

THYMIC FUNCTION AFTER HEMATOPOIETIC STEM-CELL TRANSPLANTATION FOR THE TREATMENT OF SEVERE COMBINED IMMUNODEFICIENCY

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ABSTRACT

Background Immune function can be restored in infants with severe combined immunodeficiency by transplantation of unfractionated bone marrow from HLA-identical donors or T-cell-depleted marrow stem cells from haploidentical donors, with whom there is a single haplotype mismatch, without the need for chemotherapy before transplantation or prophylaxis against graft-versus-host disease. The role of the thymus in this process is unknown.

Methods We analyzed the phenotypes of circulating T cells and the proliferative responses of peripheral-blood mononuclear cells to phytohemagglutinin in 83 patients with severe combined immunodeficiency who received allogeneic marrow transplants without T-cell ablation from related donors over an 18-year period. We also tested for the presence of episomes of T-cell antigen receptors (extrachromosomal DNA circles formed during intrathymic T-cell development) to assess thymus-dependent T-cell reconstitution.

Results Before and early after transplantation, the numbers of circulating T cells were low, with a predominance of mature CD45RO⁺ T cells (primarily resulting from the transplacental transfer of maternal cells); T-cell antigen-receptor episomes were undetectable in peripheral-blood mononuclear cells. In 73 of the infants, thymus-derived T cells expressing CD45RA and T-cell antigen-receptor episomes were detected within three to six weeks after transplantation. The mean (\pm SD) value for thymus-dependent T-cell antigen-receptor episomes peaked (at 7311 ± 8652 per microgram of peripheral-blood mononuclear-cell DNA) 1 to 2 years after transplantation and declined to low levels (less than 100 episomes per microgram of DNA) within 14 years, as compared with a gradual decline from birth to the age of about 80 years in normal subjects.

Conclusions The vestigial thymus in infants with severe combined immunodeficiency is functional and can produce enough T cells after bone marrow transplantation to provide normal immune function. (N Engl J Med 2000;342:1325-32.)

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INFANTS with severe combined immunodeficiency who receive HLA-identical bone marrow or bone marrow stem cells from a family member with whom they share an HLA haplotype (HLA-haploidentical donors) that have been depleted of T cells, without chemotherapy before transplantation or prophylaxis against graft-versus-host disease, have circulating T cells of donor origin that are phenotypically and functionally normal 90 to 120 days after transplantation.^{1,2} Although it is presumed that donor stem cells mature to become T cells in the infant's thymus, there is limited evidence that this is the case.³ Moreover, thymic tissue in infants with severe combined immunodeficiency is morphologically vestigial, weighs less than 1 g, and contains no Hassall's corpuscles or thymocytes.⁴⁻⁶ These observations have raised the question of whether the T cells are derived either from transplacentally transferred maternal T cells or from residual mature donor T cells in the graft. In recent years, the phenotypic characteristics of T cells recently released from the thymus have been identified. These CD3⁺ T cells express the surface markers CD45RA and CD62L.⁷⁻⁹ In contrast, memory T cells express the surface marker CD45RO.¹⁰ However, both mature CD45RA⁺ T cells and mature CD45RO⁺ T cells can expand outside the thymus, so CD45RA is not an unequivocal marker of newly emerged T cells.¹¹

During intrathymic differentiation, progenitor cells undergo rearrangement of T-cell antigen-receptor genes to become T cells, leading to the formation of extrachromosomal DNA circles, or episomes.¹²⁻¹⁴ These episomes can be detected in T cells that have recently developed in the thymus, whereas T cells that develop extrathymically do not contain these episomes.^{15,16} In chickens, thymectomy results in the gradual loss of T-cell antigen-receptor episomes in circulating T cells and in T cells in all peripheral lymphoid tissues.¹⁵ The same change occurs in humans after thymectomy.^{11,16} Also, the circulating T cells of infants who have the complete DiGeorge syndrome and who do not have a thymus lack these episomes, but episomes can be detected after thymic transplan-

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tation.¹⁷ Thus, the presence of episomes of the T-cell antigen-receptor gene in circulating T cells is an indication that rearrangement of the T-cell antigen-receptor gene has recently occurred in the thymus.

Our study was designed to determine whether T-cell reconstitution in infants with severe combined immunodeficiency who are given unfractionated bone marrow or marrow rigorously depleted of T cells (without chemotherapy before transplantation or prophylaxis against graft-versus-host disease) is due to the development of donor stem cells into T cells in the thymus or to peripheral expansion of mature maternal or donor T cells. Because of the lack of previous thymopoiesis and the absence of immunosuppressive therapy, bone marrow transplantation in such infants provides a unique opportunity to study the kinetics of the initial establishment of the T-cell component of the immune system.

METHODS

Study Patients

We studied 83 infants with severe combined immunodeficiency who were given unfractionated HLA-identical bone marrow transplants (7 infants), T-cell-depleted HLA-identical marrow transplants (5 infants), or HLA-haploidentical T-cell-depleted marrow transplants (71 infants), without chemotherapy before transplantation or prophylaxis against graft-versus-host-disease, over the past 18 years.² Of these 83 patients, 72 were boys; 44 had severe combined immunodeficiency due to a mutation of the gene encoding the common γ chain (an X-linked disorder), 5 had a mutation of the gene encoding Janus kinase 3 (JAK3), 2 had a mutation of the gene encoding the α chain of the interleukin-7 receptor, 12 had a deficiency of adenosine deaminase, 17 had proven autosomal recessive disease of unknown molecular type, 1 had cartilage-hair hypoplasia, and 2 had severe combined immunodeficiency of unknown molecular cause.

Donor marrow was depleted of T cells by agglutination with soybean lectin and two cycles of rosetting with sheep erythrocytes treated with aminoethylisothiuronium bromide, as described elsewhere.^{1,18,19} The mean (\pm SD) age at transplantation was 0.5 ± 0.4 year. Blood samples were obtained from the patients before transplantation and at varying intervals for up to 16 years thereafter. T-cell phenotypes and proliferative responses to mitogens were determined with the use of freshly isolated peripheral-blood mononuclear cells, as previously described.¹ Excess cells were frozen at -70°C in RPMI 1640 medium containing dimethyl sulfoxide. Blood samples obtained from 90 normal subjects (<1 year to 79 years of age) were also studied. The blood specimens were obtained with the approval of the Duke University Committee on Human Investigations and the written informed consent of the patients or their parents.

Quantitative Competitive Polymerase-Chain-Reaction Assay for T-Cell Antigen-Receptor Episomes

Polymerase-chain-reaction (PCR) analysis for T-cell antigen-receptor episomes was performed as described elsewhere.¹⁶ Briefly, DNA from 2 million to 10 million peripheral-blood mononuclear cells was isolated with the use of Trizol (Life Technologies, Gaithersburg, Md.). DNA (1 μg) was amplified at an annealing temperature of 60°C for 30 cycles and at 72°C for 30 seconds in a 50- μl reaction mixture containing 1 \times PCR buffer (Life Technologies), 1.8 mM magnesium chloride, 200 μM deoxynucleotide triphosphate, 250 nM primers,¹⁶ 2.5 μCi of [α -³²P]deoxycytidine triphosphate, 0.5 U of platinum *Taq* polymerase (Life Technologies), and 5000, 1000, 500, or 100 molecules of a standard T-cell antigen-

receptor episome. PCR amplification of the standard molecule results in a product that is 60 bp shorter than the molecule of the true T-cell antigen-receptor episome. PCR products were separated by polyacrylamide-gel electrophoresis and quantified with an imaging device (PhosphorImager, Molecular Dynamics, Sunnyvale, Calif.). The lower limit of detection was 100 T-cell antigen-receptor episomes per microgram of DNA. To determine the kinetics of thymus-derived immune reconstitution, we determined the numbers of episomes in the entire mononuclear-cell population of each sample and did not correct for the numbers of T cells.

Statistical Analysis

The patients were grouped in three categories: infants in whom T-cell function developed (defined as proliferative responses to phytohemagglutinin of more than 100,000 counts per minute per million cells) at any point (73 infants), infants in whom T-cell function never developed (after a follow-up period of at least one year after transplantation [3 infants]), and infants who had not been followed long enough for T-cell function to have developed (7 infants). At various times after transplantation, we evaluated data on the 73 infants in whom T-cell function developed, using only a single point from an individual patient in any given period. If more than one point was available for a patient in a specific period, the first point was used. Measurements of T-cell phenotype (275 measurements) and T-cell proliferative responses (432 measurements) were obtained at the following times: before transplantation (day 0); every 40 days between day 1 and day 200 after transplantation; every 100 days through day 700; every year through year 5; and every 2 years through year 15. Measurements of T-cell antigen-receptor episomes (86 measurements) were obtained at the following times: before transplantation (day 0); every 100 days between day 1 and day 300 after transplantation; every 200 days through day 700; and at years 3, 5, 7, 9, 11, and 13. The mean values for the measurements of T-cell phenotype and T-cell antigen-receptor episomes at the midpoint of each period were used for analysis. Linear and exponential analyses of the best fit for the data were performed with the use of Cricket Graph III (Computer Associates International, Islandia, N.Y.). Multiple regression analyses were performed and statistics calculated with the use of Statistica software (StatSoft, Tulsa, Okla.).

RESULTS

T-Cell Phenotypes

In normal infants, CD45RA+ cells make up the majority of peripheral T cells, whereas in normal older children and adults there are approximately equal numbers of CD45RA+ and CD45RO+ T cells.²⁰ In the 73 infants with severe combined immunodeficiency in whom T-cell function developed after bone marrow transplantation, CD45RO+ T cells predominated for the first 100 days (Fig. 1A). This could have been due to the expansion of transplacentally transferred maternal T cells or adoptively transferred mature donor T cells. The mean length of time until CD45RA+ cells became the principal type of T cell present was 140 to 180 days after transplantation, and the mean number of CD45RA+ cells was highest 350 days after transplantation (1394 ± 1232 cells per cubic millimeter). The number of CD45RA+ T cells gradually declined thereafter, but CD45RA+ cells continued to predominate over CD45RO+ cells until 12 years after transplantation (Fig. 1B). Fourteen years after transplantation, the mean number of CD45RA+ cells (measured in four patients) was 114 ± 46 cells per cubic millimeter. All 73 patients

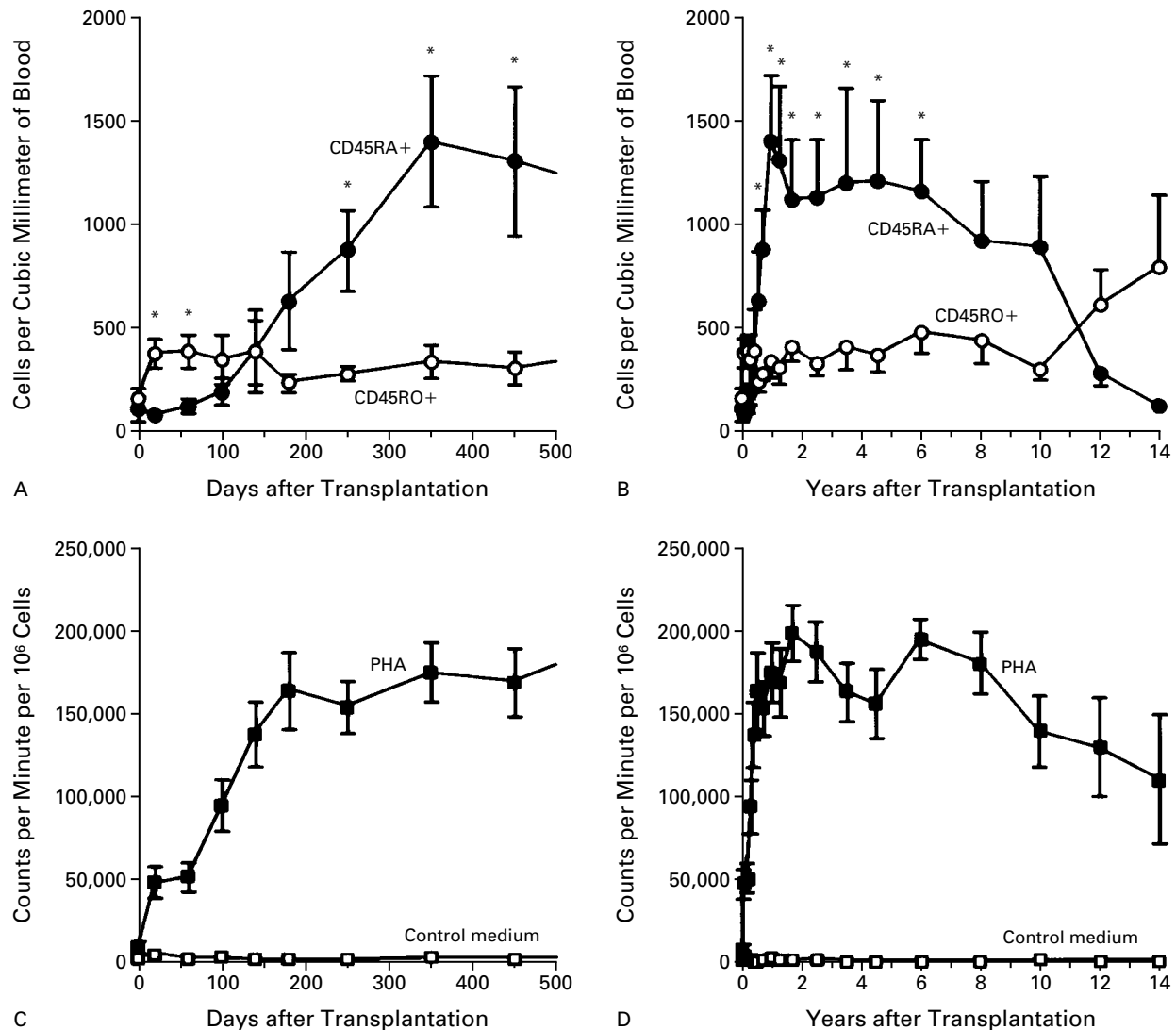


Figure 1. T-Cell Phenotype (Panels A and B) and T-Cell Proliferation in Response to Phytohemagglutinin (Panels C and D) after Successful Bone Marrow Transplantation in 73 Infants with Severe Combined Immunodeficiency.

Measurements were taken at the following times: before transplantation (day 0), every 40 days between day 1 and day 200 after transplantation, every 100 days through day 700, every year through year 5, and every two years through year 15. The mean values at the midpoint of each period were used for analysis. Between 4 and 40 measurements were evaluated in any given period. The differences between CD45RA+ T-cell levels and CD45RO+ T-cell levels were significant ($P < 0.05$) at the times indicated by an asterisk. The differences between the incorporation of [³H]thymidine in proliferating T cells in response to phytohemagglutinin (PHA) and its incorporation in response to control medium were significant ($P < 0.05$) at all times. Values are means \pm SE.

had normal T-cell function and no major or opportunistic infections.

Thirty-four infants had their CD3+, CD4+, and CD8+ T cells studied sequentially for expression of CD45RA and CD62L. Most CD45RA+ cells in infants who received transplants coexpressed CD62L; the kinetics of the development of CD45RA+CD62L+ cells were therefore not different from the kinetics of the development of cells that expressed only CD45RA (data not shown).

T-Cell Proliferation

Only T cells proliferate in response to the mitogen phytohemagglutinin. Incorporation of [³H]thymidine into the DNA of the 73 infants with severe combined immunodeficiency in whom T-cell function developed exceeded a mean of 50,000 counts per minute per million cells by 60 days after transplantation, exceeded a mean of 100,000 counts per minute per million cells by 140 days, and reached a plateau at 180 days (Fig. 1C). Thus, responsiveness to

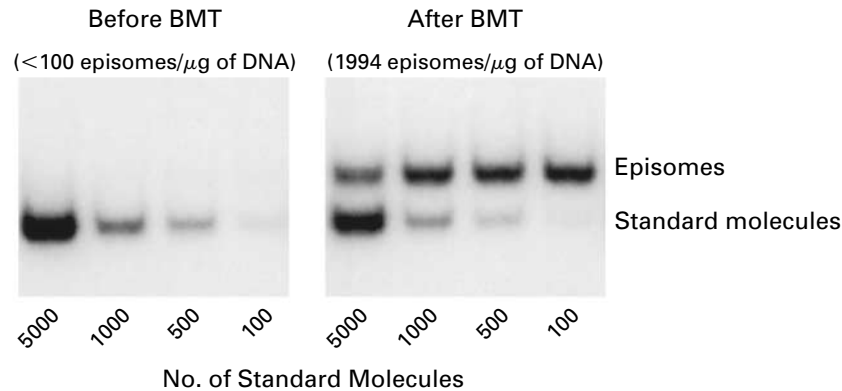


Figure 2. Appearance of T-Cell Antigen-Receptor Episomes after Bone Marrow Transplantation (BMT) in an Infant with Severe Combined Immunodeficiency. DNA was purified from the peripheral-blood mononuclear cells of Patient 2 before transplantation and 238 days after transplantation and assayed for the presence of T-cell antigen-receptor episomes by quantitative, competitive polymerase chain reaction (PCR). The autoradiographs show episomes and standard molecules amplified by PCR. The number of standard molecules in each reaction is indicated.

phytohemagglutinin developed before the appearance of CD45RA+ T cells, at a time when most of the T cells were CD45RO+ cells. Responsiveness to phytohemagglutinin declined slightly with increasing age (Fig. 1D), but even the recipients who had undergone transplantation 14 years earlier had a mean value for [³H]thymidine incorporation that was well within the normal range for our laboratory (109,623 ± 87,104 counts per minute per million cells).²

Thymic Function

Thymic tissue in infants with any form of severe combined immunodeficiency lacks thymocytes and is morphologically vestigial.^{4,5} Of the 11 infants for

whom sufficient samples of peripheral-blood mononuclear cells were available for analysis of T-cell antigen-receptor episomes before transplantation, 9 had fewer than 100 episomes per microgram of DNA (the limit of detection in our assay) (Fig. 2), indicating that T-cell development within the thymus does not occur in infants with severe combined immunodeficiency before transplantation (Table 1). Five of these 11 infants had substantial numbers of T cells (>100 cells per cubic millimeter), probably as a result of transplacental transfer of maternal T cells^{21,22}; only 2 of the 5 had detectable levels of T-cell antigen-receptor episomes (≥100 per microgram of DNA). One infant who received an unfractionated marrow transplant from an HLA-identical sibling had early T-cell function resulting from peripheral expansion of the CD45RO+ donor T cells. Reconstitution of thymus-derived T cells (those containing T-cell antigen-receptor episomes) occurred in this infant six months after transplantation, leading to a reversal of the ratio of CD45RO+ cells to CD45RA+ cells. In this infant, neither the presence of mature, transplacentally transferred maternal T cells nor the presence of adoptively transferred donor T cells from the unfractionated marrow graft prevented the later development of new T cells in the thymus.

The kinetics of thymic T-cell development in the 73 patients in whom T-cell function developed are shown in Figure 3A. T-cell antigen-receptor episomes were first detected about 100 days after transplantation. The mean peak value was 7311 ± 8652 episomes per microgram of DNA between one and two years after transplantation, after which the values declined (Fig. 3B). In the 90 normal subjects, the number of episomes declined exponentially with increasing age to undetectable levels (<100 episomes per microgram of DNA) over a period of approximately 80

TABLE 1. T-CELL COUNTS AND THYMIC FUNCTION BEFORE TRANSPLANTATION IN INFANTS WITH SEVERE COMBINED IMMUNODEFICIENCY.*

PATIENT No.	TYPE OF IMMUNODEFICIENCY	T-CELL ANTIGEN-RECEPTOR EPISOMES	CD3+ COUNT
		no./μg of PBMC DNA	cells/mm ³
1	Autosomal recessive	<100	100
2	JAK3 deficiency	<100	8268†
3	X-linked	<100	17
4	X-linked	<100	21
5	X-linked	226	651†
6	X-linked	100	365
7	X-linked	<100	15
8	X-linked	<100	42
9	X-linked	<100	570†
10	X-linked	<100	904†
11	X-linked	<100	23

*The 11 infants listed were those for whom sufficient samples of peripheral-blood mononuclear cells (PBMC) were available for analysis before transplantation.

†This patient had documented transplacental transfer of maternal T cells into the fetal circulation.

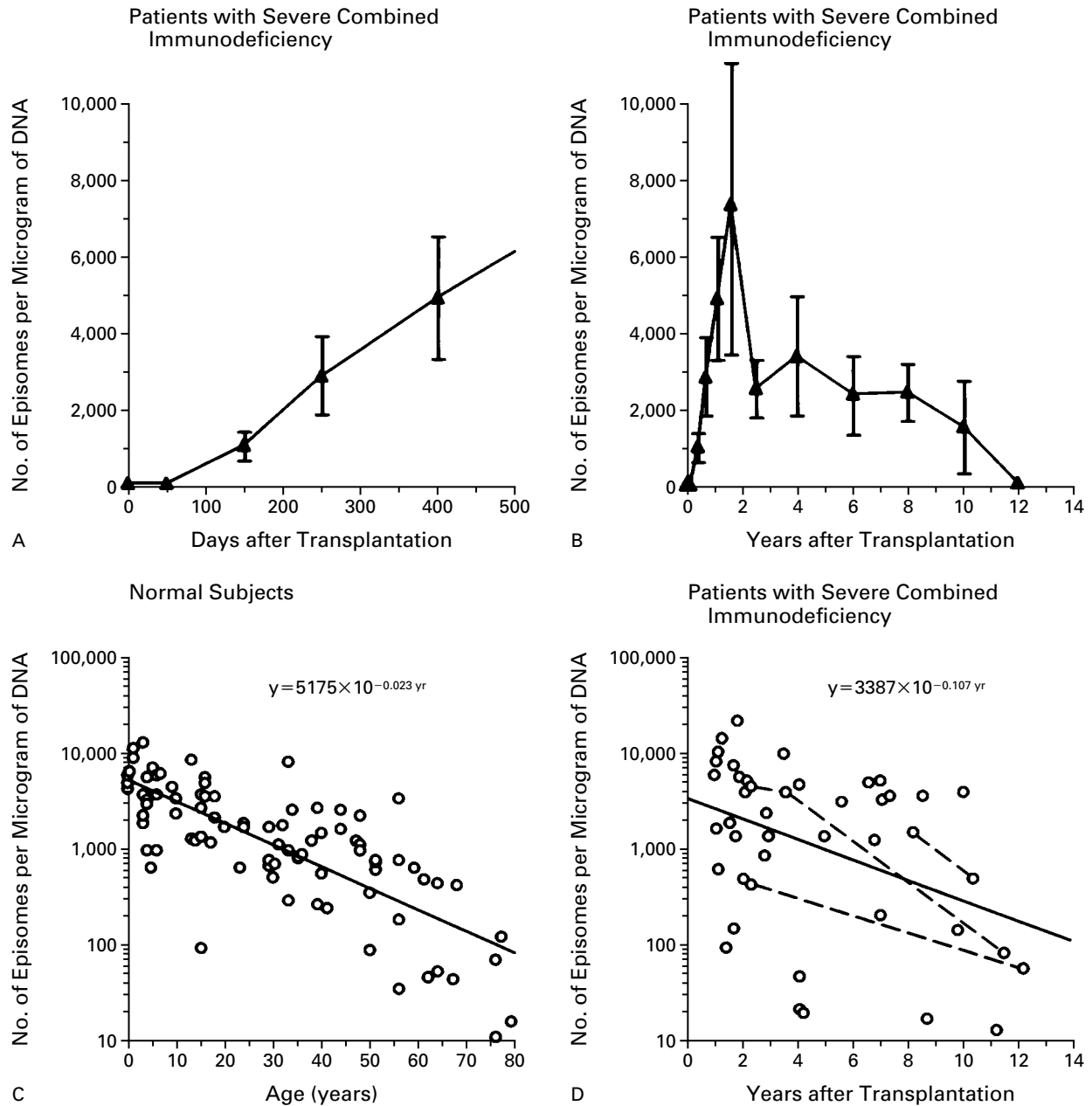


Figure 3. Kinetics of Thymic Function after Successful Bone Marrow Transplantation in Infants with Severe Combined Immunodeficiency.

Panels A and B show the mean (\pm SE) number of T-cell antigen-receptor episomes (86 measurements) at various times after transplantation in 51 infants with severe combined immunodeficiency in whom T-cell function developed and for whom samples were available for analysis. Measurements were taken at the following times: before transplantation (day 0), every 100 days through day 300 after transplantation, every 200 days through day 700, and at years 3, 5, 7, 9, 11, and 13. The mean values at the midpoint of each period were used for analysis. Between 3 and 12 measurements were evaluated in any given period. Panel C shows the number of T-cell antigen-receptor episomes in 90 normal subjects, and Panel D shows the number in 45 infants with severe combined immunodeficiency one or more years after successful bone marrow transplantation. Dashed lines represent data from selected patients for whom data were available longitudinally and after 10 years.

years (Fig. 3C). By contrast, the values in infants with severe combined immunodeficiency declined to undetectable levels by 14 years (Fig. 3D). T-cell antigen-receptor episomes were undetectable in the three infants in whom T-cell function never developed.

The kinetics of the development of responsiveness to phytohemagglutinin and the kinetics of CD45RA+ T cells and T-cell antigen-receptor episomes in the 73 infants in whom T-cell function developed are shown in Figure 4. Responsiveness to phytohemagglutinin occurred in advance of the appearance of thymic CD45RA+ T cells, at a time when CD45RO+ T cells predominated (Fig. 4A). Maximal values were reached two years after transplantation, after which the values declined more or less in parallel; however, responsiveness to phytohemagglutinin persisted the longest (Fig. 4B). The persistence of responsiveness to phytohemagglutinin is probably due to the fact that thymus-derived T cells also expand in the periphery. The generally parallel emergence and decline of CD45RA+ T cells and T-cell antigen-receptor episomes suggest that the emergence of CD45RA+ cells is a good indicator of thymic function in patients with severe combined immunodeficiency.

DISCUSSION

One of the central questions concerning methods of reconstituting immune function in infants with severe combined immunodeficiency has been whether

the small, morphologically vestigial thymus in such infants has the capacity to convert normal stem cells into immunocompetent T cells.^{4,5} It was postulated that the small size of the thymus in these infants could have been due to a lack of colonization by normal stem cells.^{23,24}

We found that, before bone marrow transplantation, infants with severe combined immunodeficiency lacked circulating T cells that had the characteristics of T cells that had recently entered the circulation from the thymus. After marrow transplantation, circulating T cells of donor origin emerged from the thymus. Previous studies have shown that some of the T cells that emerge in infants with severe combined immunodeficiency are restricted in their capacity to recognize specific antigens by the HLA haplotype of the parent who was not the donor of the transplant²⁵ and that all such T cells appear to be tolerant to the infant,²⁶ thus suggesting that both positive and negative selection has occurred in these infants.²

That the T cells that emerged after transplantation did not result from the expansion of transplacentally transferred maternal T cells is demonstrated by the fact that the maternal T cells present in infants with severe combined immunodeficiency at presentation were CD45RO+ cells and that they did not contain T-cell antigen-receptor episomes. Mature T cells that were not removed from the donor marrow in the

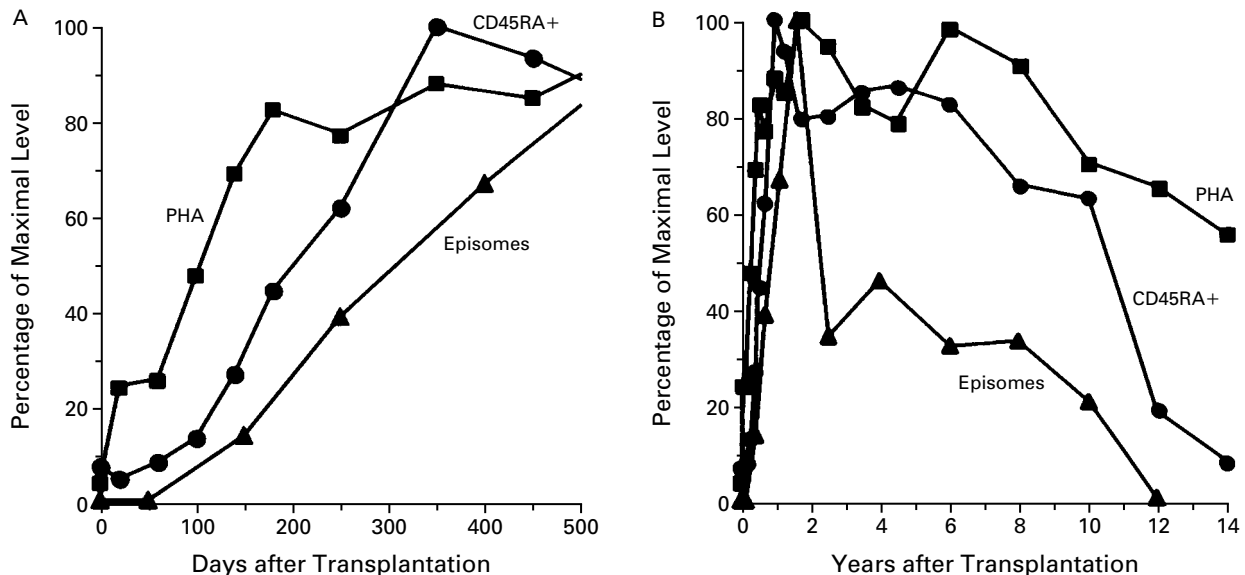


Figure 4. Kinetics of T-Cell Proliferation and Reconstitution after Successful Bone Marrow Transplantation in Infants with Severe Combined Immunodeficiency.

Shown are the levels of T-cell proliferation in response to phytohemagglutinin (PHA), peripheral-blood CD45RA+ counts, and T-cell antigen-receptor episomes as a percentage of the mean maximal level achieved during any period. The mean lengths of time needed to reach levels that were 50 percent of the maximum were 100 days for responsiveness to phytohemagglutinin, 200 days for CD45RA+ cells, and 300 days for T-cell antigen-receptor episomes.

process of T-cell depletion would also be expected to have a CD45RO⁺ phenotype and to lack T-cell antigen-receptor episomes.

The emergence of CD45RA⁺ cells is a good indicator of thymic function during the development of the immune system in patients with severe combined immunodeficiency, since the kinetics of the emergence of CD45RA⁺ cells and those of circulating T-cell antigen-receptor episomes in the transplant recipients were similar. The development of responsiveness to phytohemagglutinin occurred earlier than the appearance of CD45RA⁺ T cells, indicating that transplacentally transferred maternal T cells or adoptively transferred donor T cells can respond to this nonspecific stimulus relatively early after transplantation.

In general, dominance of CD45RO⁺ T cells persisted in the infants with the lowest numbers of T cells or the poorest T-cell function. Two infants who received cyclosporine for one month at presentation because of graft-versus-host disease caused by the transplacental transfer of maternal T cells do not as of this writing have substantial numbers of CD45RA⁺ T cells, raising the question of whether this treatment could interfere with intrathymic T-cell development.²⁷

Early reconstitution of T-cell function in one of the patients with a high number of transplacentally transferred T cells, who received an unfractionated marrow transplant from an HLA-identical sibling, was due to the expansion of adoptively transferred T cells, since T-cell proliferation in response to phytohemagglutinin preceded the appearance of circulating T-cell antigen-receptor episomes.²² Thus, peripheral T-cell expansion did not prevent T-cell development in the thymus, since new T cells developed after transplantation of unfractionated HLA-identical marrow in this infant.

In conclusion, infants with severe combined immunodeficiency have the ability to generate T cells with newly rearranged antigen receptors, and the thymus is the likely site of this process. The number of these T cells peaks in the first two years after transplantation, after which they disappear more rapidly than in normal subjects. One possible reason for the rapid decline in thymic function in these infants is that the small thymus is unable to sustain the same output as a normal thymus. Alternatively, the problem could be that there are not enough donor stem cells present to stimulate continued growth of the thymic epithelium in these infants.²⁸ Whether there will be a decline in immune function many years after transplantation is unknown. Nevertheless, T-cell reconstitution in infants with severe combined immunodeficiency occurs in the thymus and is long-lasting; many patients now between the ages of 10 and 17 years have excellent T-cell function and do not have recurrent infections.²

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