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ORIGINAL ARTICLE

Bone marrow transplantation for cartilage-hair-hypoplasia

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The association of cartilage hair hypoplasia (CHH) with severe combined immunodeficiency (SCID) has been known for more than three decades. Bone marrow transplantation (BMT) remains the only effective treatment that might cure SCID. Surprisingly little has been reported on the experience with BMT in CHH. We report here survival and long-term reconstitution of immunity after BMT in three patients with CHH. Regardless of whether a related human leukocyte antigen-matched or unrelated matched donors were used as the source of BMT, all patients are alive and well 5–20 years after BMT. Engraftment appears robust with most cells of donors origin. Repeated evaluation of the immune system showed normal cellular and humoral immunity. Our results should encourage the use of BMT in patients with CHH who have profound immunodeficiency.
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Introduction

Cartilage hair hypoplasia (CHH; OMIM 25025) was first described in the Amish by McKusick as an autosomal recessive type of metaphyseal chondrodysplasia.[1] The condition is prevalent among Finns with an estimated carrier frequency of one in 76.[2] Recently a locus responsible for CHH was mapped to chromosome 9 aiding in the discovery of mutations in the endoribonuclease RNase mitochondrial RNA processing (RMPR) in patients with this syndrome.[3–8] The exact mechanism by which RMRP abnormalities cause the various manifestations of CHH remains to be characterized.

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The syndrome is highly pleiotropic with manifestations including disproportionate short stature, hypoplasia of hair and occasionally dysplasia of bone marrow and intestinal neurons. These manifestations are variable between and within families. Similar variability has been observed for the immune aberrations which are associated with CHH. While most affected individuals have mild to moderate cellular[9–11] or humoral[12] abnormalities, some with this syndrome present with severe combined immunodeficiency (SCID).[4,5]

The immune dysfunction in the Finnish type was initially perceived to be less profound than in cases reported elsewhere. While having depressed T-cell numbers and mitogenic responses the Finnish patients appeared not to be susceptible to overwhelming viral infections and even tolerate live viral vaccines.[2–13] However, a recent review of survival in Finnish patients with CHH found increasing mortality in younger patients, suggesting that immunodeficiency was more profound than previously estimated in this population.[2] In sharp contrast, fatal varicella has been described in a few Amish patients with CHH.

Indeed, profound T-cell deficiency indistinguishable form SCID has been demonstrated in patients with cartilage hair hypoplasia.

The most effective treatment of SCID is bone marrow transplantation (BMT). Ideally, human leukocyte antigen (HLA) identical stem cells from a relative (RID) are preferred. Unfortunately, fully matched related donors are available in fewer than 20% of cases. Alternatively, T-cell depleted mismatched related donors (MMRD) or matched unrelated donors (MUD) have been used. Little is known about BMT in CHH and long-term immune reconstitution has not been previously assessed in this condition.

We describe here three patients with CHH who had profound cellular and humoral immunodeficiency. They are alive and well 6, 12 and 21 years after receiving BMT. One patient received RID BMT while the remaining two were reconstituted with bone marrow obtained from MUD.

Materials and methods

Transplantation procedures

Patients were admitted into private high efficiency particulate air (HEPA)-filtered rooms on the bone marrow transplant unit and were maintained under strict reverse

isolation procedures throughout the admission until they were discharged from the hospital. Trimethoprim/sulfamethoxazole was used for *Pneumocystis carinii pneumonitis* (PCP). Intravenous immunoglobulin (IVIG) at a dose of 600 mg/kg was infused as required to maintain IgG level above 6 g/l.

All blood products were CMV negative and irradiated.

In patients 1 and 3 who received a MUD BMT, pretransplant conditioning therapy consisted of Busulfan given over 4 days in doses of 5 mg/kg/day. This was followed by a 4-day course of cyclophosphamide (50 mg/kg/day). Graft-versus-host disease (GvHD) prophylaxis, administered to patients 1 and 3 from the day before transplant, consisted of cyclosporine A (3 mg/kg/day i.v.) or adjusted to keep a level of 150 μg/l and methylprednisolone (2 mg/kg/day i.v.) for an average of 90 days.

All patients were infused with $3–5 \times 10^8$ donor bone marrow mononuclear non-depleted cells per/kg of recipient weight. Patient 1 received granulocyte-macrophage colony-stimulating factor (GM-CSF) (5.5 mg/kg/day subcutaneously) and patient 3 received G-CSF (5.0 mg/kg/day i.v.) from the day of transplant until the absolute neutrophil count was $>1.0 \times 10^9$/l for three consecutive days.

Patient 2 received bone marrow from her HLA identical male sibling. No conditioning or GvHD prophylaxis were given.

Lymphocytes from patients were tested for HLA A, B and C as well as DR typing using standard serological methods (patients 1 and 2) or molecular analysis (patient 3).

Lymphocyte markers and T-cell proliferative responses

The surface phenotypes of blood mononuclear cells obtained by Ficoll–Hypaque density gradient centrifugation were determined by direct immunofluorescence with a fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin antibody (Tago, Burlingame, CA, USA) or FITC-conjugated MoAbs anti-CD3, CD4, CD8, CD20 and CD56 (Coulter Instruments, Mississauga, Ontario, Canada). Analysis was performed on a Coulter EPICS V flow cytometer.[14,15] Lymphocyte proliferative responses to mitogens including phytohemagglutinin (PHA) were determined by tritiated thymidine incorporation using the microtiter plate technique. All assays were performed in triplicate and were compared with those simultaneously performed on normal controls.[14,15]

Serum concentration of immunoglobulin

Serum concentrations of immunoglobulins were measured by nephelometry. Levels of serum antibodies to tetanus were measured by enzyme-linked immunosorbent assay (ELISA) and polio antibody titers were determined by complement fixation.[11,14,15]

Chimerism

Whole blood (in case of MUD BMT) or Ficoll-separated peripheral mononuclear cells (in case of RID BMT) were analyzed for donor engraftment as previously described.[16] Where a sex difference existed between donor and recipient, DNA was analyzed using a Y-specific probe (ZFX/ZFY) to confirm donor lymphocyte engraftment. In the absence of sex difference, restriction fragment length polymorphism (RFLP) analysis were performed by studying genetic polymorphism, which are due to a variable number of tandem repeats (VNTR) or (CA)n in varying regions in the genome.

Quantification of TRECs by real-time PCR

The amount of signal joint (sj) T-cell receptor excision circles (TRECs) adjusted to $CD4^+$ and $CD8^+$ T subsets, was determined by real-time quantitative polymerase chain reaction (PCR), as previously described.[15] In brief, Genomic DNA was isolated from peripheral blood mononuclear cells. For TREC PCR, the following primers and probe were used: sj-5′ forward: CACATCCCTTTCA ACCATGCT (900 nM); sj-3′ reverse: GCCAGCTGCAG GGTTTAGG (900 nM) and the oligo 5′ FAM-ACACC TCTGGTTTTTGTAAAGGTGCCCACT-TAMRA p-3′ (250 nM) as a detection probe. PCR (2 min at 50°C followed by 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min) was carried out in ABI PRISM 7900 Sequence Detector *Taq*Man system (Applied Biosystems, Rotkreuz, Switzerland). The number of TRECs in a given sample was compared to a value obtained with 10-fold serial dilutions of an internal standard provided by Dr Daniel Douek (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA).

Quantitation of T-cell receptor Vβ genes and analysis of RMRP RNA gene

Representatives of specific T-cell receptor Vβ families were detected and were quantified using flow cytometry (Coulter, Elite) as previously described.[14] Genomic DNA derived from patients' lymphocytes was analyzed by PCR amplification and direct sequencing of the RMRP RNA gene.[11] For detection of allelic mutations, the PCR fragments were cloned into TA vector and single colonies were picked and sequenced using the primers 5′-CTGAGAATGAGCCC CGTGTGGTTGG-3′, 5′-CAGCCGCGCTGAGAATGA GCC-3′, 5′-TGCTGAAGGCCTGTATCC-3′, 5′-CTAGA GGGAGCTGACGGATGACG-3′.

Results

Patients

Patient 1 was born at term and she presented at the age of 8 months with recurrent infections including severe *Pneumocystis carinii pneumonitis* (PCP). She was noted to have lymphadenopathy, generalized skin rash and failure to gain weight and was therefore given the diagnosis of Omenn's syndrome. Short limbs and sparse hair was noticed at 4 years of age.

Patient 2 was born after an uneventful pregnancy and delivery. She was referred to our center because of extensive erythroderma, failure to gain weight, chronic diarrhea and oral thrush. She was noted to have short limbs and sparse hair, after she began gaining weight following her BMT while her height remained more than 2 s.d. below the mean.

Metaphyseal dysplasia was identified when she was 3 years old.

Patient 3 was born by cesarean section due to lack of progression during delivery. She was noted to have sparse hair and very short limbs already at 2–3 months of age. Failure to 'grow lymphocytes' *in vitro* for chromosomal analysis triggered an immune work up which revealed marked lymphopenia and hypogammaglobulinemia.

There was no history of immunodeficiency or extreme short stature in any of the three extended families. Neither consanguity nor ancestors of Finnish or Amish origin were reported by the families. Patients 1 and 2 presentation with features of Omenn's syndrome has been recently reported elsewhere.[11]

Skeletal clinical features

All three patients had fine sparse hair, typical of CHH. Linear growth was also affected as expected in this syndrome. Patient 1 height was progressing along the 10% percentile of a CHH growth chart for girls, which is −5.35 s.d. below the normal range.[2] Patient 2 now 20 years old followed the 90th percentile on the CHH growth chart, while patient 3 appears to be more affected than the first 2, remaining below the 10th percentile on the CHH growth chart. Patients 1 and 2 had metaphyseal irregularity and sclerosis in the long bones as well as in the metacarpals and metatarsals.[11]

The metaphyseal changes in patient 3 were far less prominent than in the other two patients probably because of this patient's younger age at the time of evaluation. Nevertheless, metaphyseal irregularities were clearly observed in the long bones.

Genetic analysis

Mutations in the RMRP RNA gene were recently identified in patients with CHH.[3–8] We have therefore analyzed the RMRP RNA gene using genomic DNA extracted from Epstein–Barr virus (EBV)-transformed B lymphoblasts that were established from patients' peripheral blood lymphocytes.

All three patients were compound heterozygotes for RMRP RNA mutations. Patient 1 had on one allele a 28 base pair insertion (including two times duplication) in the transcription regulatory region of the RMRP gene between the TATA box and the transcription initiation site at position −9. The second allele carried a C4T point mutation. Patient 2 had a 7 nucleotide insertion at position −13(ATCTGTG) located between the TATA box and the transcription initiation site on the maternal allele and an A240C mutation on the paternal allele. Patient 3 had an 8 nucleotide insertion at position +1, which was donated by her mother and the commonly found A70G transition on the paternal allele.

Immune evaluation

All three patients had consistently profound lymphopenia and T-cell dysfunction. Flow cytometry analysis revealed markedly reduced numbers of $CD3^+$ T cells in all patients (Table 1). The numbers of B lymphocytes were within normal limits while natural killer (NK) cells were normal in patients 1 and 3 but reduced in patient 2.

Table 1 Immune evaluation before bone marrow transplantation

	Patient 1	*Patient 2*	*Patient 3*	*Normal range*
Serum immunoglobulins (g/l)				
IgG	5.6	6.2	1.0	2.3–14.1
IgM	<0.1	1.12	0.6	0.1–1.4
IgA	<0.1	<0.1	<0.1	0.1–0.8
Specific antibodies				
Tetanus (IU/ml)	0.01	<0.01	ND	>0.10
Polio	<1:8	<1:8	ND	>1:16
Lymphocyte markers (cells/μl)				
CD3	340	237	105	1900–6200
CD4	289	133	51	1300–4000
CD8	21	142	20	500–2000
CD19	170	475	480	300–3000
CD56	980	47	229	160–1100
Mitogenic responses[a] *(patient/control)*				
PHA	3/64	14/107	30/231	
Candida skin test	Negative	Negative	Negative	

Abbreviations: ND = not done; PHA = phytohemagglutinin.
[a]Expressed as stimulation index.

Mitogenic responses to PHA were profoundly depressed in all three patients (Table 1). Addition of exogenous interleukin (IL)-2 to the cultures did not rescue the proliferative response and neither did the combination of phorbol esters with the Ca_2^+ ionophore, ionomycin. Similarly, stimulation of CD3 with UCHT1 antibody in the presence or absence of exogenous IL-2 failed to stimulate the T cells of patients 1 and 2 (not shown).

The humoral immune system was also affected in these patients. Patient 1 had no detectable IgM and IgA but normal IgG on presentation. Patient 2 had no IgA and low but normal Ig and IgM levels and patient 3 had panhypogammaglobulinemia.

Together these results indicated that the patients had a combined immunodeficiency which was indistinguishable from SCID.

Bone marrow transplantation

Patient 1 received a CAMPATH treated 3/6 mismatch paternal BMT at the age of 16 months. Owing to graft failure 7 months later, a second transplant was performed by using bone marrow from a 6/6 HLA antigen identical unrelated donor. Patient 3 received as first choice bone marrow from 9/10 HLA antigen match unrelated donor. Patient 2 received bone marrow from her 6/6 HLA antigen match brother without conditioning when she was 12 months old.

The course during and immediately post-transplant was uneventful with Patients 1, 2 and 3 leaving hospital 60 days, 52 days and 47 days post-transplant, respectively. Patients 1 and 3 developed mild facial GvHD, which responded promptly to topical corticosteroids. Patient 3 had membranous conjunctivitis, presumably related to GvHD, which responded to topical cyclosporin. Patient 3 was also treated successfully with Gancyclovir for a presumed EBV infection 8 months after transplant.

Hemopoietic engraftment in patients 1 and 3 was rapid (Table 2). White blood cells rose to normal range at day 15 and day 12 post-transplant for patients 1 and 3, respectively. Similarly, patients 1 and 3 no longer needed blood transfusions at days 21 and 20 and became independent of platelet transfusion at days 12 and 32 post-transplant, respectively. Patient 2 who did not receive myeloablative conditioning did not require transfusions.

Immune reconstitution

Immune reconstitution was also rapid and was sustained for more than 15, 20 and 5 years of follow-up for patients 1, 2 and 3, respectively (Table 3). All patients have normal numbers of $CD3^+$ T cells, $CD56^+$ NK cells and $CD20^+$ B cells. In addition, all three patients have normal responses to PHA and to various antigens *in vitro*. Moreover, *in vivo* Candida skin tests were positive in all patients.

Complete recovery of the humoral immune function was noticed in all patients' with normal serum IgG, IgM and IgA levels, and excellent antibody responses to protein as well as polysaccharide vaccines. Indeed, patients 1 and 2 had uneventful episodes of chicken pox at ages 6 years and 13 years, respectively. Furthermore, all three patients have had no adverse effects to live attenuated MMR vaccine. Together these results indicate that the grafts in these patients remained robust for long periods of time.

Thymic function and T-cell repertoire

TREC analysis was performed to assess thymus production. TREC levels were 3.29×10^2, 3.53×10^2 and 2.47×10^3 for patients 1, 2 and 3, respectively, whereas control was 3.25×10^2. This level is comparable to 80 other normal control samples, indicating that all three patients had normal thymopoiesis. To analyze the T-cell repertoire we studied recently the frequency of various T-cell receptor Vβ families. All three patients had full representation of Vβ families similar to control samples obtained for healthy individuals (Figure 1).

Discussion

We have demonstrated here that BMT provides long-term immune reconstitution in profound immunodeficiency associated with CHH. Consistent with results we have obtained in other types of profound T-cell immunodeficiencies,[17,18] MUD transplants provided rapid engraftment and long-term robust and sustained immune function in our patients with CHH.

Indeed, the two patients who received MUD BMT in this report are alive and well. Serum immunoglobulin levels as well as specific antibody response to childhood vaccines are within normal levels. Similarly, the number of circulating mature B cells, NK cells and T lymphocyte subsets were all normal. *In vitro* responses to mitogens or antigens were also comparable to a healthy individual control. The function of the thymus gland also appears to be unremarkable as determined by normal TREC evaluations. In full agreement patients T-cell repertoire was complete as assessed by the diversity of the T-cell receptor β chain.

Response to conditioning with busulfan and cyclophosphamide as well as prophylaxis with corticosteroids and cyclosporine A was similar in CHH patients when compared with others receiving the same regimen.[16] In

Table 3 Immune reconstitution after BMT

	Patient 1	*Patient 2*[a]	*Patient 3*	*Normal range*
Serum immunoglobulins (g/l)				
IgG	10.2	8.4	10.9	5.3–14.4
IgM	1.0	1.1	1.2	0.3–2.4
IgA	2.5	0.6	1.2	0.5–3.6
Specific antibodies				
Tetanus	0.7	7.0	7.0	>0.10
Polio	>1:64	>1:64	>1:64	>1:16
Lymphocyte markers				
CD3	2099	1865	2075	1660–3700
CD4	749	479	1279	340–2200
CD8	838	343	767	220–1300
CD19	462	379	2208	90–1400
CD56	272	212	482	100–720
Mitogenic and antigenic responses[b] *(patient/control)*				
PHA		50/55	357/131	
Herpes simplex	101/367	110/25	14/47	
Tetanus	153/32	30/11	98/57	
Candida skin test	Positive	Positive	Positive	

Abbreviations: BMT = bone marrow transplantation; HLA = human leukocyte antigen; PHA = phytohemagglutinin.
[a]Patient 2 received an HLA matched related BMT without myeloablation.
[b]Expressed as stimulation index.

Table 2 Engraftment and GvHD after BMT

	Engraftment (day)			*GvHD*			*Chimerism*[a]
	WBC	*RBC*	*PLT*	*Grade*	*Organ*	*Treatment*	
Patient 1	9[b]	21[c]	12[b]	1	Skin, mild	Topical corticosteroid	>90% donor cells
Patient 2	NR	NR	NR		No GvHD		>90% male donor cells
Patient 3	10[b]	20[c]	32[b]	1	Mild conjunctivitis	Cyclosporin-A eye drops	>90% donor cells

Abbreviations: BMT = bone marrow transplantation; GvHD = graft-versus-host disease; NR = not relevant; PLT = platelets; RBC = red blood cells; WBC = white blood cells.
[a]Analysis of chimerism was performed on whole blood in Patients 1 and 3 or on Ficoll-separated mononuclear cells in Patient 2.
[b]Day post-transplant which WBC reached >500 or platelets >20 000.
[c]Days to transfusion independence.

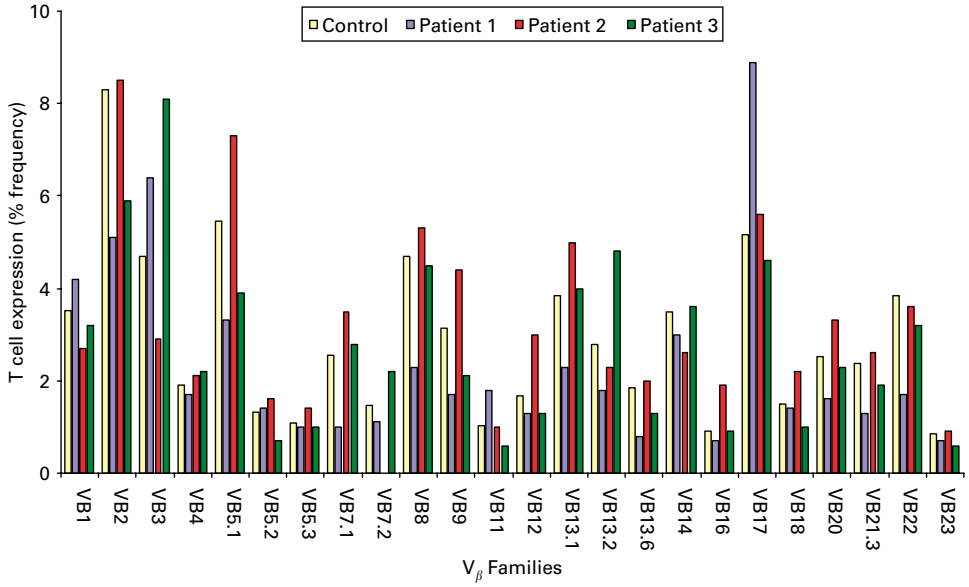

Figure 1 T-cell receptor repertoire. Relative representation of various Vβ families in the three patients' samples compared with normal control.

fact, linear growth which is attenuated in this syndrome seems to follow a typical pattern with no worsening.

Recently, myeloablation was used to facilitate engraftment of a matched related transplant in a case with CHH.[10] We show here (patient 2) that lymphocyte engraftment (more than 90% donor cells which reflects mostly T cells) and robust immune function persists 20 years post-transplant even without myeloablation when a full matched related donor is used. It is obviously expected that other hemopoietic lineages in this patient were unaffected by the bone marrow transfusion and remained of recipient origin. More importantly this patient is well and shows no susceptibility to infections, and experienced no autoimmune features or malignancy.

The need for BMT in CHH appears to be uncommon as most patients have only mild to moderate abnormalities of the immune system. In fact earlier reports from Finland suggested that profound T-cell deficiency was not a feature of CHH and that live vaccinations can be safely administered.[2,13] This was in sharp contrast with several patients we have followed over the years and reported[11] and contrary to McKusickk's[1] original reports on death from varicella in patients with CHH of Amish origin. Recent careful analysis of the Finnish population did, however, record premature deaths related to immune dysfunction.[19] Together, these results indicate that a small number of patients with CHH present early in life, likely in the first 2 years with profound T-cell deficiency which is indistinguishable from the presentation of SCID or Omenn syndrome.[11]

The diagnosis of CHH might be delayed[9,10] if the skeletal dysplasia is not extreme as it was in patient 3. Indeed, in patient 1 and 2 the diagnosis of CHH was delayed until after failure to thrive (body weight) but not linear growth was reversed after reconstitution of the immune system with BMT.

We can therefore conclude that profound immunodeficiency associated with CHH can be cured by stem cell transplantation. Replacing hemopoietic stem cells did not seem to improve the skeletal dysplasia in our patients nor did it help in another reported case.[10] This is in spite of the fact that osteoclasts which are involved in bone remodeling are of hemopoietic origin, maybe replaced in the process of BMT.

Other features associated with CHH such as the increased susceptibility to non-Hodgkin lymphoma[20] remains to be carefully studied in patients who have undergone BMT. If a comparison can be made with other immunodeficiencies such as Wiskott Aldrich syndrome[21] and Hyper-IgM syndrome[22] where BMT appears to prevent the occurrence of lymphoma it is plausible to speculate that a similar outcome might be expected in CHH.

In conclusion, we have demonstrated that long-term sustained immune function was achieved in patients with CHH and profound T-cell deficiency by means of BMT. These patients tolerated well myeloablation and engrafted rapidly with MUD transplant and suffered relatively few complications. Long-term full immune reconstitution was also obtained without myeloablation, when a matched related donor was used.

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