Leukocyte adhesion deficiency (LAD) is an autosomal recessive disease caused by mutations in the CD18 gene which codes for the \( \beta_2 \) integrin subunit. We studied two patients, the first of which had a moderate LAD phenotype and expressed only 9% of CD11/CD18 on blood leukocytes. RNA from lymphoblasts was reverse-transcribed, and the cDNA was amplified, cloned, and sequenced. An ATG to AAG alteration in the initiation codon was detected in 39 of 45 (87%) cDNA clones. This mutation was detected in the father, but not in the mother. The maternal defect was shown to be a frameshift mutation with the deletion of a single T in the aspartic acid codon at position 690 (GAT), 11 amino acids N-terminal to the beginning of the transmembrane domain. This mutation predicts a polypeptide which would terminate without transmembrane or cytoplasmic domains. The frameshift mutation was also found in the second patient who had the severe phenotype of LAD (<1% of CD11/CD18), indicating that this allele does not encode a functional protein. The partial expression in the patient with a moderate phenotype must be derived from the initiation codon mutation and may be due to a low level of initiation of translation of the CD18 mRNA at the second codon (CUG).

Leukocyte adhesion deficiency (LAD) is an autosomal recessive disease caused by deficient expression of the \( \beta_2 \) integrins (or CD11/CD18 glycoprotein complexes) on the surface of leukocytes. The characteristic features of LAD are delayed umbilical cord severance, poor wound healing, lack of pus formation, persistent leukocytosis, recurrent soft tissue and periodontal infections, and a high risk for developing recurrent life-threatening bacterial and fungal infections. Severe and moderate phenotypes of LAD have been described in which the severity of clinical infections or other complications is directly related to the degree of \( \beta_2 \) integrin deficiency (1). Severe LAD patients are at high risk for systemic and often life-threatening infections in infancy as a consequence of an almost total deficiency of CD11/CD18 complex expression on the blood leukocytes. Somewhat higher levels of CD11/CD18 complex expression on leukocytes of moderate LAD patients (2.5-10% of normal levels) account for their less severe clinical presentation and frequent survival into adulthood.

The \( \beta_2 \) integrins include LFA-1, Mac-1, and \( \alpha \) integrins. They are heterodimeric glycoproteins composed of a common 95,000-dalton \( \beta \) subunit encoded by the CD18 gene in a noncovalent association with one of three distinct \( \alpha \) subunits encoded by the CD11 gene family. LAD is caused by heterogeneous mutations in the common \( \beta \) subunit (CD18) of the leukocyte integrins. Although LAD is inherited as a single gene defect in the \( \beta \) subunit, there is a secondary absence of \( \alpha \) subunits. Biosynthesis of \( \alpha \) subunits in LAD cells is normal (2, 3), but they are not expressed on the cell surface because the \( \beta \) subunits are defective. A deficiency of CD11/CD18 complexes on the surface of the LAD leukocytes accounts for a variety of functional impairments in \textit{vitro}, including abnormalities of adhesion-dependent chemotaxis, aggregation, phagocytosis of iC3b-opsonized particles, complement or antibody-dependent cytotoxicity, and transendothelial migration (4, 5).

We have investigated the molecular nature of the \( \beta \) subunit defect in two LAD patients to further understand the basis for the two clinical phenotypes. We identified an initiation codon mutation and a frameshift mutation in a patient with a moderate phenotype. The frameshift mutation was also present in a patient with a severe phenotype.

**EXPERIMENTAL PROCEDURES**

**Patient Material**—Epstein-Barr virus-transformed lymphoblasts were obtained from a male who was described in 1979 as the 9-year-old son of healthy unrelated parents (6). He had chronic otitis media, gingivitis, periodontitis, and severe skin lesions which contained \textit{Staphylococcus aureus} and very few neutrophils even though the patient had chronic leukocytosis. Functional defects in neutrophils and the patient's clinical presentation were consistent with the subsequent diagnosis of LAD (patient 10 in Anderson and Springer (7)). The patient was classified as having the moderate form of the disease based on the severity of the clinical symptoms and the presence of approximately 9% of normal levels of CD11/CD18 complexes on the surface of f-Met-Leu-Phe-stimulated blood neutrophils as assessed by immunofluorescence flow cytometry (1). These findings were verified by immunoprecipitation of only trace amounts of a normal sized CD18 precursor protein from cell lysates and by diminished amounts of a normally sized mRNA by Northern blot analysis (patient 11 in Kishimoto et al. (2)). The severe patient is a previously unreported 5-year-old male whose clinical presentation and leukocyte studies are consistent with the severe phenotype.

**Immunofluorescent Analysis**—Indirect immunofluorescent analysis was performed on patient lymphoblasts with a FACSscan (Becton, Dickinson & Co., Mountain View, CA) using the anti-CD18 antibody.
CD18 Mutations

IB4 (generously provided by Samuel D. Wright, Rockefeller University) conjugated to biotin as described previously (8).

Isolation of RNA and the Amplification of CD18 cDNA for Cloning—Total RNA was isolated from lymphoblasts from the patients, their parents, and healthy controls (9). A 30-μg aliquot of RNA was incubated with 1.4 units of RNasin (Promega Biotec, Madison, WI), and 36 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). After 45 min at 42 °C, an additional 36 units of reverse transcriptase was added followed by an additional incubation of 45 min. The RNA was hydrolyzed by the addition of 60 μl of 0.7 M NaOH containing 40 mM EDTA and incubation at 65 °C for 10 min. The cDNA product was precipitated with ethanol and resuspended in 100 μl of water prior to amplification.

The cDNA for CD18 was amplified using the polymerase chain reaction (PCR) with primers that flank the coding region of the molecule (Fig. 1). The primers included restriction sites at their 5' end for easy cloning into an M13 vector. PCR reactions were in a volume of 50 μl and contained the following: 6.7 mM MgCl2, 67 mM Tris-HCl (pH 8.8), 10 mM β-mercaptoethanol, 10% (v/v) dimethyl sulfoxide, 170 μg/ml bovine serum albumin, 1 mM each dNTP, 16 μl of cDNA product, 0.56 μm each primer, and 5 units of AmpliTaq™ polymerase (Perkin-Elmer-Cetus). The PCR reactions were carried out for 25 cycles using a denaturation step at 94 °C for 1 min, an annealing step at 55 °C for 2 min, and an extension step at 72 °C for 4 min. The amplified fragment was collected by ethanol precipitation, digested with XbaI and HindIII, isolated from low melting agarose, and directionally cloned into M13mp18 cut with the same enzymes. For PCR analysis of M13 phage plaques, individual phage plaques were touched with a toothpick and the toothpick dipped into the reaction mix to provide template. To assay for the presence of the initiation of codon mutation, a 205-bp fragment containing the initiation codon and a single AluI site was amplified directly from phage by PCR using primers 1 and 3 (Fig. 1).

DNA Sequencing—M13mp18 clones were grown in Escherichia coli JM103. White plaques containing inserts were selected; single-stranded DNA was isolated and was sequenced using the dioxyanucleotide chain termination method (10) with Sequenase reagents (United States Biochemical Corp.). For the moderate patient, the entire coding region of the molecule was sequenced using specific oligonucleotides from the CD18 cDNA as primers.

Allele-specific Oligonucleotide Hybridization—A 499-bp segment of the CD18 sequence (base pairs 2430–2429, numbering according to Kishimoto et al. (11)) was amplified, and several clones from the patients' lymphoblasts were analyzed. The PCR product (1 μl) was mixed with 1 μl of 2.5% bromphenol blue, and 39 μl of 0.4 N NaOH containing 25 mM EDTA and spotted onto Zeta-Probe blotting membrane (Bio-Rad). Alternatively, 20 μl of M13 phage supernatants from infected cultures were spotted directly on the membrane using the Minifold II slot-blot apparatus (Schleicher & Schuell). The DNA was immobilized by baking at 80 °C in a vacuum oven for 2 h. Two 19-mer sense strand oligonucleotides were synthesized to match the normal gene sequence (CTATGTTGGATGAGAGCCCGAG) or to match the frameshift mutant sequence (CTATGTTGGAGAGAGCCCGAG). The oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (12) and were purified with NENSO®20 nucleic acid purification cartridges (Du Pont-New England Nuclear Research Products). The filters were hybridized as described elsewhere (13) at 65 °C in the presence of 10-fold molar excess of unlabeled competitor oligonucleotide (14). Filters were washed in 40 mM NaHPO4, (pH 7.2) with 1% sodium dodecyl sulfate at room temperature for 5 min and at 42 °C for 15 min and exposed to X-Omat AR film (Eastman Kodak).

RESULTS

Fluorescence-activated Cell Sorting of the Patients' Lymphoblasts—Biotin-conjugated anti-CD18 antibody and phycoerythrin-streptavidin were used to stain lymphoblasts (8). Comparisons of relative fluorescence intensities in a cell sorter revealed CD18 levels 9% of normal in the moderate patient and 1% of normal in the severe patient (Fig. 2).

The Paternal Mutation of the Moderate Patient Is in the Initiation Codon of CD18—Full-length cDNA was amplified from normal individuals and from the two patients, and the PCR products were of the expected size. The products were cloned into M13mp18, and several clones from the patients and from the controls were sequenced. Sequence analysis revealed an ATG to AAG change in the initiation codon in each of four clones from the moderate patient (Fig. 3). This mutation changes a methionine codon at position 1 to a lysine codon and is designated M1K. This change in the DNA sequence would disrupt the initiation of translation.

This single base change of a T to an A created an additional AluI site in the cDNA of the moderate patient. It was possible to screen all of the phage clones from this patient for the presence of the mutation by amplifying a small region surrounding the initiation codon. The PCR products were digested with AluI and analyzed on a polyacrylamide gel to test for the mutant (93, 70, and 42 bp) or normal (163 and 42 bp) pattern of fragments. The mutation was detected in 39 of 45 (87%) of the M13 clones from this patient. For family studies, a first strand of cDNA was synthesized from the mRNA of the moderate patient, each parent, and a healthy control, and the products were used for the amplification of the 205-bp segment containing the initiation codon. Upon digestion of

![Fig. 1. Oligonucleotides for PCR amplification of cDNA.](image1)

![Fig. 2. Immunofluorescence analysis.](image2)

![Fig. 3. Sequence analysis of cDNA clones for the initiation codon region for the moderate patient (PT) and for a normal control (NL).](image3)
the amplified product with AluI, the two bands unique to the altered initiation codon (93 and 70 bp) appeared with the amplified cDNA from the moderate patient and his father, but not with that from the mother or the control (Fig. 4).

The Maternal Mutation in the Moderate Patient Is a Single Base Deletion in Codon 690—Four of the M13 clones from the moderate patient were chosen for further analysis because they did not contain the paternal mutation as evidenced by the absence of the additional AluI site. These clones which should contain the putative maternal mutation were sequenced and were shown to contain a normal initiation codon. However, a single base deletion in the coding region of the molecule, 11 amino acids N-terminal to the start of the transmembrane domain was detected in three of four maternal clones sequenced. The T in the GAT codon of the aspartic acid at position 690 (D690) at base 2142 is present in the maternal clones (Fig. 5). This deletion creates a frameshift mutation which is expected to terminate the protein without proper transmembrane or cytoplasmic domains.

For family studies, allele-specific oligonucleotides (ASOs) of 19 bp complementary to the mutant or the wild type sequence at base 2142 were synthesized and end-labeled with [γ-32P]ATP. The cDNAs from the moderate patient and his parents were amplified and transferred to a nylon membrane for analysis with ASOs (Fig. 6). Hybridization detected the frameshift mutation in the moderate patient and his mother, but not in the father. One of the phage clones from the patient contained neither the paternal nor the maternal mutation. This single clone is thought to have arisen as the result of a DNA repair event when heteroduplex molecules formed in the PCR reaction were introduced into bacteria (15, 16).

The Severe Patient Also Has the Mutation in Codon 690—The D690 frameshift mutation was also found in four of eight cDNA clones from the severe patient (data not shown). Sequencing of cDNA clones from the parents revealed that this mutation was inherited from the father of the severe patient. The other mutant allele in the severe patient has not been identified.

**DISCUSSION**

Two patients, one with moderate phenotype and the other severe, were studied and found to be compound heterozygotes. Although unrelated, they share the same frameshift mutation just prior to the transmembrane domain. The moderate patient has also inherited from his father a mutation in the CD18 initiation codon. Both mutations would be expected to impair the synthesis or function of the protein.

The frameshift mutation would be expected to produce a truncated β subunit that is 56 amino acids smaller than a normal molecule, thus lacking a transmembrane and a cytoplasmic domain. Although the formation of αβ heterodimers occurs before the complex is transported to the cell surface (17–19), and the cytoplasmic domain and transmembrane regions are not required for heterodimer assembly (20, 21), it has been shown that regions of the cytoplasmic domain of CD18 are important in the regulation of the adhesiveness of LFA-1 to ICAM-1 in a COS cell expression system (21). However, immunofluorescent analysis showed no significant CD18 on the cell surface in the severe patient, indicating that both alleles present in this patient do not produce a significant amount of mature CD18.

In contrast, the moderate patient’s lymphoblasts express CD18 on the cell surface at a level of 9% of normal. The increased CD18 expression in the moderate patient compared to the severe patient must be the result of the mutant CD18 initiation codon. Amplification of cDNA from the moderate patient indicated that the mRNA with the frameshift mutation was less abundant, presumably due to decreased stability as has been seen for some nonsense and frameshift mutations (22, 23).

We hypothesize that the moderate phenotype is due to a low level of initiation of translation of the mRNA from the paternal allele, particularly at the second codon (CUG). The current “scanning” model of initiation of translation proposes that the 40 S ribosomal subunit binds to the upstream region of an mRNA transcript near the m7G cap structure and proceeds toward the 3' end of the transcript, searching for the first AUG triplet that will initiate translation. However, it is also important that the initiation codon be in a good context for efficient initiation to proceed. In a survey of 699 eukaryotic genes, a consensus sequence of GCCG CCN/C CCAUGG was found by Kozak (24, 25). Furthermore, the G in position +3 and the purine at position −3 was shown to be especially important in defining a good context.

Mutations in an initiation codon have been shown to play
a role in disease in the following instances: the α1 or α2 globin gene causing hemoglobin H disease (26–28), the β-globin gene causing β-thalassemia (29), the phenylalanine hydroxylase gene causing phenylketonuria (30), the α subunit of the stimulatory G protein of adenylate cyclase causing Albright hereditary osteodystrophy (31), the apolipoprotein C-II gene causing type I hyperlipoproteinemia (32), and the ornithine transcarbamylase gene causing gyrate atrophy of the retina (33). In the case of β-thalassemia, phenylketonuria, hyperlipoproteinemia, and gyrate atrophy of the retina, the phenotype is consistent with relatively complete protein deficiency. In the case of the α globin genes and the α subunit of stimulatory G protein, it is presumed that the mutant allele is inactivated, but the data did not address the possibility of low levels of initiation of translation at an alternative codon.

There are now many well characterized examples of mRNAs whose translation is initiated at non-AUG triplets (34–42). Specific precedent for the use of CUG as an initiation codon exists for the high molecular weight forms of human fibroblast growth factor (40, 41), for the int-2 gene in mouse (42). Specific precedent for the use of CUG as an initiation codon exists for the high molecular weight forms of human fibroblast growth factor (40, 41), for the int-2 gene in mouse (42).

**Table 1**

| Mutation | Comments | Ref. |
|----------|----------|-----|
| Ttgt to Tgta* splicing | Four homozygotes with moderate phenotype; 5% normal splicing | 43 |
| L149P | Moderate phenotype; other allele not identified | 45 |
| G169R | Severe phenotype; homozygous or other allele not expressed | 45 |
| R533C and K196T | Compound heterozygote; moderately phenotypic | 44 |
| M1K and D690 frameshift | Compound heterozygote; moderately phenotypic | 44 |
| GAT to GA | Four homozygotes with moderate phenotype | 43 |
| G169R | Severe phenotype; homozygous or other allele not expressed | 45 |
| R533C and K196T | Compound heterozygote; moderately phenotypic | 44 |
| G169R | Severe phenotype; homozygous or other allele not expressed | 45 |
| R533C and K196T | Compound heterozygote; moderately phenotypic | 44 |
| G169R | Severe phenotype; homozygous or other allele not expressed | 45 |
| R533C and K196T | Compound heterozygote; moderately phenotypic | 44 |

*Upper case T is the last base of the exon followed by intron sequence.*

amplification of cDNA, the mRNA from the paternal allele appears more abundant than that from the maternal allele; (iii) the context of the CUG codon is particularly favorable for initiation as shown in Fig. 7; and (iv) initiation at this CUG codon would yield an almost perfectly normal protein. It is estimated that a non-AUG codon in a good context will function at a level of approximately 3-5% of an AUG codon in the same context (35).

The molecular basis of the LAD defect has been described for only a few patients (43–45) (Table I). Homozygosity for a splicing mutation which caused skipping of a 90-bp exon resulted in a moderate phenotype due to correct splicing of a small fraction of the mutant transcripts (43). One patient with a moderate phenotype was heterozygous for a missense mutation substituting leucine for proline at position 149 (L149P), but the mutation on the other chromosome was not identified (45). One patient with a severe phenotype carried a missense mutation of arginine for glycine at position 169 (G169R). This patient was thought to be homozygous for this mutation in particular, a carrier which did not produce a stable mRNA on the other chromosome. Both the L149P and G169R mutations were shown to cause defective association of CD18 with CD11a subunit in a COS cell expression system. The mutated leucine and glycine are conserved in all known integrin β subunits. One compound heterozygote patient with a moderate phenotype carried two missense mutations, cysteine for arginine at position 593 (R533C) and threonine for lysine at position 196 (K196T) (44). It was uncertain whether one or both alleles contributed to the moderate phenotype. With the two mutations reported here, there are now four missense mutations, one splicing mutation, one initiation codon mutation, and one frameshift mutation identified as causing leukocyte adhesion deficiency (Table I). This report also represents the first incidence of a common allele among unrelated LAD kindreds. Using ASOs, it will be possible to determine the frequency of this severe allele in the population of identified LAD patients.

Acknowledgments—Isabel Lorenzo, Bonnie Hughes, and Susan Greenwood provided technical assistance. We thank Dr. Keiko Kobayashi for helpful advice and Grace Watson for preparation of the manuscript. Blood from the family with the moderate phenotype was originally provided by Dr. Douglas Biggar.

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