0031-3998/01/4904-0537
PEDIATRIC RESEARCH
Vol. 49, No. 4, 2001
Printed in U.S.A.

Impairment of the Golgi GDP-L-Fucose Transport and Unresponsiveness to Fucose Replacement Therapy in LAD II Patients

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ABSTRACT

Leukocyte adhesion deficiency type II is an autosomal recessive syndrome characterized by generalized reduction of L-fucose in glycoconjugates; the specific molecular defect is still undefined. The most important clinical symptoms include severe growth and mental retardation and severe immunodeficiency. Patients from two ethnic groups have been reported, *i.e.* Arab and Turkish. We have observed that GDP-L-fucose transport into Golgi vesicles was specifically impaired in an Arab patient, with a significant reduction of the V_{max} but no significant differences in the K_m from control and parents. GDP-L-fucose transport showed simple saturation kinetics in all samples. Transport of UDP-galactose, UDP-*N*-acetylglucosamine, and CMP-sialic acid was comparable into vesicles from the Arab patient, parents, and control. These kinetic parameters probably account for the failure to obtain any clinical and biochemical response to fucose therapy in Arab patients. This contrasts both with the distinctive kinetic properties of GDP-L-fucose transport and with the success of fucose therapy, which have been recently reported in one patient of Turkish origin. Accordingly, the biochemical properties of GDP-L-fucose transport into the Golgi are consistent with different variants of leukocyte adhesion deficiency type II that are probably the result of different molecular defects. **(*Pediatr Res* 49: 537–542, 2001)**

Abbreviations

LAD II, leukocyte adhesion deficiency type II
GMD, GDP-D-mannose 4,6-dehydratase (EC 4.2.1.47)
BCA, bicinchohinic acid

LAD II (OMIM 266265) is a rare autosomal recessive syndrome initially described in Arab children in Israel (1–5). The disease is characterized by a set of clinical symptoms that include severe growth and mental retardation, unusual facial appearance, dwarfism, neurologic abnormalities, immunodeficiency with recurrent bacterial infections, leukocytosis (with white blood cell counts ranging from 25,000 to 150,000/mm^3), and Bombay blood phenotype (1, 2). The biochemical hallmark is a lack of expression of fucosylated glycoconjugates, including H-antigen and Lewis antigens such as Lewis X (LeX) and sialyl-Lewis X (SLeX) (1). The latter molecules are the physiologic ligands for E- and P-selectin, which mediate the initial rolling of neutrophils on the activated endothelium during the recruitment of these cells to the site of inflammation (6–9). As a consequence, neutrophils from LAD II patients are unable to interact with selectins and to roll on activated endothelial cells and are impaired in the *in vivo* chemotaxis in skin-chamber assays (10–12).

The widespread severely decreased fucosylation observed in several types of glycoconjugates suggested that the molecular defect of the disease might impair the production or subsequent utilization of GDP-L-fucose rather than the activity of specific fucosyltransferases, which were found to be normal in LAD II patients (13). Recently, Karsan *et al.* (4) proposed that the defect in Arab LAD II patients could be localized in the *de novo* GDP-L-fucose biosynthetic pathway. Indeed, the activities of the two enzymes involved in the *de novo* biosynthesis, GMD (14–16) and FX protein (17), were measured; whereas FX was found to be normal, GMD activity was significantly lower in cell lysates from LAD II patients compared with controls and exhibited kinetic abnormalities (18). However, no mutation was found in the GMD cDNA isolated from LAD II

Received September 6, 2000; accepted December 19, 2000.
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Supported in part by the Italian Ministry of Scientific Research (MURST-PRIN 98, Programma di Settore Legge 95/95 "Biomolecole per la salute umana") (A.D.F.), by CNR Target Project "Biotechnology" (M.T), and by NIH Grant R37 GM30365 (C.B.H.). M.T. and A.E. are also funded by a Joint Research Project between CNR (Italy) and MOSA (Israel).

patients, and GMD protein was present in amounts comparable to control cells, suggesting that the reduced enzymatic activity might be related to an alteration of some regulatory mechanism and not to a genetic defect directly involving this protein (18). Recently, a child of Turkish origin was described with a clinical phenotype consistent with LAD II syndrome (19). Cells from this patient display hypofucosylation of cell surface glycoconjugates, with decreased expression of SLeX on neutrophils and reduced binding to E- and P-selectins (19). Decreased GDP-L-fucose transport into Golgi vesicles was detected in cells derived from this patient (20).

Despite common symptoms and biochemical abnormalities, differences between the Arab and Turkish patients were observed. The activity of GMD was not altered in the Turkish patient (21). The Arab LAD II patients and the Turkish child responded differently to replacement therapy with oral fucose: thus, whereas a significant response was observed in the Turkish patient, both as expression of selectin ligands and as improvement of the clinical symptoms (22), no effects were observed in the two Arab children even after long-term administration of high doses of fucose (5).

These differences between the Arab and the Turkish patients prompted us to study in an Arab patient the biochemical properties of those processes, downstream of GDP-L-fucose biosynthesis, that result in the fucosylation of membrane glycoconjugates. We observed that GDP-L-fucose transport activity into the Golgi is defective. The kinetic differences underlying GDP-L-fucose transport seem to reasonably account for the outcome of L-fucose replacement therapy, which was successful in the Turkish patient and unsuccessful under the same schedule of treatment with another Arab patient. The biochemical data obtained in the present study suggest that despite the similar clinical phenotype, the Arab and Turkish patients may be affected by variants of LAD II at the biochemical and molecular level.

METHODS

Materials. GDP-[^{3}H]-fucose (610 GBq/mmol), CMP-[^{3}H]-sialic acid (1.2 TBq/mmol), UDP-[^{3}H]-galactose (250 GBq/mmol), UDP-[^{3}H]-*N*-acetyl-D-galactosamine (1.85 TBq/mmol), [^{3}H]-fucose (2.9 TBq/mmol), and [^{3}H]-*N*-acetyl-D-mannosamine (370 GBq/mmol) were purchased from NEN Life Science Product, Boston, MA, U.S.A. All other chemicals were obtained from Sigma Chemical Co. Chemicals, St. Louis, MO, U.S.A.

Labeling of cells with [^{3}H]-fucose and [^{3}H]-N-acetyl-D-mannosamine. EBV-transformed lymphoblasts (0.5×10^6/mL) from an LAD II patient of Arab origin (patient Y.W., described in Ref. 2), from his parents, and from a control subject (obtained from the same geographical area), obtained as previously described (18), were incubated for 24 h in the presence of 925 KBq/mL of [^{3}H]-fucose and 277.5 KBq/mL of [^{3}H]-*N*-acetyl-D-mannosamine. Alternatively, cells at 0.2×10^6/mL were incubated for 72 h with 140 KBq/mL [^{3}H]-fucose. At the end of the incubation, cells were collected by centrifugation at $500 \times g$ for 5 min and washed three times with ice-cold PBS. Cells were then resuspended in 0.5 mL of 1% phosphotungstic acid and 0.5 N HCl and, after 30 min in ice, proteins were precipitated by centrifugation $15{,}000 \times g$. Pellets were washed twice with H_2O and then solubilized in 1 N NaOH. Acid-precipitable [^{3}H]-fucose and [^{3}H]-sialic acid were determined by scintillation counting. Protein concentration was analyzed on the solubilized pellet by BCA assay (Pierce) and by the Bradford method (23).

Activity of the GDP-L-fucose salvage pathway. Activity of the GDP-L-fucose salvage pathway was analyzed on EBV-cell cytosols. After being washed twice with PBS, cells were lysed in Tris/HCl buffer, pH 8.0, containing protease inhibitors (0.1 μg/mL pepstatin, 0.1 μg/mL chymostatin, 0.1 μg/mL leupeptin, 0.1 μg/mL antipapain, and 1 μg/mL aprotinin) by use of a Dounce homogenizer. Lysates were then centrifuged at $100{,}000 \times g$ for 1 h at 4°C. Supernatants were then incubated at 37°C at a final protein concentration of 1 mg/mL in the presence of 100 μM [^{3}H]-fucose, 5 mM GTP, 5 mM ATP, 20 mM $MgCl_2$, and 10 U/mL inorganic pyrophosphatase. At different time points, aliquots were withdrawn, extracted, and analyzed for GDP-L-fucose formation by HPLC, as previously described (17).

[^{3}H]-fucose incorporation into GDP-[^{3}H]-fucose. EBV-transformed cells at 0.2×10^6/mL were grown for 72 h in the presence of 140 KBq of [^{3}H]-fucose. After labeling, cells were washed three times with ice-cold PBS and resuspended in 400 μL of ethanol 70%. After 15 min on ice, samples were centrifuged for 5 min at $15{,}000 \times g$, and the supernatants were subjected to descending paper chromatography (Whatmann 1MM) in ethanol/ammonium acetate (1 M, pH 7.5) (6:4). The paper strips were cut into 1-cm pieces and shaken for 2 h at room temperature in 1.5 mL of water before addition of 18 mL of scintillation liquid and counting. Standard ^{3}H-labeled GDP-L-fucose and fucose were chromatographed in parallel and used to identify the corresponding compounds in samples.

Preparation of Golgi-enriched membrane fraction and transport assay of sugar nucleotides. Golgi-derived vesicles were prepared by a modification of procedures described previously (24); all procedures were performed at 4°C. For each preparation, 2.5×10^8 cells were used; cells were pelleted by centrifugation at $500 \times g$ for 5 min, washed twice with ice-cold PBS, resuspended in 3 vol of STM buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 8.5% sucrose) containing protease inhibitors, and disrupted with a tight-fitting Dounce homogenizer. Whole cells and nuclei were removed by centrifugation at $1000 \times g$ for 10 min. The supernatant was overlaid onto a cushion of 10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, and 44.5% sucrose and was then centrifuged for 1 h at $100{,}000 \times g$ at 4°C in a SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA, U.S.A.). Golgi-enriched vesicles were collected at the 8.5–44.5% sucrose interface, centrifuged again at $100{,}000 \times g$ for 30 min, and resuspended in a small volume of STM buffer. The Golgi marker enzyme sialyltransferase was assayed as described previously (25) by using asialofetuin as acceptor.

Transport assay of nucleotide sugars into Golgi-enriched vesicles was performed as described previously (26, 27) with some modifications. Assays were performed in 0.2 mL final volume using 100 μg of Golgi vesicles protein; the reaction

mixture also contained 0.5 mM 2,3-dimercaptopropanol, an inhibitor of nucleotide pyrophosphatase (28). The rate of GDP-L-fucose uptake at different concentrations was determined maintaining the amount of radioactive nucleotide sugar constant, whereas unlabeled GDP-L-fucose ranged between 0.125 and 40 μM. The other nucleotide sugars were assessed at a concentration of 20 μM. Nonspecific adsorption of the nucleotide sugars and contamination in the extravesicular space were found to be minimal in the experimental conditions used. After 5 min at 30°C, reactions were stopped by placing samples on ice and by addition of 0.25 mL of ice-cold STM buffer. Reaction mixtures were immediately spun at 100,000 $\times$ *g* for 15 min. Pellets were washed twice with 0.5 mL of ice-cold STM buffer and dissolved in 0.6 mL of NaOH 1 N; after neutralization with 0.2 mL of 4 N HCl, samples were counted by scintillation spectrometry.

Fucose administration to an LAD II patient. A male infant of Arab origin was diagnosed as suffering from LAD II at the age of 2 wk. The parents are first-degree cousins and came from the same geographic area in Israel as the other LAD II families previously described. He received a previous oral treatment with fucose from the age of 4 wk for 6 mo (starting from 2 g/day and then decreased to 1 g/day after 1 mo of therapy) (5). For the present study, fucose (500 mg/kg five times a day) was administered orally starting at the age of 11 mo by using the same protocol described by Marquardt *et al.* (22). Informed consent was obtained from the parents before the start of therapy, and the protocol was approved by the Institutional Review Board of the Rambam Medical Center. Clinical response to therapy was assessed by blood cell counts, CD15a (SLeX) expression on neutrophils, clinical evaluation of growth and neurologic involvement, and by analysis of P-selectin expression on blood cells by flow cytometry using a P-selectin-IgG conjugate (kindly provided by Dr. Marquardt) (22).

RESULTS

Fucose replacement therapy in two Arab infants with LAD II failed to improve clinical symptoms and induced no change in neutrophil counts or surface markers (5). To rule out that different protocols of administration account for the differences in response to the treatment, we started fucose therapy in the Arab patient under study by using a protocol identical to that reported by Marquardt *et al.* (22), which proved to be useful in the Turkish patient. The child was treated for more than 4 mo without any side effects. Leukocyte count was 35,000 mm^3 before therapy, and no significant change was observed during the entire treatment period (total leukocyte counts ranging from 30,000 to 50,000 mm^3 with neutrophils representing 75 to 80% of the cells). Furthermore, no H antigen on erythrocytes or CD15a on neutrophils were expressed during treatment. Binding of P-selectin-IgG conjugate to neutrophils, a sensitive marker to detect fucose expression on cell surfaces (22), was not detected in the Arab patient even after 150 d of treatment. We were also unable to observe any change in growth patterns and neuropsychologic parameters.

Fucose metabolism was investigated in EBV-transformed lymphoblasts from an Arab LAD II patient, his parents, and control. As generalized hypofucosylation is a biochemical hallmark of cells derived from LAD II patients (1, 20), we determined whether fucose incorporation into macromolecules was affected in cells obtained from this patient. EBV-transformed lymphoblasts were grown in the presence of [^{3}H]-fucose, which can be converted to GDP-L-[^{3}H]-fucose by the salvage pathway (29, 30). As shown in Figure 1, *A* and *B*, whereas the amount of radiolabeled fucose bound to acid-precipitable macromolecules was comparable in control,

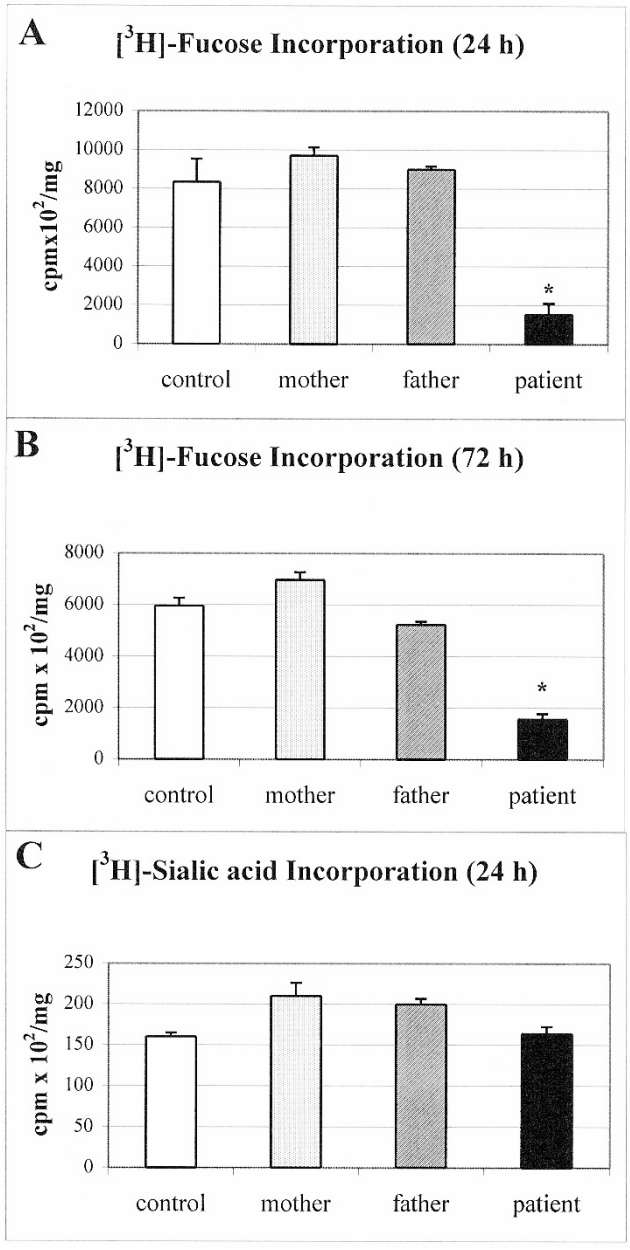

Figure 1. Radiolabeled fucose and sialic acid incorporation into macromolecules of EBV-transformed lymphoblasts. EBV-transformed lymphoblasts were grown for 24 (*panel A*) or 72 h (*panel B*) in the presence of [^{3}H]-fucose and for 24 h with [^{3}H]-*N*-acetyl-D-mannosamine (*panel C*). Macromolecules were precipitated by phosphotungstic acid, and the incorporated radioactivity was determined by scintillation spectrometry. Values represent the mean $\pm$ SEM from three different experiments. *$p < 0.005$.

mother, and father, incorporation into macromolecules in patient cells was severely impaired (20% of that observed in the other samples). The decreased incorporation of fucose was specific, because labeling with [^{3}H]-*N*-acetyl-D-mannosamine, a specific precursor for sialic acid synthesis (31), was similar in control and patient samples (Fig. 1*C*). Consistent with these data, no significant differences were observed in the activity of the GDP-L-fucose salvage pathway in the cytosolic fraction of EBV lymphoblasts from control, mother, father, and patient (0.39, 0.44, 0.71, and 0.57 nmol·h^{-1}·mg^{-1} protein, respectively).

To determine the relative cytosolic concentrations of GDP-L-fucose, cells were labeled for 72 h with [^{3}H]-fucose until a steady state labeling of the GDP-L-fucose pool was reached. As shown in Figure 2, GDP-L-[^{3}H]-fucose in patient cells was 60% higher than in control, mother, and father. These results, together with the observation that no decrease in fucosyltransferase activity was observed in the Arab patients (13), suggest that the mechanism responsible for hypofucosylation observed in patient cells is not the result of decreased cytosolic production of GDP-L-fucose in the cytosol and may likely be upstream of fucosyltransferases, *i.e.* impaired transport into the Golgi lumen.

To determine directly whether or not GDP-L-fucose transport was compromised in this patient, a Golgi-enriched fraction was obtained from EBV-transformed lymphoblasts, and transport of several nucleotide sugars was measured *in vitro*. Figure 3*A* shows that transport of GDP-L-fucose into the Golgi apparatus of patient cells was approximately 30% of that observed for mother, father, and control. Decrease was specific, because transport of CMP-sialic acid (Fig. 3*B*), UDP-galactose (Fig. 3*C*), and UDP-*N*-acetyl-galactosamine (Fig. 3*D*) was comparable in all samples. These results also demonstrate that the integrity of Golgi vesicles was comparable in every Golgi preparation and that the defect in GDP-L-fucose transport detected in the patient was not a consequence of broken

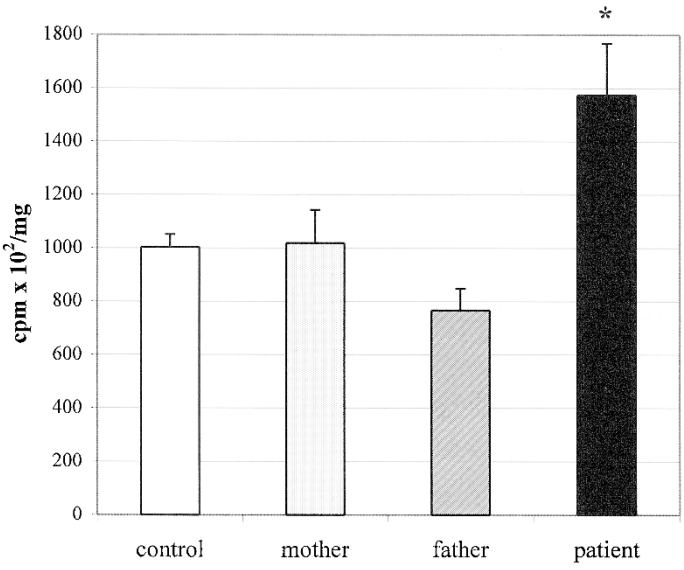

Figure 2. Radiolabeled fucose incorporation into GDP-L-fucose by EBV-transformed lymphoblasts. EBV-transformed lymphoblasts were grown for 72 h in the presence of 140 KBq/mL of [^{3}H]-fucose. After labeling, cells were washed three times with ice-cold PBS and extracted in 70% ice-cold ethanol. The supernatant was then subjected to descending paper chromatography, and the amount of radiolabel corresponding to GDP-L-fucose was determined by scintillation spectrometry. Results are the mean ± SEM of three independent determinations. *$p < 0.05$.

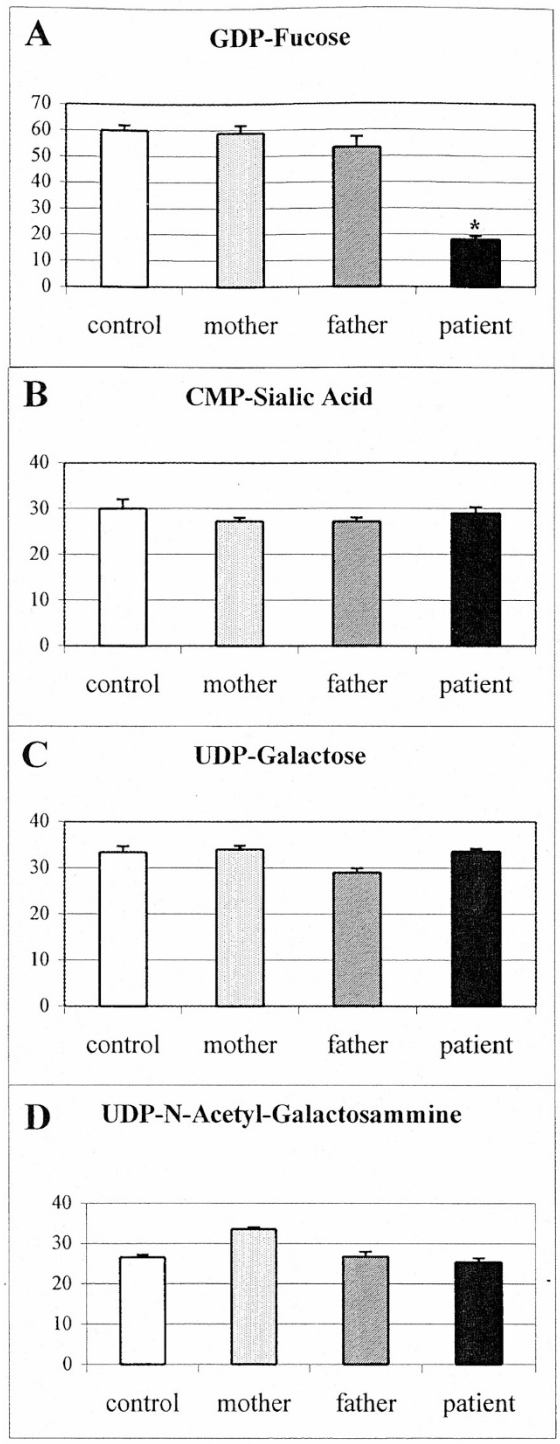

Figure 3. Transport of nucleotide sugars into Golgi-enriched vesicles of EBV-transformed lymphoblasts. Transport of GDP-[^{3}H]-fucose (*A*), CMP-[^{3}H]-sialic acid (*B*), UDP-[^{3}H]-galactose (*C*), and UDP-[^{3}H]-*N*-acetyl-galactosamine (*D*) into the Golgi-enriched fraction was determined for normal control, an LAD II patient, and his parents. Incubations were performed using 20 μM nucleotide sugars for 5 min at 30°C. Results are expressed as pmol·min^{-1}·mg^{-1} of protein and are the means of three separate experiments. *$p < 0.005$.

vesicles. Vesicles from all samples were enriched approximately 10-fold in the activity of sialyltransferase over the starting homogenate (data not shown), showing that the preparations were of comparable purity (25).

The GDP-L-fucose transport activity in all samples was saturable with the presence of only one component in the corresponding saturation curves. For the control Golgi vesicles, an apparent K_m determined by nonlinear regression of 9.5 ± 2.5 μM and a V_{max} of 56.6 ± 3.3 pmol·min^{-1}·mg^{-1} of protein was obtained (Fig. 4, *A* and *B*). Although Golgi vesicles obtained from EBV lymphoblasts of both father and mother showed kinetic parameters of GDP-L-fucose transport comparable to control vesicles, those derived from the LAD II patient displayed a significant decrease in the V_{max}, which was 18.9 ± 2.8 pmol·min^{-1}·mg^{-1} (Fig. 4), whereas the K_m of patient cells was comparable to those measured in control and parent samples as demonstrated by the double reciprocal plot (Fig. 4*B*).

DISCUSSION

LAD II syndrome is a rare autosomal recessive genetic disease initially confined to the Arab population in Israel (1–5). The four Arab patients diagnosed to date are from the same village, and some of the parents are related. Given the restricted geographical area in which these cases were found, it is likely that these patients have a common genetic defect. The recent identification in Germany of an infant of Turkish origin with a clinical presentation consistent with LAD II (19) indicates a broader distribution of the disease and suggests that the real incidence may be underestimated. Our study was motivated by the completely different outcomes of fucose replacement therapy, *i.e.* successful in the Turkish patient (22) and unsuccessful in Arab patients (five and this study). Indeed, although the main clinical symptoms that identify LAD II syndrome are similar in the Arab and Turkish patients, some differences in the clinical phenotype were observed (1, 2, 5, 19). Importantly, activity of GMD was reduced in cell lysates from Arab patients (18), whereas it was normal in the Turkish patient (21). Taken together, these data would be consistent with different molecular defects in the Turkish and Arab patients.

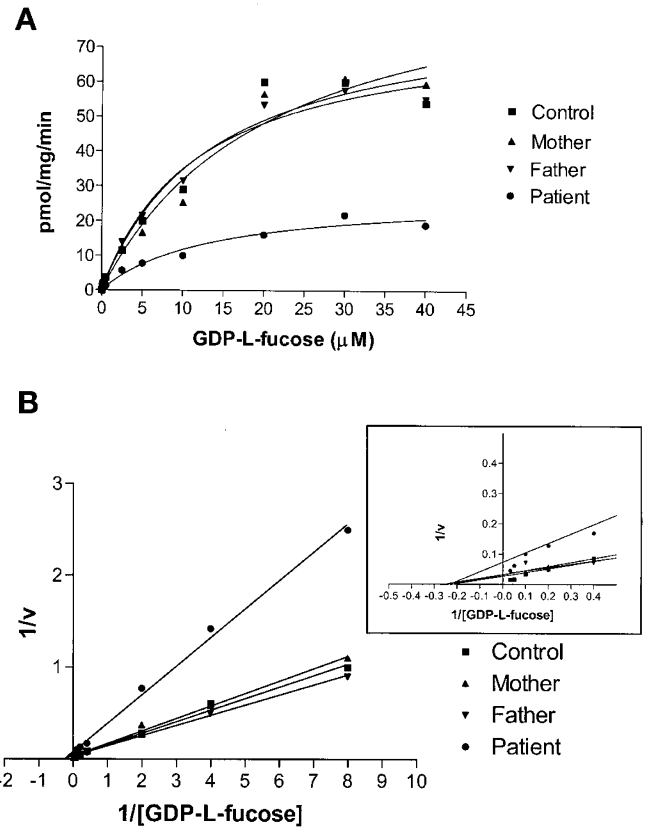

Figure 4. Rate of GDP-L-fucose transport into Golgi-enriched vesicles of EBV-transformed lymphoblasts *vs* GDP-L-fucose concentrations in the reaction medium. (*A*) Initial rates of transport were determined using increasing concentration of the unlabeled nucleotide sugar while maintaining constant the amounts of GDP-L-[^{3}H]-fucose. Incubations were performed for 5 min at 30°C. Results are the mean ± SEM of three independent determinations. (*B*) Double-reciprocal plot of the data presented in *A*. An enlarged view of the intercepts with *x* and *y* axes is shown in the *insert*.

Our biochemical studies show a decreased expression of ^{3}H-labeled fucose in macromolecules of EBV-transformed lymphoblasts and demonstrate that this decrease is not correlated with alterations in GDP-L-fucose salvage pathway activity. We have found that GDP-L-fucose transport was specifically impaired in Golgi vesicles from this Arab patient compared with the control and parents, displaying a significant and specific reduction in the V_{max} of the transport activity. GDP-L-fucose transport showed only one saturable component with a K_m of approximately 9 μM for control, patient, and parents. This result is consistent with the kinetic patterns and apparent K_m values previously measured for the rat liver Golgi transport of GDP-L-fucose (27, 32) and of other nucleotide sugars into the Golgi apparatus (33).

A defective import of GDP-L-fucose into Golgi vesicles has also been detected in the Turkish LAD II patient described in Germany (20). However, the kinetic parameters reported in that study for GDP-L-fucose transport in fibroblast-derived Golgi vesicles for both control and LAD II patient are different compared with our data. That study showed two-component kinetics with an initial saturable component followed by a nonsaturable one. The apparent K_m for the saturable component in the control was 0.7 μM, whereas the overall affinity of the transporter for GDP-L-fucose in Golgi vesicles from the patient was not estimated and appeared to be decreased in the saturable component compared with the control (20). It is not clear whether the biphasic patterns observed for GDP-L-fucose transport in the study with the Turkish LAD II cells also occur with other nucleotide sugars. Previous studies by our group using fibroblasts have always shown Michaelis-Menten kinetics (24, 34). The differences observed in transport characteristics cannot be explained by the different source of Golgi vesicles (EBV lymphoblasts *versus* fibroblasts). Preliminary data obtained in our laboratory, using Golgi vesicles from both control and LAD II patient fibroblasts, have shown kinetic parameters similar to those observed for EBV cells with an identical K_m value (our unpublished results).

Lubke *et al.* (20) proposed that the biochemical defect in the Turkish patient may be due to an abnormal K_m of the GDP-L-fucose transporter, as incubation of the patient fibroblasts with

1 mM fucose but not 1 mM mannose overcame the fucosylation defect (20). If the ability of exogenous fucose to correct the phenotype relies on increasing the cytosolic concentration of GDP-L-fucose and thereby on overcoming the consequences of an increased K_m, as inferred by Lubke *et al.* (20), then the failure of the Arab children to respond to fucose replacement therapy is consistent with our observation that the affinity of GDP-L-fucose transport in the Arab patients is comparable to his parents and the control. Addition of 10 mM fucose to EBV lymphoblasts, fibroblasts, and endothelial cells derived also from Arab subjects resulted in the expression of fucosylated epitopes on the cell surface (4). However, we speculate that the effects seen at high concentrations, approximately 50–100-fold higher than the maximal serum levels detected *in vivo* after administration of high doses of fucose (5), are not physiologic.

A decrease of GDP-L-fucose availability for the fucosyltransferases within the Golgi lumen explains the generalized reduction of fucose in the different types of glycoconjugates. However, the fact that whereas some fucosylated antigens are absent, cells derived from the patient incorporate low but detectable levels of fucose into macromolecules, indicates that not all fucose-containing glycoconjugates are comparably decreased. This is most likely the consequence of different K_m values of fucosyltransferases catalyzing the addition of fucose to selected acceptors under limiting supply of GDP-L-fucose in the lumen of the Golgi apparatus (34). At the same time, the defective import into the Golgi causes an increased cytosolic concentration of this nucleotide sugar, which may be responsible for the alterations in the kinetic properties of GMD observed in cell lysates from LAD II Arab patients (18), through feedback inhibition. In fact, GDP-L-fucose has been shown in our laboratory to behave as a potent noncompetitive inhibitor of human recombinant GMD, with a K_i of approximately 11 μM (16).

Currently, no information is available on either the gene or amino acid sequence of the mammalian GDP-L-fucose transporter. This specific transporter has been purified from rat liver and identified as an 80-kD protein with 39 kD subunits as determined by SDS-PAGE (27), strongly suggesting a homodimeric structure; this is in agreement with previously published structures for other Golgi transporters (35). Identification of the human gene encoding for the GDP-L-fucose transporter should eventually clarify whether the defect in LAD II affects the transporter structural gene, its promoter, or other regulatory components of its activity and will also elucidate the molecular mechanisms underlying possible variants of this disease.

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