µM	µM/h/mg	%
GDP-fucose	14	335	15	114	34
H type 1	56	426	51	182	43
H type 2	1690	47	1330	22	46

individuals by immunochemical and mass spectrometry structural studies (29). The amounts of Le^a or Le^b glycosphingolipids detected in these individuals were in the order of 1% of the normal Lewis glycosphingolipids found in Lewis-positive individuals (30). Such a low level of antigen production is not detectable by most typing techniques, and it is within the background level of detection for the COS-7 cells transfected with mutant Lewis constructions. Therefore, it is possible that the cognate enzymes of mutant inactivating alleles are able to produce small amounts of antigen which are not detected by current techniques. A similar quantitative problem can apply to L2 and to any of the other FUT3 mutants described (Table V).

TABLE V
Summary of all reported mutations affecting the coding region of FUT3, which can modify the cognate Lewis enzyme activity and give erythrocyte Le(a−b−) phenotype

Nucleotides		Amino acids		Geographic area	Ref.
Position[a]	Change	Position[a]	Change		
59	T → G	20	Leu → Arg	Indonesia	This study (L1)
				Japan	(10, 11)
314	C → T	105	Thr → Met	Sweden	(9)
508	G → A	170	Gly → Ser	Japan	(10, 11)
1007	A → C	336	Asp → Ala	Japan	(11)
1067	T → A	356	Ile → Lys	Indonesia	This study (L2)

[a] Position numbering according to Ref. 8.

Alternatively, other enzymes encoded by FUT4, FUT5, or another still unknown gene, may be responsible for the synthesis of the small amounts of Le^a or Le^b epitopes found in genuine Lewis-negative individuals.

The presence of small amounts of Lewis antigens in Lewis-negative individuals shed light on one of the most mysterious differences between ABO (31) and Lewis systems. The ABO alloantigens are a clear-cut, all-or-none alloantigen system, where all the individuals lacking A or B antigens have the corresponding anti-A or anti-B alloantibodies in plasma. This is not the case among Lewis-negative individuals, where the presence of anti-Lewis antibodies is not the rule. Only a small proportion of Lewis-negative individuals have natural anti-Lewis antibodies in plasma, and they are usually of lower titer than the anti-A or anti-B natural antibodies (32). Homozygous L1/L1 have Lewis antigens in secretions; therefore, anti-Le^a or anti-Le^b would be autoantibodies, and, as expected, these antibodies were not found (Table II). However, even the so-called genuine Lewis-negative individuals can still make small amounts of Lewis antigens in tissues, and this fact accounts for the low incidence of natural anti-Lewis antibodies among genuine Lewis-negative individuals.

The Lewis enzyme is the only α(1,3)-fucosyltransferase that also has α(1,4)-fucosyltransferase activity and therefore can use both type 1 and type 2 precursor acceptors, although it works better on H type 1 (apparent K_m ~ 50 µM) than on H type 2 (apparent K_m ~ 1500 µM) (Table IV). These results are in good agreement with the specificity of Lewis enzyme purified from human milk (33) and from the human A431 cell line (34), which has been shown to be able to make Fucα1→3GlcNAc, Fucα1→4GlcNAc, and Fucα1→4Glc linkages, but the apparent K_m for Galβ1→3GlcNAc (type 1 precursor) was 10 times better than the apparent K_m for Fucα1→2Galβ1→4Glc (H type 2-like).

The expression of Lewis enzyme activity in human tissues starts only during the last trimester of gestation (35), where it replaces a myeloid variant of α(1,3)-fucosyltransferase. This switch of myeloid to Lewis enzyme expression has been well documented in renal collecting portions (36).

The available data suggest that the human fucosyltransferase genes are organized in clusters located on different chromosomes (37). FUT1[2] and FUT2 reside on the long arm of chromosome 19; FUT3, FUT5, and FUT6 reside on the short arm of chromosome 19 (12); and FUT4 resides on the long arm of chromosome 11 (38). All but FUT4 have been found to be polymorphic. In the majority of cases, single point mutations in the catalytic domain and in double dose inactivate the enzyme, as described here for L2.

The sequence of FUT3 alleles from Le(a−b−) individuals has shown different mutations in Japan and Europe. Furthermore, each of these mutant alleles was only present in double dose in about half (Japan) and one-third (Sweden) of Lewis-negative

[2] FUT1–FUT6 are the fucosyltransferase gene names registered in the Genome Data Base.

individuals, suggesting heterogeneity of the molecular basis of these FUT3 defects. By contrast, 18 of 19 Indonesian salivary Lewis-negative individuals had the L2 mutation in double dose, suggesting a high degree of homogeneity of the FUT3 defect in Java, probably secondary to a founder effect on this island. The L1 mutation seems more widely distributed in the world, since we found it in Indonesia, and two independent groups have found the same mutation in Japan (Table V).

The particular effect of the L1 mutation, related to its location in the transmembrane domain of the cognate enzyme, constitutes a new form of regulation of differential expression of fucosyltransferases in tissues. The "down-modulating" effect of the L1 mutation may be even more pronounced in the physiological situation, where this enzyme is expressed under control of its endogenous promoter, and not, as studied here, under the control of a strong promoter on a multicopy episome. Thus, under lower, physiological expression levels, homozygosity for the L1 mutation may yield enzyme activity sufficient to create detectable levels of Lewis antigens in saliva, yet not sufficient to support the process that allows Lewis antigen adsorption by erythrocytes.

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