RAPID COMMUNICATION

Transfer of the ADA Gene Into Human ADA-Deficient T Lymphocytes Reconstitutes Specific Immune Functions

By Giuliana Ferrari, Silvano Rossini, Nadia Nobili, Daniela Maggioni, Angela Garofalo, Raffaella Giavazzi, Fulvio Mavilio, and Claudio Bordignon

Peripheral blood lymphocytes obtained from a patient affected by adenosine deaminase (ADA) deficiency and severe combined immunodeficiency were infected with a retroviral vector containing two copies of a human ADA minigene and injected into bg/nu/xid (BNX) immunodeficient mice. Six to 10 weeks after injection, human T cells were cloned from the spleens of recipient animals and analyzed for proliferative potential, T-cell surface markers, expression of ADA activity, integration of retroviral sequences, T-cell receptor (TCR) gene rearrangement, and specificity of antigen recognition. Effective gene transfer and expression restored proliferative potential in vitro and long-term survival in vivo. All clonable human T lymphocytes obtained from the spleen of recipient animals had high levels of vector-derived ADA enzyme activity and showed predominantly the CD4+ phenotype. Retroviral integrations and TCR-β gene rearrangements demonstrated the presence of a variety of different clones in the spleens of recipient mice. Furthermore, the combined analyses of vector integration and TCR rearrangement provided evidence that a circulating progenitor cell was transduced by the retroviral vector, giving rise to different and functional TCRs. Evaluation of antigen-specificity demonstrated both alloreactive and foreign antigen specific immune responses. These results suggest that restoration of enzyme activity in human ADA-deficient peripheral blood T cells by retroviral-mediated ADA gene transfer allows in vivo survival and reconstitution of specific immune functions. Therefore, retroviral vector-mediated gene transfer into circulating mononuclear cells could be successful not only in maintaining the metabolic homeostasis, but also for the development of a functional immune repertoire. This is a fundamental prerequisite for the usage of genetically engineered peripheral blood lymphocytes for somatic cell gene therapy of ADA deficiency. © 1992 by The American Society of Hematology.

DEFICIENCY OF adenosine deaminase (ADA) results in severe combined immunodeficiency (SCID), a lethal disorder treated with either allogeneic bone marrow transplantation1-2 or enzyme replacement therapy.3,4 Somatic cell gene therapy of this disease is currently on clinical trial after approval of a protocol involving use of autologous peripheral blood lymphocytes (PBLs) in which a normal ADA gene is transduced by a retroviral vector.5,6 Several studies in murine, nonhuman primate, and human cells had indicated that retroviral vectors can be used for efficient gene transfer and expression of the human ADA gene.7-13 We recently demonstrated that ADA-deficient (ADA-) PBLs from SCID patients transduced with a retroviral vector for human ADA survived and developed in immunodeficient mice with efficiency similar to that of PBLs obtained from normal individuals.14 Human cells were not detected in mice injected with uninfected ADA-PBLs, indicating that intracellular synthesis of vector-derived enzyme was necessary for long-term survival in vivo. Furthermore, preliminary indications were obtained that expression of normal levels of enzyme activity could restore some immune functions in ADA- PBLs.14

In this report, we analyze in detail human T cells rescued from the spleens of bg/nu/xid (BNX) immunodeficient mice reconstituted with PBLs from an ADA- SCID patient, after gene transfer with the retroviral vector DCA.15 Human T cells were expanded and cloned in vitro by phytohemagglutinin (PHA)/interleukin-2 (IL-2) or antigen stimulation and analyzed for ADA expression, retroviral integration, T-cell receptor (TCR) gene rearrangement, and specificity of antigen response. Our results show that restoration of ADA activity in ADA- PBLs by gene transfer allows reconstitution of specific immune functions such as proliferative response to alloantigens, tetanus toxoid (tt), and Tick-borne encephalitis virus (Fruhsommer-Meningoencephalitis virus, FSME) viral antigens. Different rearrangements of the TCR-β genes were demonstrated in all different T-cell clones. This, together with the analysis of retroviral integrations, provided evidence that gene transfer in a T-cell progenitor may be achieved by transduction of mononuclear cells obtained from the peripheral blood of patients affected by ADA- SCID.

MATERIALS AND METHODS

Cell preparation and virus infection. ADA- PBLs were obtained from a 4-year-old ADA- SCID patient under parental informed consent. The diagnosis was confirmed by immunologic tests and dosage of ADA activity in the mononuclear peripheral blood cells and erythrocytes, which ranged between 0.2% and 1.1% of normal controls. At the time of the described experiments, the patient was treated with pegylated-enzyme (PEG-ADA; Enzon, South Plainfield, NJ) replacement therapy and had normal lymphocyte counts. PBLs were obtained by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient separation and subjected to multiple infection cycles with amphotropic, cell-free viral stock in the presence of polybrene (4 μg/mL), at high vector to cell ratio (2 to 5 cfu/cell) under PHA/IL-2 stimulation (2 μg/mL of purified PHA, Wellcome

From www.bloodjournal.org by guest on October 25, 2017. For personal use only.
Laboratories, Dartford, UK; 100 U of recombinant human (rh) IL-2, Roche, Nutley, NJ). Structure of the DCA retroviral vector, containing two copies of a human ADA minigene in the proviral form, and derivation of a high-titer amphotropic producer cell line were previously described. After completion of the multiple infection cycles, PBLs were resuspended in phosphate-buffered saline (PBS) and injected into recipient mice.

Mice. NIH-Beige-Nude-XID (bg/bg nu nu x4/t4, BNX) mice were bred and maintained in accordance with institutional guidelines in our animal facility. Throughout these experiments, mice were used at the ages of 6 to 10 weeks and received no additional cytoreduction or immunosuppression before reconstitution with human cells. Animals were injected intraperitoneally (IP) with a cell dose of 2 to 10^4 PBLs/animal, and human cell survival was monitored by dosing human IgG serum levels. Six to 10 weeks after injection, mice were killed and peripheral blood and spleen were used for further analyses.

T-cell cloning and phenotyping. Cell suspensions were obtained by mincing and filtering mouse spleens through a sterile gauze. Cells were cultured in Terasaki microplates with RPMI-1640 supplemented with 2 mmol/L L-glutamine, 1% nonessential amino acids, 1% Na pyruvate, 50 mg/mL kanamycin (all from GIBCO, Grand Island, NY), 5% human serum (HS), and 100 U/mL rhIL-2, under limiting dilution conditions, according to the method of Lanzavecchia. Cell concentrations (10^4, 10^5, 10^6, 10 cells/well) were chosen according to the expected frequencies of human cells in the spleens of reconstructed animals, as assayed by DNA dot-blot analysis and hybridization to human Alu sequences. After 1 to 2 weeks positive wells were scored and cells transferred to 96-well flat-bottom plates. The clones were maintained in complete RPMI-medium supplemented with 5% HS and 100 U/mL rhIL-2 and restimulated every 2 to 3 weeks with irradiated (3,000 R) allogeneic PBLs. Phenotype of clones was determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated anti-human-CD4 (T4) and CD8 (T8) monoclonal antibodies (MoAbs) (Coulter Immunology, Hialeah, FL), Isotype-matched FITC-conjugated MoAbs were used for all analyses.

Thin-layer chromatography (TLC) analysis of ADA activity. ADA enzyme activity was analyzed by the [14C]-adenosine to [14C]-inosine conversion assay followed by TLC. Cell lysates from individual clones (~ 2 x 10^6 cells) were normalized for protein content. Positive and negative controls were, respectively, lysates from normal PBLs and uninfected, IL-2-stimulated ADA- PBLs, because IL-2 stimulation is reported to increase the efficiency of ADA expression in ADA- cells. After 1 to 2 weeks positive wells were scored and cells transferred to 96-well flat-bottom plates. The clones were maintained in complete RPMI-medium supplemented with 5% HS and 100 U/mL rhIL-2 and restimulated every 2 to 3 weeks with irradiated (3,000 R) allogeneic PBLs. Phenotype of clones was determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated anti-human-CD4 (T4) and CD8 (T8) monoclonal antibodies (MoAbs) (Coulter Immunology, Hialeah, FL), Isotype-matched FITC-conjugated MoAbs were used for all analyses.

DNA analysis. High molecular weight DNA was isolated from T cells by standard phenol-chloroform extraction, digested to completion in 5-μg aliquots, with restriction enzymes (GIBCO-BRL, Grand Island, NY), electrophoresed in a 0.8% agarose gel at 1.5 V/cm in Tris-acetate-EDTA buffer, transferred to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK) by Southern capillary blotting,19 and hybridized to 10^6 dpm of 32P-labeled probe. DNA probes were a 1.2-kb HindIII-Smal fragment of pSV2neo20 and the YT-2 full-length cDNA clone of the human TCR-β,21 Filters were washed under high stringency conditions and exposed to a Kodak X-AR5 film (Eastman Kodak, Rochester, NY) at -70°C.

Antigen-specific stimulation and proliferative assay. For in vivo stimulation, 50 μg/mL of tetanus toxoid (Swiss Serum Institute, Bern, Switzerland), together with 5 x 10^5 ti-pulsed irradiated ADA- autologous (to the human donor) PBLs as antigen-presenting cells, were injected IP into a recipient BNX mouse 6 weeks after injection of DCA-infected ADA- PBLs. Two weeks later, spleen cells were obtained and 10^5 cells/well were stimulated in 96-well flat-bottom plates in complete RPMI-medium supplemented with 5% HS, with 5 x 10^6 tt-pulsed irradiated (3,000 R) autologous PBLs per well, and 20 μg/mL tt. Growing T-cell lines were maintained in complete RPMI-medium with 5% HS and 100 U/mL rhIL-2, and restimulated every 2 to 3 weeks under the same conditions. For proliferation assay 4 x 10^4 T cells were cultured with 10^6 irradiated autologous PBLs, either untreated or pulsed overnight with tt (20 μg/mL) or FSME (0.5 μg/mL; IMMUNO AG Wien, Austria) in 200 μL complete RPMI-medium with 10% fetal calf serum (FCS) in 96-well flat-bottom microplates. After 48 hours the cultures were pulsed with 1 μCi/well of 3H-thymidine (Amersham, specific activity 5 Ci/mmol/L) and the radioactivity incorporated was measured after an additional 16 hours. To analyze proliferative responses to an allogeneic stimulator, T cells were cultured with irradiated human 5 x 10^6 allogeneic PBLs for 5 days, and proliferation was tested as above.

RESULTS
We recently used a human PBL/immunodeficient mouse model22-24 demonstrating that efficient ADA gene transfer and expression was the necessary prerequisite for long-term in vivo survival of ADA deficient PBLs.25 To analyze whether this gene transfer procedure would restore the functional properties of the transduced lymphocytes, we extended the use of this model. Thirty-eight clones were obtained in two separate cloning experiments. All clones were positive for vector-derived ADA activity, and they were analyzed for the expression of cell surface antigens. Positivity to either the human CD4 or CD8 was observed. The relative proportion of CD4+ and CD8+ cells was comparable with that shown by 89 clones obtained from the spleens of four controls BNX mice injected with normal adult PBLs from two different donors (Table 1), thus suggesting that the gene transfer procedure by itself did not modify the representative repertoire of transduced cells. This was further supported by the preliminary evaluation of TCR-Vβ genes usage by anchored polymerase chain reaction (PCR)25 in PBLs before and after vector transduction (data not shown).

Table 1. Cell Surface Phenotype of Human T-Cell Clones Obtained From BNX Mice After Reconstitution With DCA-Transduced Human ADA- PBLs or Normal Untransplanted Human PBLs

To determine the number and site of vector integrations in transduced cells, a number of clones was expanded for Southern blot analysis of proviral sequences integrated in genomic DNA. Specifically, DNAs were digested with XbaI, which cuts in both 5' and 3' LTRs of DCA, and with HindIII, which does not cut in the proviral genome, and hybridized to a Neo-specific probe (Fig 1b). A single 5.3-kb band was detected, indicating that the proviral DNA is integrated as an excised unit into the human chromosome. This was further supported by the preliminary evaluation of TCR-Vβ genes usage by anchored polymerase chain reaction (PCR)25 in PBLs before and after vector transduction (data not shown).

Normal PBLs: donor no. 1 646 11 1 1 1 1 1 1
Normal PBLs: donor no. 2 631 38 22 38 (B2) DCA-infected ADA- PBLs 751 3

Proportion of CD4+ and CD8+ clones was determined by flow cytometry using FITC-conjugated anti-human-CD4 and CD8 MoAbs. Isotype-matched FITC-conjugated MoAbs were used for all analyses.
Fig 1. Southern blot analysis of integration of DCA proviruses in T cells cloned from a BNX mouse reconstituted with DCA-transduced ADA-PBLs. (a) HindIII restriction pattern of six representative clones (left) and XbaI, BamHI, and BglI patterns of two clones showing identical integration bands (right). A NeoR specific DNA fragment was used as probe. DNA from the DCA-producer AM-12 line is shown as positive control (C). Bands corresponding to rearranged proviruses are indicated by asterisks. A λ-phage HindIII digest was used as size marker. Sizes are in kilobase pairs. (b) Schematic map of integrated DCA proviral genome. 19 Human ADA promoter (arrowhead-shaped) and cDNA sequences inserted in the 5' and 3’ long terminal repeats (5’ LTR and 3’ LTR) and the NeoR cDNA are indicated. Black boxes denote LTR sequences. XbaI(X), BamHI(B), and BglI(Bg) restriction sites in the DCA provirus are indicated. The 5.3-kb diagnostic XbaI fragment and the NeoR probe used for hybridization are shown below the DCA map.

band, corresponding to the expected size for the intact integrated provirus, was observed in the XbaI digest of all clones (data not shown). When DNA was digested with HindIII, one major band (eg, in the CD4+ clones no. 56, 45, 70, and 82 and in the CD8+ clone no. 15) or two major bands (eg, in the CD4+ clone no. 89) were obtained, indicating the presence of a very low number of integrated provirus per cell, as well as the essentially clonal nature of the cells expanded in vitro (Fig 1a). In one case (clones no. 70 and 82) the HindIII pattern appeared identical, including the presence of minor bands of lower molecular weight, probably caused by integration of rearranged proviruses (Fig 1a). The restriction patterns obtained by XbaI, BamHI, and BglI, which all cut twice in the proviral genome (Fig 1b), confirmed the presence of rearranged proviruses of the same apparent molecular weight in both clones. This was considered a marker of clonality, and therefore indicated that the two clones have identical genotype.

The heterogeneity of the transduced clones was evaluated by analysis of TCR-β rearrangement using HindIII and EcoRI restriction patterns, which in most cases discriminate between germline and rearranged TCR-β constant chain genes.26,27 This analysis showed that the patient is heterozygous for an HindIII polymorphism26 that gives rise to an ~14-kb band in addition to the 8.5- and 3.8-kb bands containing the β2 and β1 loci, respectively (Fig 2). A variety of TCR-β rearrangements was found in the analyzed clones, indicating the presence of a repertoire in the population of the transduced T lymphocytes. Interestingly, clones no. 70 and 82, which share a common proviral integration pattern (Fig 1a), showed two different patterns of TCR-β germline rearrangement. It is relevant to notice that both clones showed functional TCR activity because both responded specifically to α-antigen stimulation (data not shown). These results provide strong evidence that vector transduction occurred in a circulating T-cell progenitor in a maturation stage preceding TCR rearrangement. Maturation of this cell in at least two different progeny

Fig 2. Southern blot analysis of TCR-β rearrangements in DCA-transduced T-cell clones. HindIII (left) and EcoRI (right) restriction patterns are shown, after hybridization to a full-length human TCR-β cDNA. DNA from an Epstein-Barr virus-transformed lymphoblastoid cell line (C) represents control germline patterns. Size markers are as in Fig 1.
We recently used a human PBL/immunodeficient mouse model, demonstrating the potential application of transduced PBLs for gene therapy of ADA deficiency. PBLs derived from an ADA-SCID patient were infected in vitro with the retroviral vector DCA and injected into immunodeficient BNX mice. In these animals we demonstrated long-term survival of human B and T cells. To analyze the population of vector-transduced ADA− human cells surviving in BNX mice, we expanded human T lymphocytes from the spleens of two animals by in vitro stimulation with PHA and IL-2. All but one of the T-cell clones obtained showed high levels of vector-derived ADA activity, as measured by adenosine to inosine conversion (Fig 3B).

**Table 2. Antigen-Specificity of Human CD4+ Lymphocytes Obtained From the Spleen of BNX Mice Injected With DCA-Transduced ADA+ PBLs**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Medium</th>
<th>Autologous APCs</th>
<th>Allogeneic Stimulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>727G2</td>
<td>-tt</td>
<td>160</td>
<td>2,760</td>
</tr>
<tr>
<td>727G36</td>
<td>+tt</td>
<td>1,260</td>
<td>15,313</td>
</tr>
<tr>
<td>727F1</td>
<td>-FSME</td>
<td>593</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>+FSME</td>
<td>593</td>
<td>2,870</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>593</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>593</td>
<td>2,870</td>
</tr>
</tbody>
</table>

Representative experiments performed with three different human T-cell lines (G2, G36, and F1) from the spleen of the same recipient mouse (727) are shown. ADA− PBLs used to reconstitute the 727 recipient mouse were obtained from a patient undergoing enzyme replacement therapy. The donor patient, in treatment with PEG-ADA (see Materials and Methods), had been vaccinated against both antigens. The rationale of the experiment was to demonstrate transduction, and therefore survival in vivo, of human antigen-specific T cells by using tt or FSME for stimulation and expansion in vitro. Table 2 shows representative experiments performed with three different human T-cell lines (G2, G36, and F1) from the spleen of the same recipient mouse. Depending on in vitro challenge with antigen plus antigen-presenting cells, antigen restricted proliferation against tt, FSME, and alloantigens was obtained. Finally, to analyze the frequency of antigenic response at clonal level, a reconstituted BNX mouse was injected with tt plus tt-pulsed irradiated PBLs from the patient as antigen-presenting cells. Two weeks later, spleen cells were obtained and restimulated in vitro, always in the presence of tt-pulsed, irradiated PBLs. The large majority (90.9%) of T-cell clones obtained showed specific proliferative response to tt-pulsed, but not to un pulsed, APCs (Fig 3A). All of these T-cell clones also showed high levels of vector-derived ADA activity, as measured by adenosine to inosine conversion (Fig 3B).
transduction further supports the view that vector-mediated gene transfer does not impair the repertoire of the transduced cells. Moreover, we show that gene transfer may be achieved in progenitors by infection of PBMCs with retroviral vectors. This body of evidence should further support the assumption that gene therapy of ADA deficiency into peripheral blood lymphocytes should lead to successful and lasting development of a functional T-cell repertoire.

REFERENCES

15. Hantopoulopoulo PA, Sullenger BA, Ungers G, Gilboa E: Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. Proc Natl Acad Sci USA 86:3519, 1989

ACKNOWLEDGMENT

Special thanks go to Paola Panina and Antonio Lanzavecchia for help in the cloning of human T cells from the spleen of recipient mice; to A. Ugazio, L. Notarangelo, and the staff of the Pediatric Clinic of the University of Brescia for the continuing collaboration during this study; to C. Hansen for the BNX breeding pair; and to Coulter Italy for help and support.
Transfer of the ADA gene into human ADA-deficient T lymphocytes reconstitutes specific immune functions

G Ferrari, S Rossini, N Nobili, D Maggioni, A Garofalo, R Giavazzi, F Mavilio and C Bordignon