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Correction of CD18-Deficient Lymphocytes by Retrovirus-Mediated Gene Transfer

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Leukocyte adhesion deficiency (LAD) is an inherited disorder of leukocyte function caused by derangements in CD18 expression. The genetic and functional abnormalities in a lymphocyte cell line from a patient with LAD have been corrected by retrovirus-mediated transduction of a functional CD18 gene. Lymphocytes from patients with LAD were exposed to CD18-expressing retrovirus and enriched for cells that express CD11a and CD18 (LFA-1) on the cell surface. Molecular and functional analyses of these cells revealed (i) one copy of proviral sequence per cell, (ii) viral-directed CD18 RNA that exceeded normal endogenous levels, (iii) normal quantities of CD11a and CD18 protein on the cell surface, and (iv) reconstitution of LFA-1–dependent adhesive function.

LEUKOCYTE ADHESION DEFICIENCY (LAD) is a rare autosomal recessive disorder that is characterized by profound abnormalities in leukocyte function and recurrent, life-threatening infections (*1, 2, 3*). Leukocytes from affected patients are deficient in three transmembrane glycoproteins that mediate many adhesion-dependent interactions in the immune system. Current management of LAD patients is aimed at treating infectious episodes with appropriate antibiotics. However, many patients die of sepsis in childhood despite aggressive supportive therapy. Partial correction of leukocyte dysfunction has been achieved in a few patients after allogeneic bone marrow transplantation from partially histocompatible normal siblings (*4*).

The glycoproteins deficient in LAD are normally heterodimers formed between one of three CD11 subunits and a common CD18 subunit; they are called LFA-1 (CD11a and CD18), Mo1 (CD11b and CD18), and p150,95 (CD11c and CD18) (*5*). Each type of subunit is encoded at a different genetic focus. The first clue concerning the specific molecular defect underlying LAD came from experiments on the biosynthesis of CD11 and CD18 subunit proteins in metabolically radiolabeled cells (*6, 7*). Leukocytes from LAD patients synthesized diminished or structurally abnormal CD18 subunit molecules suggesting that the primary defect is an abnormality in CD18 expression or function. Direct support of this hypothesis was provided by analyses of DNA and RNA from LAD patients, which identified mutations in the gene encoding CD18 (6 to 8). The observation that LAD patients are deficient in the expression of all three heterodimers suggests that CD11 processing and transport to the membrane is linked to expression of normal CD18 subunits.

An alternative approach to the treatment and potential cure of LAD is somatic gene therapy (*9*). Studies in somatic cell hybrids suggest that the introduction of a functional CD18 gene into LAD cells may reconstitute CD11 and CD18 expression on the cell surface (*10*). Clinical severity in LAD is correlated with residual CD11 and CD18 expression, suggesting that significant clinical improvement may be realized with less than complete gene reconstitution (*3*). The relative success of allogeneic bone marrow transplantation in treating LAD indicates

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that the hematopoietic stem cell would be an appropriate target cell for CD18 gene transfer (*4*). Finally, the availability of an authentic canine model of LAD should facilitate the development and testing of new genetic therapies (*11*). We describe the use of recombinant retroviruses to correct the genetic and functional defects in lymphocytes from an LAD patient.

Complementary DNA sequences spanning the entire human CD18 coding region were cloned into a retroviral vector depicted in (Fig. 1A). The vector, pEMO-CD18, contains a single transcriptional unit that is initiated at the 5′ long terminal repeat (LTR) sequence (*12*). The pEMO-CD18 plasmid was transfected into an amphotropic retroviral packaging cell line (*13*) and 25 stably transfected clones were isolated in an attempt to identify the highest virus-producing cell line (*14*). The stable transfectants were screened for production of pEMO-CD18–derived virus by Southern (DNA) blot analysis to detect and quantify proviral sequences in infected populations of cells (*15, 16*). Murine fibroblastoid cells (NIH 3T3 cells) were exposed to viral stocks from each producer cell line. Genomic DNA from each population of infected fibroblasts was isolated, digested with Kpn I, and analyzed by Southern blotting with human CD18 as a probe (*15, 16*). NIH 3T3 cells infected with virus from the highest producing cell line contained approximately one-tenth of a copy of proviral sequences per cell (Fig. 1B) (*14*). The resulting proviral-derived Kpn I restriction fragment was of the correct size (3.6 kb), an indication that no detectable rearrangements had occurred. Analysis of RNA from duplicate plates of cells revealed high levels of viral-directed CD18 (Fig. 1C).

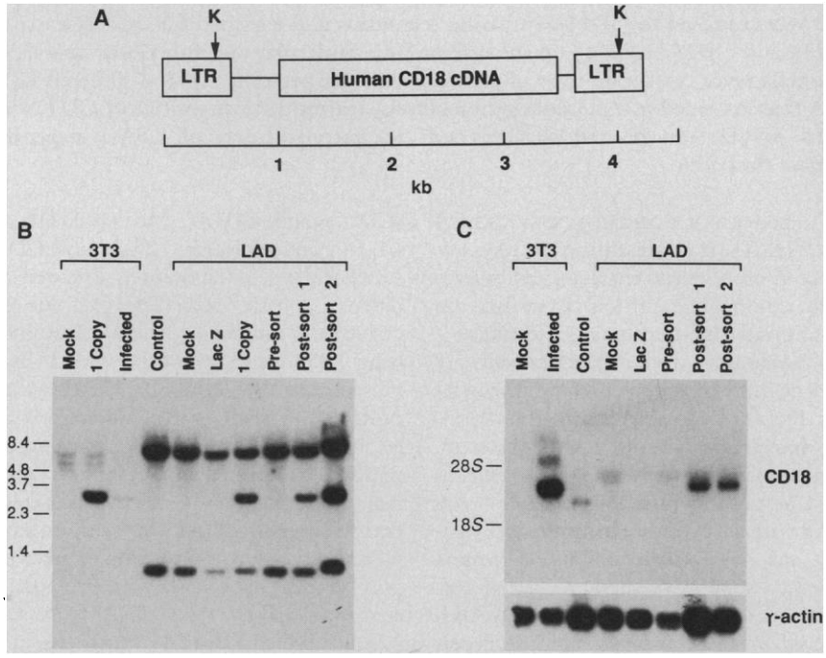

Fig. 1. (**A**) Schematic representation of the pEMO-CD18 vector. A full-length cDNA clone was cloned into an LTR-based vector which was used to make amphotropic virus (*12*). K represents Kpn I sites, and LTR represents long terminal repeat sequences. (**B**) Southern blot analysis. Total cellular DNA (10 μg) was digested with Kpn I, fractionated by agarose gel electrophoresis, and transferred to Zetabind (*15, 16*). Filters were hybridized with human CD18 cDNA probes that had been ^{32}P-labeled to high specific activity by the random priming method (*30*). DNA was obtained from 3T3 cells (lane mock, DNA from mock-infected cells; lane 1 copy, DNA from mock-infected cells supplemented with 7.5 pg of pEMO-CD18 plasmid; and lane infected, DNA from cells infected with virus from 70-18), from EBV-transformed lymphocytes derived from a normal adult (lane control), and from LAD lymphocytes [lane mock, DNA from mock-infected cells; lane lacZ, DNA from cells exposed to the lacZ-expressing virus; lane 1 copy, DNA from mock-infected cells plus 7.5 pg of pEMO-CD18 plasmid; and cells exposed to the virus from 70-18 and analyzed directly (lane pre-sort), after one round of FACS (lane post-sort 1), and after two rounds of FACS (lane post-sort 2)]. Molecular weight markers are noted as kilobases along the left margin. (**C**) Total cellular RNA (10 μg) from each population of cells was isolated, separated on formaldehyde gels, transferred to nitrocellulose paper, and hybridized with human CD18 cDNA probes as described (*15, 16*). RNA samples were isolated from 3T3 cells that were mock-infected (lane mock) or infected with virus from 70-18 (lane infected). Analyses were also performed on RNA from an EBV-transformed lymphocyte cell line derived from a normal adult (lane control), and an LAD lymphocyte cell line that was mock-infected (lane mock) or infected with virus from 70-18 and analyzed directly (lane pre-sort), following one round of FACS (lane post-sort 1), or two rounds of FACS (lane post-sort 2). The filter was stripped and reprobed with a labeled cDNA containing γ-actin sequences as described (*15*). Representative autoradiograms of the filter probed with the CD18 probe (top) and γ-actin probe (bottom) are presented.

Genetic complementation of LAD was studied with a previously described in vitro model system in which lymphocytes from normal and affected patients are immortalized with Epstein-Barr virus (EBV) prior to experimentation (subsequently referred to as control or LAD lymphocytes, respectively). The LAD lymphocyte cell line was derived from a patient (Z.J.S.) with profound abnormalities in leukocyte function and recurrent life-threatening infections [patient 9 in (*2*)]. A control cell line was derived from lymphocytes of a normal adult. Flow cytometry revealed easily detectable expression of CD18 and CD11a on the cell surface of control lymphocytes (Fig. 2, I and J) and virtually no expression on the cell surface of LAD lymphocytes (Fig. 2, A and B). RNA blot analysis revealed the expected 3.4-kb CD18 transcript in control lymphocytes and no corresponding transcript in LAD lymphocytes (Fig. 1C) (*17*).

Retrovirus-mediated gene transfer into EBV-transformed lymphocytes was optimized with an amphotropic virus that expresses a convenient reporter gene, the lacZ gene (*18*). These studies indicated that retroviral infection is most efficient when lymphoblasts are cocultivated with a virus-producing cell line for 72 hours (*19*). LAD lymphocytes were cocultivated with the cell line producing the CD18-expressing retrovirus (70-18) and subsequently analyzed by indirect immunofluorescence and flow cytometry for expression of CD18 and CD11a on the cell surface (*20, 21*). After cocultivation, a small subpopulation of fluorescent lymphocytes was detected with monoclonal antibodies (MAbs) to both CD18 and CD11a (approximately 2 to 4% of total cells) (Fig. 2, C and D). The culture was enriched for this subpopulation of cells by two rounds of fluorescent-activated cell sorting (FACS) with MAbs specific for CD18 and CD11a, sequentially (*21*). Flow cytometric analysis revealed two populations of cells with equal representation (an unstained population and a highly immunofluorescent population) following the first sort (Fig. 2, E and F), and a fairly uniform population of highly fluorescent cells after the second sort (Fig. 2, G and H).

Infected LAD lymphocytes were analyzed for the presence of proviral DNA sequences and human CD18 RNA before and after each round of FACS (described as pre-sort, post-sort 1, and post-sort 2). Enrichment of the infected population of LAD lymphocytes for cells that express surface CD18 and CD11a was associated with a coordinate enrichment in the frequency of proviral sequences (Fig. 1B) and amount of viral-directed CD18 RNA (Fig. 1C). This sug-

gests that the selected phenotype (that is, CD18 and CD11a expression on the cell surface) results from the expression of the retroviral-transduced CD18 gene. As was expected, the proportion of cells expressing CD11a and CD18, as measured by flow cytometry, accurately predicted the frequency of retroviral infection, as measured by Southern blot analysis (*22*). For example, after the second round of FACS, the cells uniformly expressed CD11a and CD18 (Fig. 2, G and H) and contained approximately one proviral copy per cell (Fig. 1B). These cells also express levels of viral-directed CD18 RNA exceeding that of the endogenous transcript in normal lymphocytes (Fig. 1C). The viral-directed transcript is longer than endogenous CD18 mRNA because of sequences that have been added to the 5′ and 3′ untranslated regions of the chimeric viral RNA (Fig. 1A).

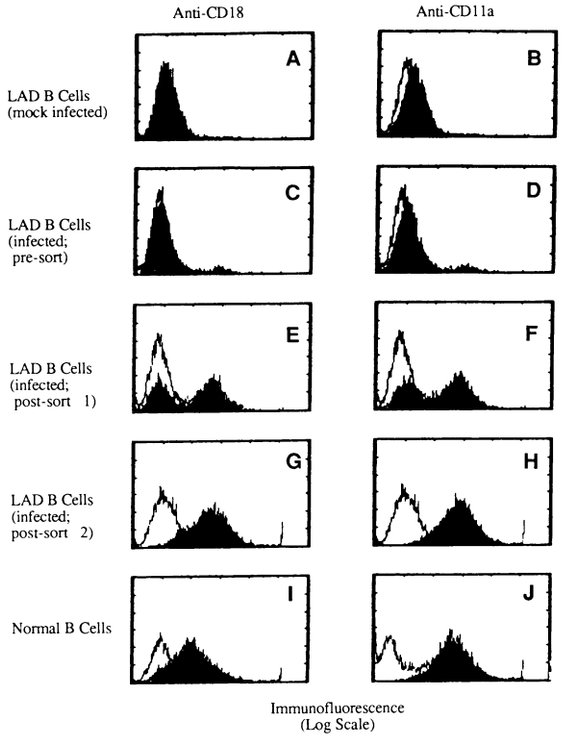

Fig. 2. Flow cytometric analysis of normal and LAD lymphocytes. Cell surface expression of CD11a and CD18 was measured in EBV-transformed lymphocytes by indirect immunofluorescence staining and flow cytometric analysis (*20, 21*). Each cell population was assayed for cell surface expression of CD18 with a monoclonal antibody to CD18 (anti-CD18) [CLB-LFA-1/1 (*31*), first column] and CD11a with an anti-CD11a MAb [2F12 (*32*), second column]. These cell lines included LAD lymphocytes that were mock-infected (**A** and **B**) or infected with virus from 70-18 and analyzed directly (**C** and **D**), after the first FACS selecting for CLB-LFA-1/1–positive cells (**E** and **F**), or after the second FACS selecting for 2F12–positive cells (**G** and **H**). An EBV-transformed lymphocyte cell line derived from a normal adult was also analyzed (**I** and **J**). Data are presented as individual histograms of cell frequency versus immunofluorescence (log scale). In each histogram, indirect immunofluorescence staining with the CD18 and CD11a reagents (blackened histogram) is compared to indirect immunofluorescence staining by isotype-identical negative controlled MAb (open histogram).

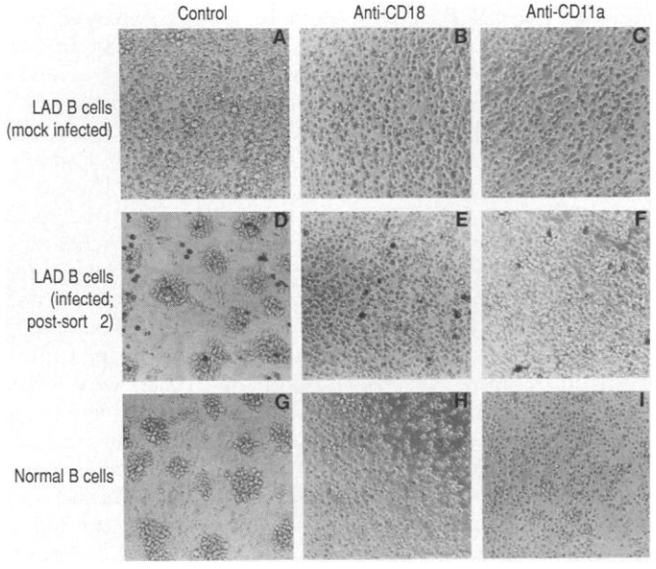

Fig. 3. Qualitative assay of CD11a- and CD18-modulated lymphocyte aggregation. Cell lines were incubated with PMA for 60 min in the presence of various MAbs (first column, isotype-identical control MAb; second column, anti-CD18 MAb, CLB-LFA-1/1; and third column, anti-CD11a MAb, 2F12) in the form of ascites (1:100 dilution) as described previously (*23*). Cells were then visualized through an inverted Nikon Diaphot microscope. Representative micrographs are presented. Analyses were performed on LAD lymphocytes that were mock-infected (**A**, **B**, and **C**) or infected with 70-18 virus and sorted twice (**D**, **E**, and **F**), as well as normal lymphocytes (**G**, **H**, and **I**).

Expression on the cell surface of functional CD11a-CD18 heterodimers was assayed on the basis of LFA-1–mediated aggregation of phorbol myristate acetate (PMA)–stimulated lymphocytes (*23*). Exposure of normal lymphocytes to PMA leads to the formation of large cellular aggregates (Fig. 3G). This aggregation is dependent on LFA-1 expression because it can be blocked with MAbs to either CD18 or CD11a (Fig. 3, H and I). Lymphocytes derived from LAD patients did not exhibit aggregation (Fig. 3A). Exposure of retroviral-transduced LAD lymphocytes to PMA resulted in the formation of large aggregates of cells indicating that expression of the recombinant CD18 gene fully reconstituted homotypic aggregation (Fig. 3D). This conclusion is supported by the observation that MAbs to CD11a and CD18 block aggregation in these cells (Fig. 3, E and F).

LAD has been considered a candidate for bone marrow–directed gene therapy (*1, 2*). Our experiments contribute to the development of such therapies because they demonstrate the feasibility of reconstituting normal adhesion function in cultured LAD cells by retrovirus-mediated transduction of a normal CD18 gene. The vector used in our study drives expression of CD18 from a viral LTR which contains transcriptional elements that possibly confer expression in a constitutive and nonrestricted manner. Identical results have been obtained with a higher titer amphotropic virus derived from a vector that expresses CD18 from another strong promiscuous promoter, the 5′ flanking sequences of the chicken β-actin gene (*24*). These kinds of vectors, in fact, may be sufficient to reconstitute the normal regulation of CD11 and CD18 expression that occurs during the inflammatory response in vivo because the majority of this regulation is posttranscriptional (*25*). However, the CD18 gene appears to be transcriptionally regulated in a lineage-specific manner and during myeloid development (*26*). The potential effects of ectopic and unregulated expression of CD18 on hematopoietic development and function, such as may occur in a recipient of genetically modified stem cells, must ultimately be studied in an appropriate animal model.

A major obstacle to the use of gene therapy for LAD now becomes efficient retrovirus-mediated gene transfer into the human hematopoietic stem cell. We and others have developed methods for efficiently infecting murine hematopoietic stem cells with replication-defective virus; however, progress in the human system has been slow (*16, 27*). Recent advances in our understanding of human hematopoiesis (*28*) and

the availability of putative human stem cell assays (*29*) provide an important framework to begin studies on gene transfer into human stem cells. Additional improvements in gene transfer may be realized through the use of higher titer amphotropic viruses or the development of techniques to enrich for transduced LAD stem cells such as those based on cell surface expression of CD11 and CD18.

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12. Viral sequences were derived from a wild-type Moloney provirus in which sequences between the Pst I site at nucleotide 563 [see (*33*) for numbering] and the Cla I site at nucleotide 7674 were removed and replaced with synthetic Bam HI linkers (*33*). A full-length human CD18 cDNA clone was reconstructed in puc18 using two non-overlapping Eco RI fragments derived from previously described λgt11 clones (*34*). Digestion of this plasmid with Hind III (located in the polylinker next to the 5′ Eco RI site) and Nae I (located in the 3′ untranslated region) released a 2500-bp fragment that spanned all of the CD18 coding sequence. The 5′ Hind III site and 3′ Nae I site were converted to Bcl I sites with synthetic oligonucleotide linkers and the fragment was cloned into the Bam HI site of the retroviral vector. The B2 mutation was introduced into the primer binding site (*35*).
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Cell Cycle Dependence of Chloride Permeability in Normal and Cystic Fibrosis Lymphocytes

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Cystic fibrosis (CF) is a genetic disease characterized by abnormal regulation of epithelial cell chloride channels. Nonepithelial cells, including lymphocytes and fibroblasts, may exhibit a similar defect. Two independent techniques were used to assess the macroscopic chloride permeability (P_{Cl}) of freshly isolated B lymphocytes and of B and T lymphocyte cell lines. Values for P_{Cl} increased specifically during the G_1 phase of the cell cycle and could be further enhanced by increasing intracellular adenosine 3′,5′-monophosphate (cAMP) or calcium. In lymphocytes from CF patients, regulation of P_{Cl} during the cell cycle and by second messengers was absent. Characterization of the cell cycle–dependent expression of the chloride permeability defect in lymphocytes from CF patients increases the utility of these cells in the analysis of the functional consequences of mutations in the CF gene.

MANY OF THE SALT TRANSPORT ABnormalities of cells from patients with CF are due to impaired second messenger activation of Cl^- permeability (P_{Cl}) (*1*). Patch-clamp studies of airway cells reveal a defect in the activation of an outwardly rectified Cl^- channel, both in response to agonists that act by means of cAMP as measured during cell-attached recording and in excised, inside-out membrane patches exposed to cAMP-dependent protein kinase. In single-channel studies, a similar Cl^- channel was identified in B and T lymphocyte cell lines that was also defective in its activation (*2*). However, these results were difficult to confirm, either because of low channel density (*2*) or failures in channel activation by agonists (*3*). To avoid the sampling problems inherent in single-channel measurements, we used two independent techniques to assess the macroscopic Cl^- permeabilities in normal lymphocytes and those from CF patients: fluorescence digital imaging microscopy (FDIM)

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