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Hematopoietic Cell Transplantation with Cord Blood for Cure of HIV Infections

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A B S T R A C T

Hematopoietic cell transplantation (HCT) using *CCR5*-Δ32/Δ32 stem cells from an adult donor has resulted in the only known cure of human immunodeficiency virus (HIV) infection. However, it is not feasible to repeat this procedure except rarely because of the low incidence of the *CCR5*-Δ32 allele, the availability of only a small number of potential donors for most patients, and the need for a very close human leukocyte antigen (HLA) match between adult donors and recipients. In contrast, cord blood (CB) transplantations require significantly less stringent HLA matching. Therefore, our hypothesis is that cure of HIV infections by HCT can be accomplished much more readily using umbilical CB stem cells obtained from a modestly sized inventory of cryopreserved *CCR5*-Δ32/Δ32 CB units. To test this hypothesis, we developed a screening program for CB units and are developing an inventory of *CCR5*-Δ32/Δ32 cryopreserved units available for HCT. Three hundred such units are projected to provide for white pediatric patients a 73.6% probability of finding an adequately HLA matched unit with a cell dose of $\geq 2.5 \times 10^7$ total nucleated cells (TNCs)/kg and a 27.9% probability for white adults. With a cell dose of $\geq 1 \times 10^7$ TNCs/kg, the corresponding projected probabilities are 85.6% and 82.1%. The projected probabilities are lower for ethnic minorities. Impetus for using CB HCT was provided by a transplantation of an adult with acute myelogenous leukemia who was not HIV infected. The HCT was performed with a *CCR5*-Δ32/Δ32 CB unit, and posttransplantation in vitro studies indicated that the patient's peripheral blood mononuclear cells were resistant to HIV infection.

INTRODUCTION

Infection with the human immunodeficiency virus type 1 (HIV-1) requires both a CD4 receptor and a chemokine receptor, principally chemokine receptor 5 (CCR5) [1]. In humans, the CCR5 protein is encoded by the *CCR5* gene located on the short arm (p) of chromosome 3 at position 21. Certain populations have inherited the Δ-32 mutation, which results in the genetic deletion of a portion of the *CCR5* gene. Homozygous carriers of this mutation (*CCR5*-Δ32/Δ32) are resistant to HIV-1 infection [2-4]. In 2009, Hütter et al. [5] reported long-term control of HIV infection by hematopoietic cell transplantation (HCT) using peripheral blood stem cells from a *CCR5*-Δ32/Δ32 donor. More than 5½ years after this treatment, the patient is still off antiretroviral medication, and in the analysis of peripheral blood cells and different tissue samples, including gut, liver, and brain, no viral load or proviral DNA could be detected [6]. Allers et al. [7] reported that these results strongly suggest cure of HIV is achieved in this patient. Deeks and McCune [8] commented that "the HIV research community is hesitant to use the word 'cure,' but this single case could very well be the first example to fit the bill."

Although it is compelling, this concept of performing HCT using stem cells from *CCR5*-Δ32/Δ32 adult donors of bone marrow or peripheral blood for treatment of HIV-infected patients cannot be readily generalized. This is true because the prevalence of the homozygous variant allele is low, that is, only about .8% to 1% of individuals of northern European descent have the *CCR5*-Δ32/Δ32 genotype [9,10], and it is much less prevalent in other ethnic groups [11]. Furthermore, most patients in need of an HCT have only a small number of potential donors from among registries of adult donors because a very close human leukocyte antigen (HLA) match for 8 of 8 or 7 of 8 high-resolution alleles at four loci

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(A, B, C, DRB1) is required between adult donors and recipients [12]. Hütter and Thiel reported no further cases of HCT for HIV-infected patients because of the inability to match such patients with adult *CCR5*-Δ32/Δ32 donors despite a diligent search for "patient number 2" [13], and to date, no other such patients have been reported.

In marked contrast, HCT using umbilical cord blood does not require such stringent HLA matching between donor and recipient [14]. Acceptable HLA-matched units include those that are matched at 4 of 6, 5 of 6, or 6 of 6 alleles at 3 loci using low-resolution testing at the A and B loci and high-resolution testing at the DRB1 locus, disallowing 2 mismatches at the same locus for 4 of 6 matching.

Our hypothesis, therefore, is that cure of HIV by HCT can be readily accomplished only by using *CCR5*-Δ32/Δ32 umbilical cord blood stem cells. To test this hypothesis, we developed a cord blood screening program and are developing an inventory of *CCR5*-Δ32/Δ32 units.

While testing was being performed, one of the units subsequently found to be *CCR5*-Δ32/Δ32 was discovered to have been released for HCT for an adult patient with acute myelogenous leukemia. This provided us the opportunity to document that a *CCR5*-Δ32/Δ32 cord blood unit resulted in donor cell engraftment and that the patient's peripheral blood mononuclear cells after transplantation were resistant in vitro to infection with HIV-1.

MATERIALS AND METHODS

Developing an Inventory of CCR5-Δ32/Δ32 Cord Blood Units

Samples of cord blood units for testing were primarily from white donors in the inventories of StemCyte International Cord Blood Center (hereafter referred to as simply StemCyte) and from numerous collaborating cord blood banks: St. Louis Cord Blood Bank; Carolinas Cord Blood Bank at Duke University; University of Colorado Cord Blood Bank; MD Anderson Cancer Center Cord Blood Bank; the Barcelona, Spain Cord Blood Bank; and the Sydney, Australia Cord Blood Bank. Samples from collaborating cord blood banks were sent to StemCyte, usually as about 500 μL of blood from the pre- or postprocessing sample. DNA is extracted from these samples, and the DNA specimens were then tested for *CCR5*-Δ32 at the City of Hope Medical Center to determine if the units were homozygous, heterozygous, or wild type. Some cord blood samples were sent from the collaborating cord blood banks as DNA.

DNA isolation was carried out using QIAamp DNA Mini and QIAmp DNA Blood Mini Kits (Qiagen Inc, Valencia, CA). These are designed for purification of an average of 6 μg of total DNA from 200 μL of whole human blood and up to 50 μg of DNA from 200 μL of buffy coat.

Genotype analysis was performed at the City of Hope Medical Center on DNA preparations (1 μL, 100 ng DNA) extracted from cord blood using a polymerase chain reaction (PCR)-based assay for homozygosity of *CCR5*-Δ32 base pair deletion.

DNA oligonucleotides were purchased from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA).

Primer 1 TTCATTACACCTGCAGCTCTC
Primer 2 CCTGTTAGAGCTACTGCAATTAT
Primer 3 TGCAGCTCTCATTTTCCATACATTA

To identify the *CCR5* genes with an internal 32—base pair deletion (*CCR5*-Δ32 allele), the DNAs were amplified using a real-time PCR on a C1000 Thermocycler (BioRad, Bio-Rad Laboratories, Hercules, CA). A total of 1.5 μL of genomic DNA was used as template, and each PCR was carried out in a 25-μL volume in 1x SYBR Green supermix (170-8882, Bio-Rad) with 15 pmol each of primer 2 and primer 3 for 3 minutes at 94°C (1 cycle); 20 seconds at 94°C, 1 minute at 68°C (30 cycles); and 7 minutes at 72°C (1 cycle).

A second round of PCR was performed on samples that amplified with the primer 2 and 3 pairs, which is specific for the deletion. The second round used primers 1 and 2, which are positioned outside of the deleted region, to allow us to discriminate whether the deletions are homozygous or heterozygous. Reaction mixtures consisted of 15 pmol of each primer, 1.5 μL of the first round amplicons, .2 μM dNTP mix, 3 mM $MgCl_2$, and 1U Taq gold DNA polymerase (Applied Biosystems-Life Technologies, New York, NY) in a total reaction volume of 25 μL at 95°C for 3 minutes (1 cycle); 95°C for 45 seconds, 60°C for 45 seconds, 72°C for 45 seconds (40 cycles); and 72°C for 7 minutes (1 cycle). After the PCR amplification, the products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The products from deletion-containing samples generate either a single band of 157 base pair from the homozygous deletions (Δ32/Δ2) or two bands of 189 and 157 base pair from the heterozygous samples.

Projections of the Probability of Finding an Appropriately HLA-Matched Cord Blood Unit with an Adequate Cell Dose for a Patient in Need of an HCT

HLA match rates for an inventory of 300 *CCR5*-Δ32/Δ32 cord blood units were estimated with population HLA haplotype frequencies using a population genetic model developed by Kollman and colleagues previously applied to project match rates for planning the National Cord Blood Inventory [15,16]. Simulation of HLA genotypes of patients and cord blood units not yet collected requires the use of HLA haplotype frequencies, which were calculated at the allele-family level for HLA-A and -B and allele level for DRB1 in a cohort of 679,519 white European donors from the Be The Match Registry [17]. We simulated cord blood units using distribution of cord blood unit total nucleated cells (TNCs) of units banked in the Be The Match Registry and simulated patients using separate distributions of weights of pediatric (under age 16 years) and adult (age 16 years and over) patients who have searched the Be The Match Registry. We incorporated the dose requirement into the model by calculating the percentage of cords with the commonly used minimum adequate TNC dose of 2.5×10^7/kg for each simulated patient based on his or her weight and considered matched units with inadequate dose as unavailable. The simulated HLA genotypes consisted of a pool of 10,000 simulated European-American patients searching 10 replicate simulated European-American 300-unit cord blood unit inventories, with reported match rates averaged over the results from the 10 inventories. This model assumes that HLA haplotypes in patients and *CCR5*-Δ32/Δ32 variant cords are drawn from the same overall European-American HLA frequency distribution. However, because the geographic distribution of the CCR5 variant is centered over Northern Europe [18], the HLA match rates for European-American patients are slightly higher than the projection if the patient has Northern European ancestry and lower if the patient has Southern European ancestry.

The *CCR5*-Δ32/Δ32 units in the special inventory are primarily from white donors, and it is true that patients in need of transplantation more commonly find a matched donor from among those in their own ethnic group. However, this is not invariably true, and HLA matched donors are not uncommonly found among those in other ethnic groups. To assess match rates for minority populations, we simulated 10,000 African American, Mexican Hispanic, and Chinese American patients searching European-origin cord inventory replicates.

Recent data indicate that a minimum cord blood cell dose of $\geq 1 \times 10^7$ TNCs/kg body weight is adequate when a combined haploidentical and cord blood transplantation is performed [19]. Accordingly, similar projections were also carried out using this minimum cell dose.

Testing the Cells In Vitro of a Patient with Acute Myelogenous Leukemia Who underwent Transplantation with CCR5-Δ32/Δ32 Cord Blood Stem Cells for Resistance to HIV Infection with Laboratory Strains BAL and NL4-3

Peripheral blood samples were collected from an HIV-negative patient with acute myelogenous leukemia on day +123 post-HCT at a time when chimerism studies indicated complete engraftment by *CCR5*-Δ32/Δ32 cells. The cells were cryopreserved and, when thawed, were shown to have >90% viability by trypan blue exclusion and 7- amino-actinomycin D flow cytometry. The Ficoll-hypaque—separated peripheral blood mononuclear cells from the transplantation recipient, a normal adult control, and *CCR5*-Δ32 heterozygous and homozygous cord blood units were tested for susceptibility to infection with the laboratory strains HIV1 BAL, and the transplantation recipient's peripheral blood mononuclear cells were also infected with NL4-3 at multiplicity of infection of .01 in vitro after standard phytohemagglutinin stimulation. Supernatants were collected and measured for p24Ag (Perkin-Elmer, Inc, Waltham, MA) at 3 and 7 days postinfection. No additional peripheral blood mononuclear cells were added to cultures during the 7-day incubation. Patient cells were tested for *CCR5*-Δ32/Δ32 chimerism and again for viability by 7-amino-actinomycin D by flow cytometry at the termination of the experiment.

RESULTS

Identifying CCR5-Δ32/Δ32 Units in Inventories of Cryopreserved Cord Blood Units

Thus far, we have identified 134 cord blood units that are *CCR5*-Δ32/Δ32s after having tested approximately 18,000 units, primarily from white patients, for an incidence of approximately .7%. In addition, we searched 8,000 Asian

Table 1
Projected HLA Match Rates with a 300-Unit Inventory of *CCR5*-Δ32/Δ32 Cord Blood Units

Ethnic Group	Adult Patients	Pediatric Patients
Whites	*Includes need for TNCs of 2.5 × 10⁷ cells/kg*	
	6 of 6 matches: .01%	6 of 6 matches: .01%
	5 of 6 matches: 4.5%	5 of 6 matches: 10.6%
	4 of 6 matches: 27.9%	4 of 6 matches: 73.6%
	Includes need for TNCs of 1 × 10⁷ cells/kg	
	6 of 6 matches: .09%	6 of 6 matches: 1.01%
	5 of 6 matches: 10.7%	5 of 6 matches: 10.8%
	4 of 6 matches: 82.1%	4 of 6 matches: 85.6%
Minority	*Includes need for TNCs of 2.5 × 10⁷ cells/kg*	
African American	4 of 6 matches: 9.9%	4 of 6 matches: 28.6%
Mexican American	4 of 6 matches: 14%	4 of 6 matches: 44.1%
Chinese American	4 of 6 matches: 2.7%	4 of 6 matches: 12.3%
	Includes need for TNCs of 1 × 10⁷ cells/kg	
African American	4 of 6 matches: 31.6%	4 of 6 matches: 34.1%
Mexican American	4 of 6 matches: 48.9%	4 of 6 matches: 52.5%
Chinese American	4 of 6 matches: 13.9%	4 of 6 matches: 15.7%

TNC indicates total nucleated cell.

units in the inventory of StemCyte Taiwan but found no homozygous units. Testing of an additional approximately 25,000 cord blood units from whites is expected to increase the special inventory to about 300 units.

Probabilities of Finding Adequately Matched Cord Blood Units with an Adequate Cell Dose in an Inventory of 300 CCR5-Δ32/Δ32 Cord Blood Units

Table 1 indicates the projected probabilities of finding an adequately HLA-matched unit with a TNC count of $\geq 2.5 \times 10^7$/kg or with a TNC count of $\geq 1 \times 10^7$ cells/kg in an inventory of 300 *CCR5*-Δ32/Δ32 units for pediatric and adult white patients and for patients of other ethnic groups. Projected match rates for white patients using a minimum necessary TNC count of $\geq 2.5 \times 10^7$/kg were 73.6% for pediatric patients and 27.9% for adults. Using a minimum necessary TNC count of $\geq 1.0 \times 10^7$/kg, the projected match rates were 82.1% for adults and 85.6% for pediatric patients. Probable match rates were significantly lower for patients of minority ethnic groups (Table 1).

Testing the Cells of the Patient Who underwent Transplantation with a CCR5-Δ32/Δ32 Unit for Resistance to HIV Infection

As seen in Figure 1A, the recipient's peripheral blood mononuclear cells on day +123 showed no significant HIV replication with in vitro infection with either of the laboratory strains of HIV1, BAL (CCR5 tropic), or NL4-3 (CXCR4) compared with a normal control. Figure 1B shows a comparison of HIV replication in vitro with the laboratory CCR5 strain HIV-1BAL for cord blood peripheral blood mononuclear cells from CCR5 wild-type, heterozygous, and homozygous units. There was no detectable HIV replication in vitro in the *CCR5*-Δ32/Δ32 cord blood cells compared with significant replication in the heterozygous and wild-type cord blood cells.

DISCUSSION

Long-term control of HIV infection has been accomplished by Hütter et al. [5] with HCT using peripheral blood stem cells from a *CCR5*-Δ32/Δ32–matched unrelated adult donor. The patient has remained without any evidence of HIV infection for more than 5½ years after discontinuation of antiretroviral drug therapy [13]. However, using *CCR5*-Δ32/Δ32 stem cells from adult donors in bone marrow or peripheral blood stem cell transplantations cannot be readily generalized as a treatment option because of the rarity of the variant allele and the need for a very close HLA match between recipient and donor. As noted, the unsuccessful attempt by Hütter et al. to perform an HCT for a second patient confirms the futility of this approach [13]. In contrast, umbilical cord blood transplantations require significantly less stringent requirements for HLA matching.

Accordingly, our hypothesis is that cure of HIV infections by HCT can be much more readily accomplished using umbilical cord blood stem cells obtained from a modestly sized inventory of cryopreserved *CCR5*-Δ32/Δ32 cord blood units. Furthermore, the numerous reports of essentially equivalent posttransplantation outcomes using cord blood as compared with the results of bone marrow and peripheral blood cell transplantations lend impetus to this approach [20-26]. Despite these reports, there are plentiful data indicating that cord blood transplantation is significantly underutilized [27]. Starting in 2003, StemCyte cord blood inventories were screened for *CCR5*-Δ32/Δ32 donors.

In addition, the transplantation of a *CCR5*-Δ32/Δ32 cord blood unit to an adult patient with acute myelogenous leukemia as part of a double cord blood transplantation provided the opportunity to collect data that indicated engraftment of the *CCR5*-Δ32/Δ32 unit as the dominant unit. At a time when chimerism studies indicated 100% engraftment by the *CCR*5-Δ32/Δ32 unit, in vitro studies indicated that the patient's peripheral blood mononuclear cells were resistant to HIV1 BAL and NL4-3 strains (Figure 1A, B).

Projections regarding the probability of finding an appropriately HLA-matched unit with an adequate cell dose for a white patient using a population of simulated patients are based on the HLA frequencies within the US white population and the cell doses of units presently in the special inventory. We arbitrarily chose an inventory of 300 *CCR5*-Δ32/Δ32 units for our initial calculations and are developing such an inventory. Collaboration among numerous cord blood banks makes the development of a special inventory of this size eminently feasible, and, if needed, additional units can readily be added. Gonzales et al. [11] estimated that there are approximately 400,000 cord blood units cryopreserved around the globe, among them 2,000 to 4,000 *CCR5*-Δ32/Δ32 units.

The projections took into consideration the need for an adequate cell dose, which is generally accepted to be in the range of $\geq 2.5 \times 10^7$ TNCs/kg. The projections indicate that an inventory of 300 units would provide a 73.6% probability of finding an adequately HLA-matched unit for white pediatric patients and a probability of 27.9% for white adult patients (Table 1). Also, since Liu et al. [19] reported that a cord blood cell dose as low as 1×10^7 TNCs/kg body weight is adequate for cord blood transplantations done in association with a haploidentical transplantation, we have made projections using this as the minimum necessary cell dose. These projections indicate the probability of finding an adequately HLA-matched unit for 85.6% of white pediatric patients and 82.1% for white adult patients (Table 1). The use of combined haploidentical and cord blood transplantations [19] provides important, and probably essential, advantages when considering use of cord blood transplantation to cure HIV in adults. Finding two adequately matched units from an inventory of 300 cord blood units would be very problematic, whereas the use of a single cord blood unit combined with a haploidentical transplantation is much more feasible. Further, in such transplantations, engraftment is rapid, and chimerism studies have shown that cells from the cord blood unit almost always are the only ones present several months posttransplantation [19]. However, it must be noted that combined haploidentical–

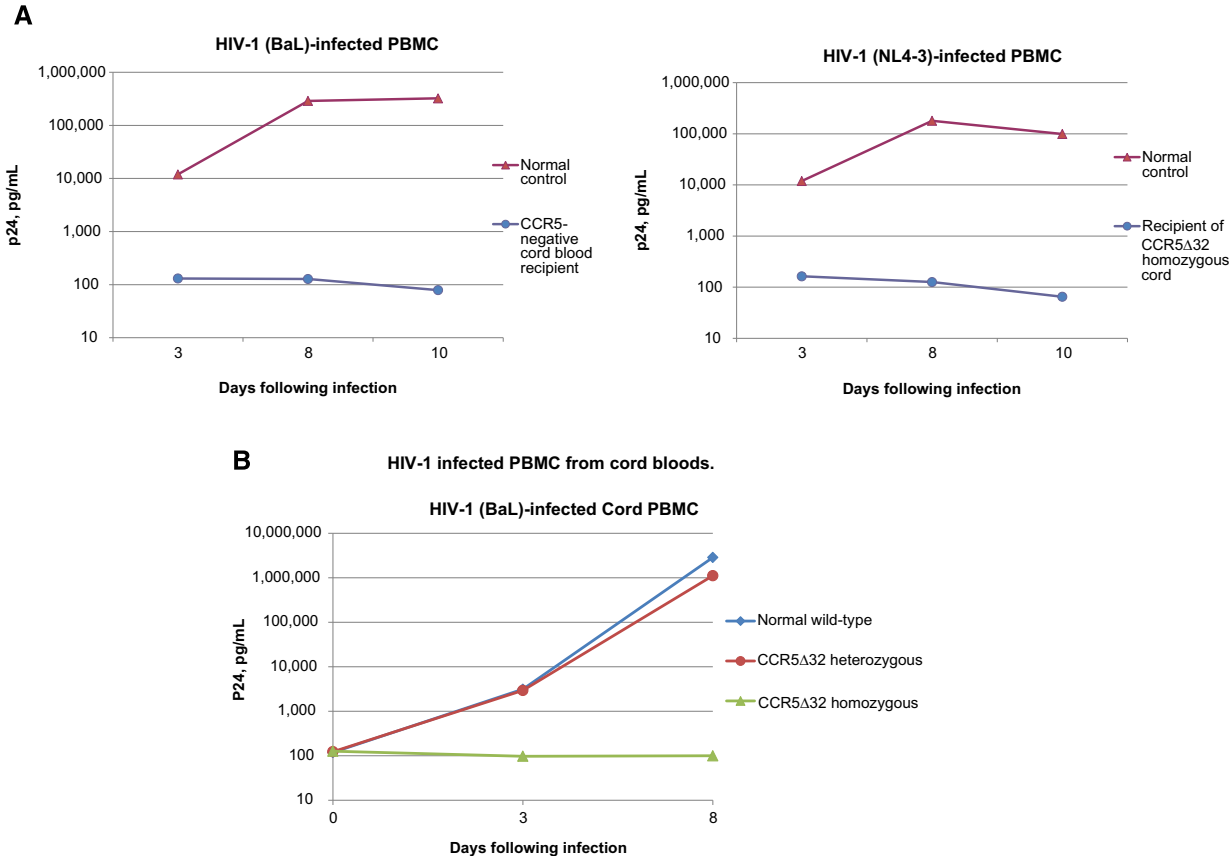

Figure 1. (A) In vitro study of infectivity by HIV-1 of peripheral blood mononuclear cells (PBMCs) in the posttransplantation period from an HIV-uninfected patient who underwent transplantation with a *CCR5*-Δ32/Δ32 cord blood. The recipient's PBMCs showed no significant infection with either lab strains of HIV-1, BAL (CCR tropic), or NL4–3 (CXCR4) compared with a normal adult control. (B) A comparison of HIV replication in vitro with the laboratory CCR5 strain HIV-1 BAL for cord blood PBMCs from normal CCR5 wild-type, heterozygous, and homozygous units. There was no detectable replication in vitro in the *CCR5*-Δ32/Δ32 cord cells compared with significant replication in the heterozygote and wild-type cord cells.

cord blood transplantations are not commonly performed at this time in the United States, and the adequacy of a cell dose of $\geq 1 \times 10^7$TNCs/kg has not yet been confirmed.

To assess match rates for minority populations, we simulated 10,000 African American, Mexican Hispanic, and Chinese American patients searching European-origin cord inventory replicates. As expected, the projected probabilities are significantly lower (Table 1).

The most obvious patient population for the transplantation of *CCR5*-Δ32/Δ32 cord blood units is that group of patients who are in need of an HCT for a hematological malignancy or other indication and are also infected with HIV. The incidence of patients meeting these criteria has not been determined and would appear to be relatively small. However, Hütter and Zaia [28] pointed out that "the lifetime expectancy of HIV-infected patients has improved substantially, but nevertheless the incidence rate of malignancies in these patients has increased considerably. Therefore, it can be assumed that there will be a rising necessity for HIV-1 infected patients with malignancies for allogeneic HCT." Krishnan and Forman also indicate that the incidence of Hodgkin's lymphoma and non-Hodgkin's lymphoma is increased in HIV-infected patients compared with the general population [29]. AIDS-related malignancies remain a leading cause of mortality in HIV-infected patients [30].

Many physicians still automatically consider HIV status a barrier to transplantation, and transplant centers often exclude these patients from their protocols [30]. However, experience during the last 25 years indicates successful HCT in HIV-infected patients with hematological disease, including not only leukemia and relapsed lymphoma but also successful treatment of nonmalignant disorders such as aplastic anemia [28]. The outcome of allografted HIV-positive patients is probably only negligibly poorer in comparison with HIV-negative patients [28].

An alternative approach to cure of HIV infection is offered by CCR5-targeted gene therapy of either T cells or stem cells, using siRNA-based therapeutics, as recently reported [8,29,31-33]. Another highly innovative approach relies on engineered zinc finger nucleases specific for the *CCR5* gene [34]. Zinc finger nucleases are a powerful tool that can be used to edit the human genome ad libitum [35]. The technology has experienced remarkable development in the last few years with regard to both the target site specificity and the engineering platforms used to generate zinc finger proteins [35]. To date, clinical benefit has not been demonstrated in clinical trials [32], but recent progress in the field provides optimism that some of the promises of gene therapy may finally be realized [36,37].

In addition to HCT for HIV-infected patients with a hematological malignancy or other indication for transplantation, selected patients with AIDS who have no other illness should also be considered for a clinical trial of *CCR5*-Δ32/Δ32 cord blood transplantation. The optimism of antiretroviral treatment is dampened by the current impossibility of viral eradication, the sustained medication with

complex regimens, the potential toxic effects, and the prevalence of drug-resistant isolates [38]. Patients with AIDS who are responding poorly to antiretroviral regimens and are informed of the significant risks of HCT and the potential benefits should be allowed to participate in a clinical trial of HCT if an appropriately HLA-matched *CCR5*-Δ32/Δ32 unit of adequate cell dose is available.

The seemingly logical approach to a cure using adults who volunteer to be bone marrow/peripheral blood donors and who are found to have the variant CCR5 allele is an improbable solution. The testing for *CCR5*-Δ32/Δ32 at the time donors are registered with the National Marrow Donor Program is expensive and not likely to be effective because of the statistical improbability of finding both the high-grade HLA match, as is required when HCT is done using stem cells from adults, and a *CCR5*-Δ32/Δ32 genotype in the donor. Thus, it is more reasonable to suggest large-scale testing of newly donated and currently inventoried cord blood for *CCR5*-Δ32/Δ32 units.

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