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Long-Term Expression of Gene Introduction Into Normal Human T-Lymphocytes by Retroviral-Mediated Gene Transfer

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Abstract Human T-lymphocytes are long lived, easily accessible, mature, and capable of proliferation. They are theoretically a suitable target for retroviral mediated gene transfer. To test this hypothesis, normal human T-cells obtained from bone marrow and peripheral blood were stimulated with phytohemagglutinin (PHA) and infected 24 h later with the retroviral vector N2 which carries the bacterial neo gene. T-lymphocytes were propagated in culture for up to 14 weeks with interleukin-2 (IL-2). Analysis by whole cell RNA dot/blot using a single stranded RNA probe demonstrated persistent expression of the neo gene. Preliminary functional studies revealed that both helper and suppressor functions were preserved in the infected cells in culture. These results demonstrate that normal T-cells are capable of long-term expression of genes introduced by retroviral mediated gene transfer and are potential target cells for somatic gene therapy.

Key words: bone marrow, peripheral blood, gene transfer, IL-2, neo gene

Transferring new genetic information into hematopoietic cells provides a powerful approach to understanding the events governing the developmental program of normal hematopoietic stem cells. Furthermore, the correction of certain genetic defects may be possible by the insertion of a functional gene into reconstituting human hematopoietic cells, since the hematopoietic system is arranged as a hierarchy where the mature cells arise from a pool of rare stems cells. Both of these long-term goals require high-efficiency gene transfer technologies. Retrovirus vectors offer the most efficient method of introducing genes into hematopoietic cells at the present time [1–3]. Although most work has been carried out using murine hematopoietic cells, retroviruses carrying selectable genes have been transferred into progenitor cells from several species including human [4–7].

In most of these reports gene transfer and expression have been assayed by selecting for drug-resistant colonies which grow in culture over the course of 14 days [3]. The murine system has provided the most information regarding long-term expression [8]. While several studies have demonstrated gene expression in long-term reconstituted animals up to 1 year, long-term expression has been a problem with a number of other retroviral constructs. Although the exact nature of this apparent down regulation of gene expression is not known, it may be due to differentiation of stem cells [9,10].

The development of protocols to test the feasibility of gene therapy requires that the newly introduced genes be expressed for long periods of time in the appropriate cell population. The problem is that most human progenitor cell assays only test for expression over 14 days. Long-term marrow culture, which assays for stem cells that are capable of giving rise to generally myeloid cells, can be maintained for 6–8 weeks. However, since ADA-deficient severe combined immunodeficiency (SCID) will likely be an important model system [11,12] in which to test gene therapy, it will also be important to assess long-term expression in lymphoid cells, specifically T-cells. In this report we have demonstrated high-efficiency gene transfer and long-term expression in normal bone marrow derived T-cells up to 14 weeks in continuous culture. These infected T-cells retain normal T-cell phenotype and function. Gene transfer into T-cells or their progenitors may provide a gene therapy

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alternative to pluripotent stem cells. These experiments lay the foundation for considering this alternative.

METHOD AND MATERIAL

Retroviral Vector

The N2-vector containing the neo gene, which confers resistance to the antibiotic G418, has been described in detail [14]. Infectious but replication defective virus was produced by initial transfection of the vector into PSI-2 helper cells with subsequent infection of the amphotropic helper cell line PA317 by virus containing supernatants [13,14]. Amphotropic viral titres were $\geq 2 \times 10^5$/ml when assayed on the human ovarian carcinoma cell line HOC-7 as previously described [14].

Infection Protocol

Peripheral blood or bone marrow was obtained from normal volunteers after informed consent had been given. Light-density (less than 1.077) mononuclear cells were collected by Ficoll-Hypaque density gradient centrifugation. Cells were suspended in IMDM (Gibco, Grand Island, NY) with 10% fetal calf serum (Flow Labs, Mississauga, Ontario), 1% PHA (Wellcome, Dartford, England) and were either cocultivated with irradiated (1,500 rads) PA317N2 or PA317 fibroblasts for 24 h or exposed to equal volumes of supernatant from PA317N2 or PA317 fibroblasts [7].

Long-Term T-Cell Cultures

Cells were maintained in liquid culture using a medium of RPMI 1640 (Gibco, Grand Island, NY), 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin. Aliquots of cell cultures exposed to the neo containing virus were grown in G418 in varying concentrations from 400 μg/ml to 1,000 μg/ml (actual G418 concentrations) for periods of time ranging from 1 to 4 weeks. All cultures were supplemented with interleukin-2 (Cetus, Emeryville, CA), 20 units/ml twice weekly. Cultures were propagated up to 16 weeks and then were harvested for RNA analysis [15].

Whole Cell RNA Blotting

The technique has been described earlier [4]. Briefly, cells were harvested, counted, and doubling dilutions were made starting with a minimum of 1×10^6 cells. Cells were then washed in PBS, suspended in TE, and lysed with 0.5% Non-idet NP40 (BDH Chemicals, Poole, England). After denaturation in 2 M formaldehyde (Anachemia, Montreal, Canada), and 6 × SSC (final concentration), the lysate was applied to a Nytran (Schleicher and Schuell, Keane, NH) membrane using a Bio-Rad dot/blot apparatus. Subsequently, the membrane was baked in vacuo at 80°C.

Probe

Single strand neo-antisense RNA labelled with 32P-alpha-UTP (800 Ci/mmol, Amersham, Arlington Heights, IL) was generated with SP6 polymerase (Promega Biotech, Madison, WI) from a pSP65 plasmid (Promega Biotech, Madison, WI) containing the 1.6 kb Bam HI–Hind III neo insert cloned from pBRneo. The probe was purified by Elutip-R (Schleicher and Schuell, Keane, NH) chromatography using the manufacturer's specifications. Approximately 1×10^6 cpm of probe was added to each ml of hybridization buffer. Prehybridization and hybridization were done according to the method of Amasino [16], except an ambient temperature of 60°C was used. After washing, the membrane was blotted dry, put in plastic resealable bags, and exposed to film in −70°C, with a Dupont Crone intensifying screen (Dupont, Wilmington, DE).

Surface Markers of Cultured T-Lymphocytes

Phenotyping was carried out on both infected and noninfected cultured cells using a FACS Cell Sorter [17]. Antibodies utilized in this study were 3A1 (CD7), a mouse anti-human Ig, courtesy of Dr. A. Sullivan, McGill University; OKT11 (CD2), OKT4 (CD4), and OKT8 (CD8) from Ortho Diagnostic Systems, Raritan, NJ; Leu 7 and Leu 11 (CD16) from Becton Dickinson Immunocytometry Systems, Mountain View, CA; and B4 (CD19) and B1 (CD20) from Coulter Corp., Hialeah, FL.

B- and T-Lymphocyte Collaboration Studies

Infected and noninfected cultured T-cells, at two concentrations (10^4/ml and 10^5/ml), were incubated with normal fresh unseparated mononuclear cells (10^6/ml) and B-cells (2×10^5/ml) for 7 days. The supernatant was harvested and IgG and IgM production measured using an ELISA assay. Normal fresh unseparated mononuclear cells were obtained from peripheral blood of an unrelated donor following separation on a Ficoll-Hypaque gradient and were resuspended

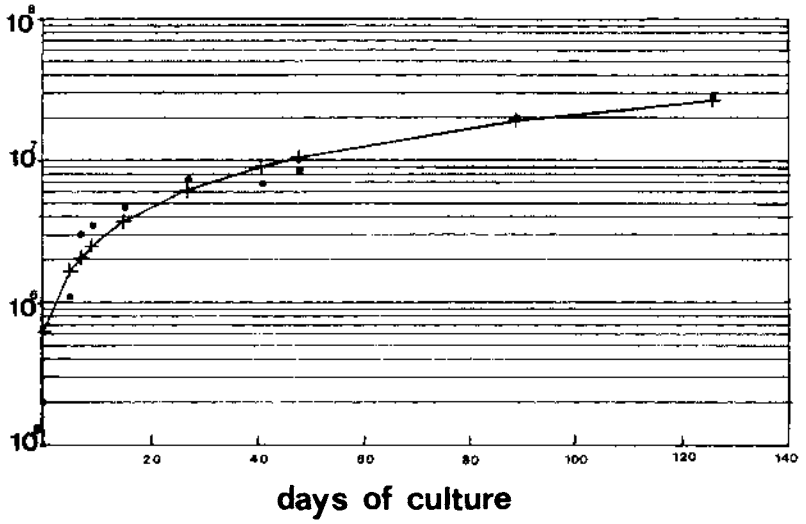

Fig. 1. Growth characteristics of long term T-lymphocyte cultures from neo-virus infected bone marrow cells in the absence of G 418 selection.

in MEM (Gibco, Grand Island, NY) with 10% FCS. B-cells were obtained from the mononuclear layer after sheep red blood cell (SRBC) rosetting. Briefly, a 10% suspension of SRBC in Hank's solution was incubated in neuraminidase for 1 h at 37°C. Cells were then washed and resuspended in MEM with 10% FCS to make a 1.5% solution of neuraminidase treated SRBC. Equal volumes of unseparated mononuclear cells and treated SRBC were mixed, spun, incubated, resuspended in MEM with 10% FCS, and layered on Ficoll-Hypaque. Cells at the interface were collected, resuspended, and added again to an equal volume of neuraminidase treated SRBC. The cells were spun and the pellet was incubated overnight at −4°C. Following incubation, the cells were resuspended in MEM with 10% FCS and layered onto Ficoll-Hypaque. B-cells were collected from the interface.

ELISA Assay

One hundred microliters of supernatant was added to protein-coated polyvinylchloride plates. Following incubation and washing, 100 μl of light chain goat anti-human Ig conjugated to alkaline phosphatase was added to each well and incubated at 37°C; 1 mg/ml diethanolamine was added to each well and read in an ELISA reader.

RESULTS

Retroviruses require cells to be cycling in order to infect them efficiently and since progenitor cells are generally out of cycle, bone marrow was infected with viral supernatants using the protocols outlined in Method and Material following stimulation with PHA. The infected cells were plated under assay conditions that permit the growth of T-cell colonies in the presence of G418 to select for those T-cells precursors that contain and express the neo gene. Approximately 15% of the mononuclear bone marrow cells incubated with virus were resistant to 1,000 μg/ml G418, while uninfected T cells were not. The gene transfer efficiency following infection with viral supernatants or by cocultivation was identical. Therefore, infection with viral supernatants was used in all subsequent experiments. Viral supernatants overcome the problem of the potential loss of progenitor cells on the fibroblast monolayer during cocultivation.

Bone marrow, after infection with neo virus, was cultured under conditions optimal for T-cells in the presence and absence of G418. Uninfected bone marrow was used as a control. The growth curve of the result in T-cells growing in the absence of G418 is shown in Figure 1. This curve shows that cell growth was initially exponential but after 7–10 days entered a linear phase of growth that persisted throughout the 130–150 days the cultures were followed. There was no difference in the rate of growth between the infected and uninfected cells.

In order to confirm that the neo gene was in the cultured cells, RNA whole cell blotting was performed 7–10 days following infection. As shown in Figure 2 significant levels of neo spe-

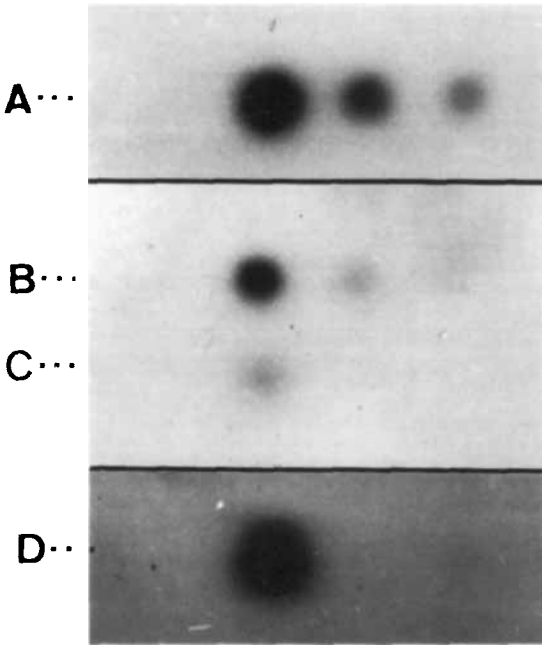

Fig. 2. Whole cell lysis RNA dot/blot probe with ^{32}P labeled single strand neo (N2-vector) antisense RNA. Serial doubling dilutions. Left column: 1 × 10^6/ml. **A**: 16-week cultures of N2 infected T cells, unselected. **B**: 8-week cultures of N2 infected T cells, unselected. **C**: Uninfected T-cells. **D**: N2 infected, preselected, OCIM2 cells (5 × 10^5/ml) grown in 800 μg/ml G 418.

cific message were detected in the population of infected cells grown in the absence of any in vitro selection. Significant neo expression was also detected in the infected and unselected population of T-cells at 8 weeks and 16 weeks in culture. The negative control was uninfected T-cells, while the positive control was a clone of OCIM2, human erythroleukemia cells carrying the same N2 vector. These results demonstrate significant levels of neo expression in bone marrow-derived T-cells up to at least 16 weeks in continuous culture in the absence of any selection.

To confirm the T-cell phenotype of the population of infected cells grown under the conditions described in method and material, cells were immunophenotyped at varying time intervals using a panel of monoclonal antibodies, including anti CD7, CD2, CD4, CD8, CD16, and Leu7 (Fig. 3); 74 to 93% of cells were positive for the CD2 antigen, confirming that these cultures were predominantly T-cell cultures. CD4/8 ratios were lower than those reported for normal fresh bone marrow and peripheral blood, ranging between 0.12 and 0.40. In some cultures the T4/T8 ratio decreases with duration of incubation. In cultures analyzed twice with an intervening 6 week time interval, one uninfected culture showed a decline in the CD 4/8 ratio from 0.40 to 0.28 while another infected culture maintained a stable ratio of 0.22. No significant population of Leu 7 and CD16 positive cells could be detected. Less than 6% of bone marrow-derived cultures expressed B cell antigens 4 to 6 weeks after infection.

To determine if these cultured lymphocytes retained their capacity to stimulate or suppress immunoglobulin production when mixed with fresh unrelated donor B-cells, propagated T-cells were cultured at two cell concentrations with fresh B-cells from normal donors and the level of immunoglobulin production was measured. Significant levels of IgM were secreted by the B-cells in these mixed lymphocyte reactions using T-cells obtained from culture at 4 and 9 weeks. Suppressor function was also retained, manifested by a decrease of immunoglobulin production at a concentration of 10^4 T cells when cultured with fresh unseparated mononuclear cells. These results confirm that not only did these cells have the surface phenotype characteristic of cultured T-cells but they also retained their helper and suppressor functions.

DISCUSSION

The selection of a target cell for clinically applicable retroviral gene transfer has proven, to date, to be a difficult task. If the goal of gene therapy is to provide the host with a gene previously lacking or aberrant, then the new gene should be expressed for the life span of the recipient at a physiological level in order to be beneficial. This requires that the target cell be accessible for infection, be permissive of infection, and be able to maintain long-term expression of the newly introduced gene following infection. Maintenance of expression, in turn, depends upon the activity of the genome in the region of integration of the new gene as well as the longevity and proliferative capacity of the target cell. Hematopoietic stem cells are accessible, efficiently infected [6,7,11], and capable of proliferation but long-term expression has been difficult to achieve in animal models [8,9,10,12,18]. In one murine model, integration of the SV40 promoter linked neo gene into spleen colonies was shown to be 100% by Southern blot but expression was lost [8]. Canine hematopoietic stem

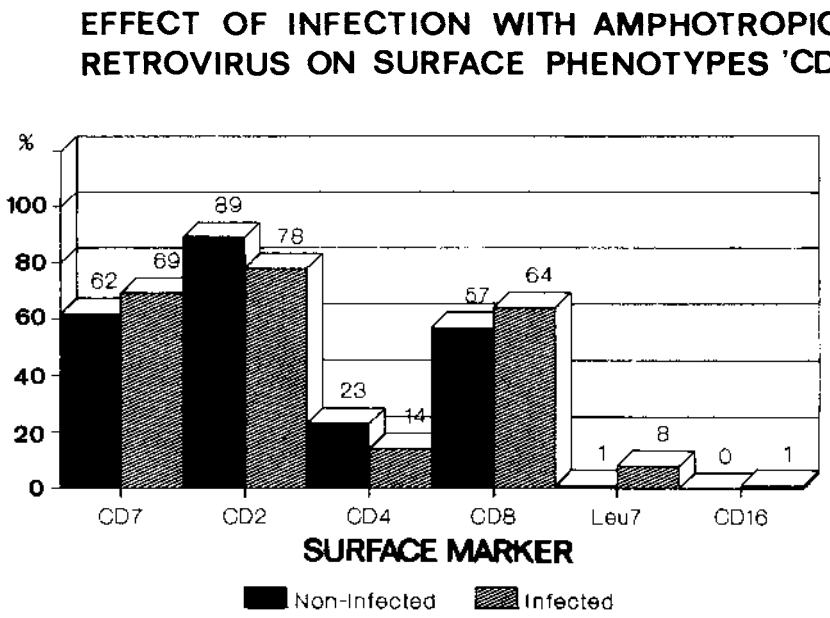

Fig. 3. Phenotypic profiles of neo-virus infected and noninfected T-cells.

cells infected with the dihydrofolate reductase gene lost expression of that gene even when selective pressure was applied with methotrexate [9]. Cynomolgus macaque monkeys receiving bone marrow infected with the human ADA gene showed only short-term expression of a low level of the ADA gene following reconstitution [10]. Possible explanations for loss of gene expression include loss of infected clones, low infection rates of pluripotent stem cells, viral integration at a genomic site, which becomes inactive as stem cells differentiate, inhibition of viral promoters, and low frequency of progenitor cells [3,10,11,18].

Our study focuses on the T-lymphocytes as a possible target cell for gene therapy. A priori, T-cells possess many characteristics desirable in a target cell. T-cells are easily accessible by leukapheresis, a simple procedure with a low morbidity giving a high yield of mononuclear cells. Subsequently, the T-cell population can be expanded from these cells using PHA and IL-2 stimulation [15].

T-cells are fully differentiated yet capable of proliferation. This characteristic of T-cells obviates the need to test the hypothesis that genomic inactivation secondary to differentiation causes loss of expression. The proliferative capacity of T-cells without differentiation suggests that, short of clonal loss or selection in vivo, gene expression should be maintained.

Concerning the longeivity of T-cells, Jerne argues on the basis of labelling experiments in rat and mouse models that the immune system is comprised of a relatively stable population of cells once established [19]. More recent evidence that this is indeed the case is found in the literature on patients exhibiting T-cell chimerism following either fetal liver and thymus or HLA haplotype mismatch bone marrow transplantation. These human chimerics, transplanted without cytotoxic preparation, for severe combined immunodeficiency syndrome, exhibit donor T-cells but recipient B-cells and monocytes [20–25]. At least one of these children has maintained this stable chimerism for more than 10 years [20]. These data suggest that the stable T-cell population in these chimerics arises from and is perpetuated by the committed lymphocyte population infused at transplantation rather than through continuous renewal by stem cells.

Our experiments revealed that T-cells are easily infected and able to maintain long term expression of the new gene without disruption of normal T-cell function.

We have shown that the transfer of genetic information, the NEO gene in this case, can be

easily accomplished using either cocultivation or supernatant infection with a retroviral vector. Further, we have shown that T-cells are able to maintain expression of the NEO gene for at least 4 months as evidenced by continuous growth in G418 and by whole cell RNA dot/blotting.

Phenotyping results of the cultured T-cells suggest that the culture technique does tend to select for CD8+ cells over time [26]. This selection probably reflects the difference in IL-2 receptor level among T-cell subsets, which ultimately regulates the proliferative capacity of the mitogen stimulated T-cells. Malek has shown, in the mouse, that adequate levels of IL-2 receptors could be generated following mitogen stimulation in 50–70% of L3T4−Ly2+ cells but similar receptor levels in the L3T4+Ly2− cells could only be induced in the presence of accessory cells. As our T-cell cultures do not include accessory cells, our selection for the L3T4−Ly2+ human counterpart reflects the data in mice. It is important to note, however, that while the culture conditions did tend to expand preferentially CD8+ subsets, both helper and suppressor functions of the cells were preserved. Both infected and uninfected T-cells were able to stimulate polyclonal immunoglobulin production in B-cells and suppress production of immunoglobulin in the presence of unseparated mononuclear cells. Thus, while subset selection (i.e., CD4/CD8 ratio) does ultimately occur in the culture system, function of all subsets is maintained.

We have demonstrated that for both theoretical and experimentally supported considerations, the T-cell is well suited as a target cell for gene transfer and shows promise as the target cell for the clinical correlate of gene transfer, i.e., gene therapy.

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