

AGE, THYMOPOIESIS, AND CD4+ T-LYMPHOCYTE REGENERATION AFTER INTENSIVE CHEMOTHERAPY

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Abstract Background. Inadequate reconstitution of CD4+ T lymphocytes is an important clinical problem complicating chemotherapy, human immunodeficiency virus infection, and bone marrow transplantation, but relatively little is known about how CD4+ T lymphocytes regenerate. There are two main possibilities: bone marrow-derived progenitors could reconstitute the lymphocyte population using a thymus-dependent pathway, or thymus-independent pathways could predominate. Previous studies have suggested that the CD45RA glycoprotein on CD4+ T lymphocytes is a marker for progeny generated by a thymus-dependent pathway.

Methods. We studied 15 patients 1 to 24 years of age who had undergone intensive chemotherapy for cancer. The absolute numbers of CD4+ T lymphocytes in peripheral blood and the expression of CD45 isoforms (CD45RA and CD45RO) on these lymphocytes were

studied serially during lymphocyte regeneration after the completion of therapy. Radiographic imaging of the thymus was performed concomitantly.

Results. There was an inverse relation between the patients' ages and the CD4+ T-lymphocyte counts six months after therapy was completed ($r = -0.92$). The CD4+ recovery correlated quantitatively with the appearance of CD45RA+CD4+ T lymphocytes in the blood ($r = 0.64$). There was a higher proportion of CD45RA+CD4+ T lymphocytes in patients with thymic enlargement after chemotherapy than in patients without such enlargement (two-sided $P = 0.015$).

Conclusions. Thymus-dependent regeneration of CD4+ T lymphocytes occurs primarily in children, whereas even young adults have deficiencies in this pathway. Our results suggest that rapid T-cell regeneration requires residual thymic function in patients receiving high-dose chemotherapy. (N Engl J Med 1995;332:143-9.)

DEPLETION of CD4+ T lymphocytes is an important clinical problem in bone marrow transplantation¹⁻³ and human immunodeficiency virus (HIV) infection.⁴ Yet the mechanisms by which CD4+ T lymphocytes regenerate are poorly understood. In 1961 Miller discovered the importance of the thymus in T-cell development in his studies of neonatally thymectomized mice.⁵ Since then, extensive studies have elucidated the role of the thymus in fetal T-cell development,⁶ and there has been a general acceptance of the view that the thymus plays an ongoing part in T-cell generation. This view underlies the concept that HIV infection of the thymus contributes to the depletion of CD4+ T lymphocytes in the acquired immunodeficiency syndrome (AIDS).^{7,8} Similarly, the idea that immunocompetence after bone marrow transplantation requires at least partial HLA matching between donor and recipient⁹ assumes that T-cell regeneration occurs by means of a thymic pathway.

The postnatal role of the thymus is unclear, however. The absence of immunodeficiency in children and adults who have undergone thymectomy^{10,11} and the observation that the human thymus undergoes spontaneous involution at a relatively young age imply that T-cell populations may be maintained by mechanisms

largely independent of the thymus. It has thus been suggested that thymus-independent pathways may be important for the generation and maintenance of T cells,¹² and these pathways have been invoked to explain T-cell regeneration after bone marrow transplantation.^{13,14} Clarifying the relative importance of the thymus-dependent and thymus-independent pathways may be an important step in devising strategies to improve the reconstitution of CD4+ T lymphocytes in disease states.

Because there is a substantial decrease in thymic size during puberty and thymic rebound has been reported in children after chemotherapy,¹⁵ we have postulated that the contribution of the thymus to the regeneration of CD4+ T lymphocytes may vary with the patient's age. Previous work has shown that CD4+ T lymphocytes exported from the thymus express the surface antigen CD45RA.¹⁶⁻¹⁸ We investigated CD45 isoform expression on CD4+ T lymphocytes in patients 1 to 24 years of age during the regeneration of T cells after intensive cytotoxic chemotherapy. We found age-related differences in CD4+ T-lymphocyte regeneration and evidence that children and young adults recovering from cytotoxic chemotherapy have different rates of thymopoiesis.

METHODS

Patients and Protocols

Fifteen patients with histologic evidence of cancer were enrolled in studies conducted by the National Cancer Institute to treat brain tumors (Pediatric Branch [PB] protocol 90-C-211), sarcoma (PB 86-C-169 and 93-C-125), and non-Hodgkin's lymphoma (PB 89-C-41 and 93-C-207). In each study, the dose of cyclophosphamide was substantial, ranging from 1.2 to 4.5 g per square meter of body-surface area

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per cycle. The chemotherapy administered in protocols PB 90-C-211, 86-C-169, and 89-C-41 has been described in detail elsewhere.¹⁹ Patients treated in protocols PB 93-C-125 and 93-C-207 received sequential cycles of cyclophosphamide-containing drugs at doses of 2.4 and 1.2 g per square meter per cycle, respectively, administered at least every 21 days. All the protocols were approved by the institutional review board of the National Cancer Institute, and informed consent was obtained from all patients or their parents before enrollment in the study.

No patient had detectable involvement of bone marrow with tumor. Three patients received radiation therapy: Patient 6 received 6000 cGy to the right forearm, Patient 7 received 6600 cGy to the buttocks and pelvis, and Patient 12 received 3000 cGy to the cranium and spine. Patient 1 had HIV infection acquired from his mother; before the development of lymphoma, his only manifestations of disease were chronic eczema and thrombocytopenia. All the patients were rendered free of detectable neoplastic disease during chemotherapy. Twelve patients remained free of disease, whereas Patients 1, 9, and 14 relapsed 6, 18, and 8 months after therapy, respectively.

Flow Cytometry

Specimens of peripheral blood were obtained during routine clinic visits and were handled according to established clinical guidelines. The specimens were stained for flow cytometry with the whole-blood lysis technique and analyzed with a FACScan (Becton Dickinson, San Jose, Calif.) with Lysis II software.¹⁹ The monoclonal antibodies used included anti-CD3 (Leu-4) and anti-CD4 (Leu-3) (Becton Dickinson), anti-CD45RO (UCHL1) (Dako, Carpinteria, Calif.), and anti-CD45RA (Alb11) (Gentrak, Plymouth Meeting, Pa.). Irrelevant antibodies of the IgG1, IgG2a, and IgG2b subclasses were used to ascertain background staining. CD4+ T lymphocytes were defined as cells positive for both CD4 and CD3. CD4+ T lymphocytes with exclusive expression of CD45RA were designated as bearing high-molecular-weight isoforms of CD45, and those with exclusive expression of CD45RO were designated as bearing low-molecular-weight

isoforms. To calculate absolute numbers of each lymphocyte subgroup, the percentage of cells staining positive was multiplied by the absolute count of peripheral-blood lymphocytes as determined by a Coulter counter (Coulter, Hialeah, Fla.) followed by a differential leukocyte count in a blood sample obtained simultaneously.

Radiographic Imaging

As part of routine follow-up of their diseases, all the patients except Patient 12 underwent radiographic imaging that included imaging of the thymus. Patients 4, 6, 7, 9, 10, 11, and 15 underwent computed tomographic (CT) scanning of the chest, and Patients 1, 2, 3, 4, 5, 8, 10, 13, 14, and 15 underwent scanning with gallium-67. The images of the thymus were analyzed serially for most patients from the time of presentation until one year after the completion of therapy. Patients 1 and 8 were followed for 6 months after therapy, and Patient 6 was followed for 10 months. The mean (\pm SE) number of times the patients (except Patient 12) were studied for the occurrence of thymic rebound in the year after therapy was 4.3 ± 0.5 .

Radiographic evidence of thymic rebound was sought by the radiologist and the nuclear-medicine physicians. At the time of the analyses, these investigators were unaware of the degree of CD4+ T-lymphocyte recovery in individual patients. Thymic volumes were calculated from CT images as described elsewhere,²⁰ with thymic rebound defined as at least a doubling of the thymic volume measured at presentation. In the patients studied with gallium-67, increased mediastinal uptake of gallium consistent with thymic rebound was assessed as described elsewhere.¹⁵ Thymic rebound was defined as an uptake of gallium-67 in the anterior mediastinum not observed previously and having the characteristic size, shape, and location of thymic activity. Patients who underwent imaging by both methods were analyzed by each, with concordant results in all cases.

Because neither CT scanning nor gallium scanning can reliably distinguish thymic rebound from recurrent tumor in the thymic region,²¹ the radiographic studies were analyzed serially to ascertain that the thymus had returned to the size observed at presentation.

Table 1. Depletion and Recovery of CD4+ T Lymphocytes and Occurrence of Thymic Rebound in the Study Patients.*

PATIENT No.	AGE	TUMOR TYPE/MEDIASTINAL INVOLVEMENT	CD4+ COUNT			RA:RO RATIO		THYMIC REBOUND	MAXIMAL CD4+ COUNT†	MONTHS TO MAXIMAL COUNT‡
			BEFORE THERAPY	NADIR‡	AFTER 6 MO§	AFTER 6 MO§	MAXIMAL¶			
	yr		per mm ³						per mm ³	
1	1	NHL/No	756	8	822	6.45	6.45	Yes	1543	7
2	3	NHL/No	755	32	590	1.40	15.3	Yes	672	10
3	7	NHL/No	ND	172	733	0.06	0.95	Yes	1179	10
4	11	NHL/Yes	520	114	385	1.00	1.00	Yes	687	13
5	13	NHL/No	ND	63	314	0.27	0.76	No	561	9
6	13	Sarcoma/No	1743	71	628	2.87	2.87	Yes	628	6
7	14	Sarcoma/No	514	76	329	0.90	2.14	Yes	662	12
8	18	NHL/No	ND	119	213	0.51	0.51	No	213	6
9	19	Sarcoma/No	935	224	342	0.06	0.13	No	342	6
10	19	NHL/No	ND	199	199	0.10	0.19	No	907	7
11	21	Sarcoma/No	615	135	201	0.17	0.17	Yes	3931	15
12	23	Brain/No	740	9	54	0.02	0.30	ND	433	15
13	24	NHL/No	ND	83	131	0.45	0.45	No	376	18
14	24	NHL/Yes	205	30	47	0.02	0.02	No	93	8
15	24	NHL/No	ND	81	123	0.07	0.66	No	309	12

*NHL denotes non-Hodgkin's lymphoma, and ND not determined.

†After the completion of therapy.

‡Values are the lowest CD4+ T-lymphocyte counts recorded, generally occurring at or near the completion of chemotherapy. Values were obtained at the time of hematologic reconstitution after the preceding cycle of chemotherapy and no more than 48 hours before the ensuing cycle. Normal counts are 1000 to 1800 per cubic millimeter for patients one to six years of age and 700 to 1100 per cubic millimeter for patients seven years of age or older.¹⁸

§Values were obtained 6 \pm 1 months after the completion of chemotherapy. CD45RA is expressed by 66 to 77 percent of CD4+ T lymphocytes in normal subjects 1 to 6 years of age, 55 to 67 percent of CD4+ T lymphocytes in subjects 7 to 17 years of age, and 32 to 49 percent of CD4+ T lymphocytes in subjects \geq 18 years of age.¹⁸

¶Values are the highest ratios of CD45RA to CD45RO (RA:RO ratios) recorded within one year after the completion of therapy. The maximal ratios occurred at the time of the longest follow-up in all patients except Patients 1 and 2, in whom they occurred six months and three months, respectively, after the completion of therapy.

Such resolution of thymic enlargement in the absence of clinical or radiographic evidence of recurrent tumor was regarded as sufficient evidence that the increase in size was benign.

Statistical Analysis

Spearman correlation coefficients were calculated, and the Wilcoxon rank-sum test was used to compare measurements between patients. All P values are two-sided.

RESULTS

Fifteen patients 1 to 24 years of age underwent intensive chemotherapy for cancer with regimens containing oxazaphosphorines. Table 1 contains data on each patient before, during, and after treatment. Although CD4+ T-lymphocyte counts were depressed in some patients at the time of presentation, serial measurements showed further depletion after chemotherapy, as reported elsewhere.¹⁹ The mean (\pm SE) CD4+ T-lymphocyte count after the completion of chemotherapy in the 15 patients was 94.4 ± 17.1 per cubic millimeter. The extent of depletion was not related to the age of the patients ($r = 0.13$).

Age-Related Differences

CD4+ T lymphocytes were analyzed serially for at least six months after the completion of chemotherapy. Younger patients had greater recovery of CD4+ T lymphocytes six months after chemotherapy than older patients, who had persistent severe depletion of CD4+ T lymphocytes (Fig. 1). There was an inverse correlation between the CD4+ T-lymphocyte count six months after therapy and the patient's age ($r = -0.92$). CD4+ T-lymphocyte counts can vary with age in normal people,¹⁸ but the severely depleted counts in our older patients six months after therapy were well below normal values for their age (as shown in the notes to Table 1). Analysis of the net increase in CD4+ T-lymphocyte counts from the completion of therapy until six months later (as derived from data in Table 1) showed a similar inverse relation to age ($r = -0.90$).

Regeneration of CD45RA+CD4+ T Lymphocytes

CD45RA is expressed on recent emigrants from the thymus,¹⁶⁻¹⁸ and work with T-cell regeneration in an animal model has shown that CD4+ T lymphocytes expressing high-molecular-weight isoforms of CD45 (CD45RA) are regenerated from bone marrow progenitors through a thymus-dependent pathway. By contrast, CD4+ T cells derived from thymus-independent pathways express low-molecular-weight isoforms of CD45 (CD45RO) almost exclusively.²² In this series of patients, CD45RO was consistently expressed on essentially all CD4+ T lymphocytes during and immediately after intensive chemotherapy. The reason for this chemotherapy-associated change in phenotype is not known, but the phenomenon has been reported previously.¹⁹ Because of it, newly generated CD45RA+CD4+ T lymphocytes could be readily detected.

To evaluate whether the age-related differences in

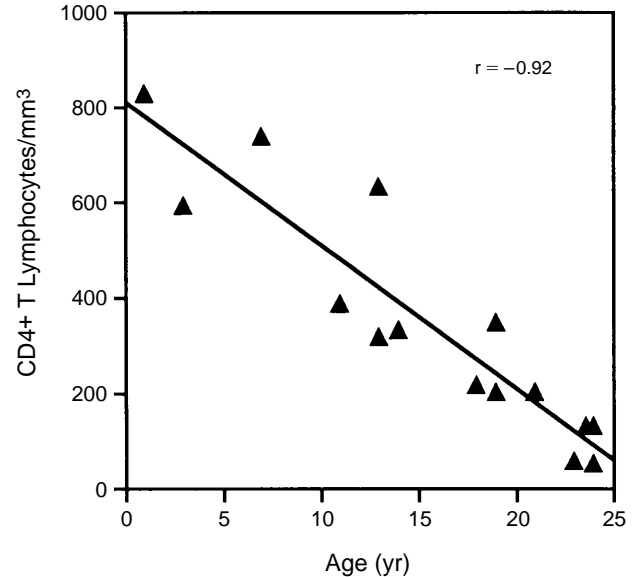


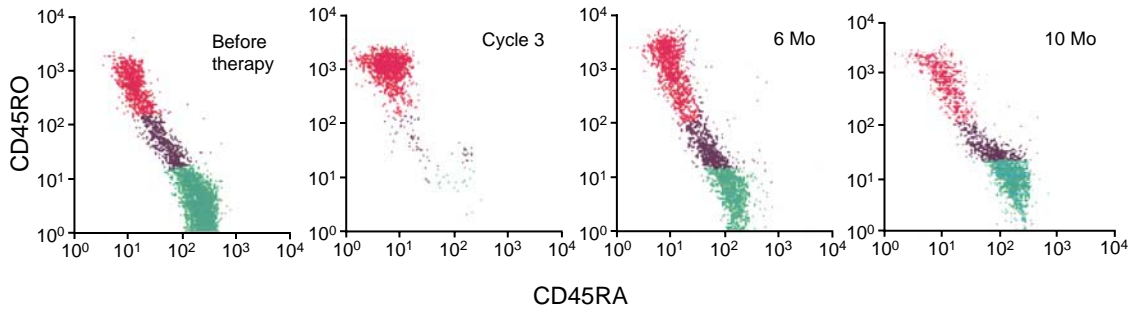
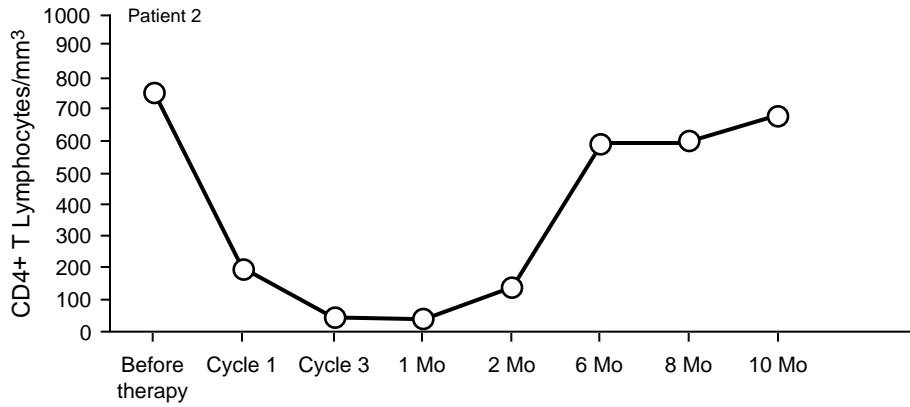
Figure 1. Relation between Age and Reconstitution of CD4+ T Lymphocytes.

Absolute CD4+ T-lymphocyte counts were measured in the peripheral blood of patients approximately six months after the completion of chemotherapy. The correlation coefficient was calculated by the Spearman rank-correlation method.

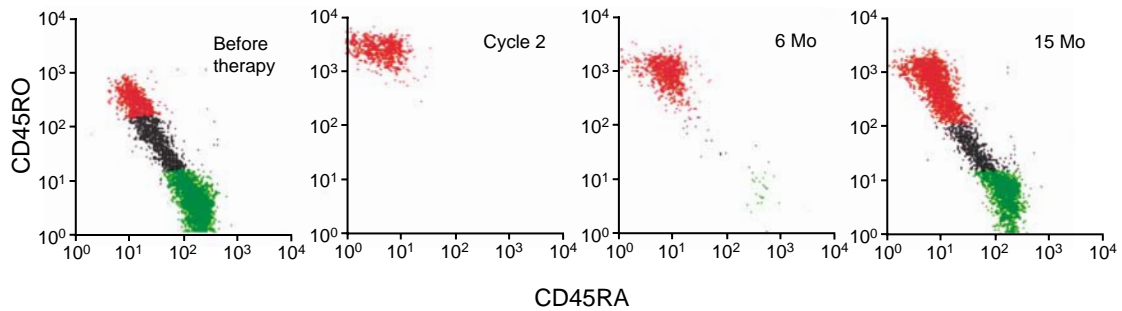
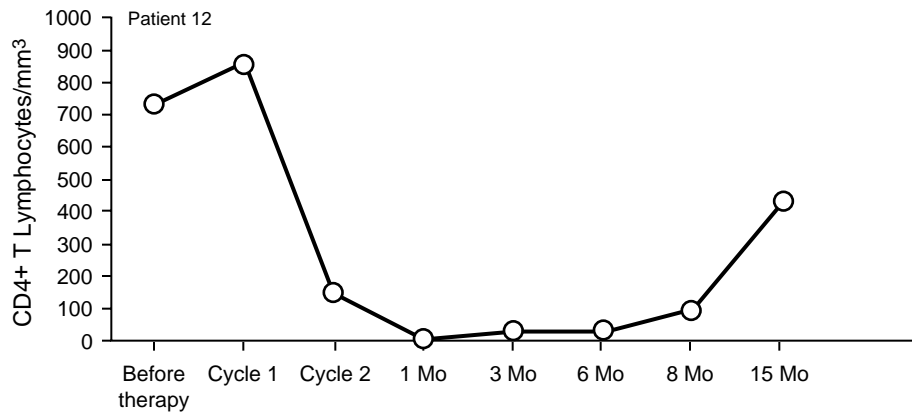
the rate of CD4+ T-lymphocyte recovery after chemotherapy could be due to differing rates of thymopoiesis, we prospectively evaluated the expression of CD45 isoforms on CD4+ T lymphocytes during regeneration after chemotherapy. Data from two patients are shown in Figure 2. Panel A shows data from a three-year-old (Patient 2). During and immediately after chemotherapy, almost all CD4+ T lymphocytes expressed CD45RO (ratio of CD45RA to CD45RO [RA:RO ratio], 0.03). Six months after the completion of chemotherapy, increased numbers of CD45RA+CD4+ T lymphocytes appeared (RA:RO ratio, 1.4), at the same time as an increase in the absolute CD4+ T-lymphocyte count. In contrast, Panel B shows the persistence of CD45RO on essentially all CD4+ T lymphocytes in a 23-year-old (Patient 12) for six months after chemotherapy (RA:RO ratio, <0.01). Eight months after therapy, a few CD45RA+CD4+ T cells were seen (RA:RO ratio, 0.30), at the same time as a slight increase in the CD4+ T-cell count. Fifteen months after chemotherapy, increased numbers of CD45RA+CD4+ T lymphocytes were observed (RA:RO ratio, 0.96) in association with an increase in the number of CD4+ T lymphocytes. Figure 3 shows the correlation between the RA:RO ratio and the net increase in the CD4+ T-lymphocyte count six months after chemotherapy ($r = 0.64$).

The recovery of CD4+ T lymphocytes in one patient (Patient 3) did not follow a typical course with regard to the RA:RO ratio and the number of CD4+ T lymphocytes. The recovery of the CD4+ T-lympho-

A



B



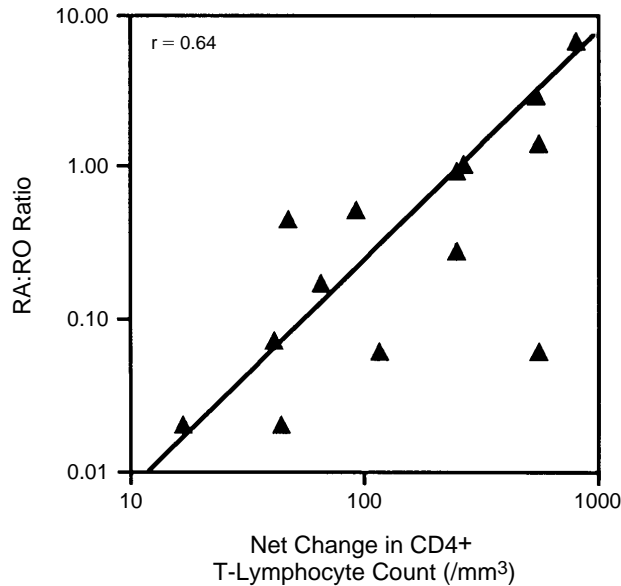


Figure 3. Relation between the RA:RO Ratio and the Recovery of CD4+ T Lymphocytes.

The net change in the CD4+ T-lymphocyte count six months after the completion of therapy was calculated by subtracting the nadir (Table 1) from the count six months after therapy. The RA:RO ratio was ascertained by flow cytometry six months after therapy. The data point for Patient 10 is not shown (the net change was zero), but this information was used in calculating the correlation coefficient.

cyte count was brisk by three months after chemotherapy, but low numbers of CD45RA+CD4+ T lymphocytes persisted. The patient had a complicated course because of recurrent bowel obstruction that required bowel resection approximately six months after chemotherapy. After his recovery from surgery, high RA:RO ratios appropriate for his age appeared, along with a further increase in the CD4+ T-lymphocyte count. These events suggest that lymphocyte activation related to clinical events may confound changes in RA:RO ratios.

Radiographic Evidence of Thymic Rebound

Serial images of the thymus by CT scanning, radio-nuclide imaging, or both were obtained in 14 patients. Figure 4 shows CT scans of two patients at presentation and three months after chemotherapy. In an 11-year-old (Patient 4), the large anterior mediastinal mass after therapy aroused concern about recurrent lymphoma and prompted an open biopsy of the thymus. Histologic analysis revealed normal thymic tissue. In contrast, CT scans of a 19-year-old (Patient 10)

showed no increase in thymic size three months after therapy.

Table 1 shows the maximal RA:RO ratio in each patient during the period after therapy when the images of the thymus were obtained. The Wilcoxon rank-sum test revealed that the patients with radiographic evidence of thymic rebound had significantly higher maximal RA:RO ratios than those without rebound during that period ($P=0.015$). The patients with radiographic evidence of thymic rebound were younger than those without such evidence ($P=0.017$), and six months after the completion of therapy they had higher CD4+ T-lymphocyte counts ($P=0.011$) and higher net increases in the counts ($P=0.007$).

DISCUSSION

Our evidence suggests that a thymopoietic pathway of CD4+ T-lymphocyte regeneration is important throughout childhood. However, the thymus appears to have a diminished capacity for regeneration of CD4+ T lymphocytes as it involutes with the approach of adulthood. Nevertheless, several patients who were at least 18 years of age eventually had increased CD4+ T-lymphocyte counts that correlated with the appearance of sizable numbers of CD45RA+CD4+ T lymphocytes. Therefore, the capacity for regeneration of CD4+ T lymphocytes after chemotherapy appears to diminish with age, but it is unclear whether there is an age after which the thymus loses its regenerative capacity completely. Further studies in older adults are needed to address this issue. Our finding suggests, however, that any therapy or disease state that depletes CD4+ T cells may have a more profound effect in older patients than in children.

With regard to the chemotherapy-induced depletion of CD4+ T lymphocytes, four of the eight patients in this study who were at least 18 years old at the time of presentation had opportunistic infectious complications¹⁹ during their prolonged periods of CD4+ T-cell lymphopenia. In contrast, such complications developed in only one of the seven patients in this study who were under 18 years of age. The number of patients we evaluated was too small to permit us to ascertain the true incidence of opportunistic complications in relation to age in patients undergoing chemotherapy, but our findings suggest that in the setting of intensive chemotherapy, thymic production of CD4+ T lymphocytes may help protect patients from clinically important immunodeficiency.

The information presented here may help to further our understanding of the causes of inadequate recovery

Figure 2. Changes in the Number of CD4+ T Lymphocytes and in CD45 Isoforms during T-Cell Reconstitution.

Panel A shows changes in the number of CD4+ T lymphocytes and CD45 isoforms (CD45RA and CD45RO) during chemotherapy and 1, 2, 6, 8, and 10 months thereafter in a three-year-old patient (Patient 2). Panel B shows data for a 23-year-old patient (Patient 12). Three-color flow cytometry was used to gate on CD4+ T lymphocytes, and two-color immunofluorescence dot plots are shown. CD45RA (green dots) is the high-molecular-weight isoform and CD45RO (red dots) the low-molecular-weight isoform, both expressed by CD4+ T cells. Black dots represent CD4+ T cells with intermediate expression of both CD45RA and CD45RO. Values during chemotherapy were measured at the time of hematologic reconstitution after the cycle noted.

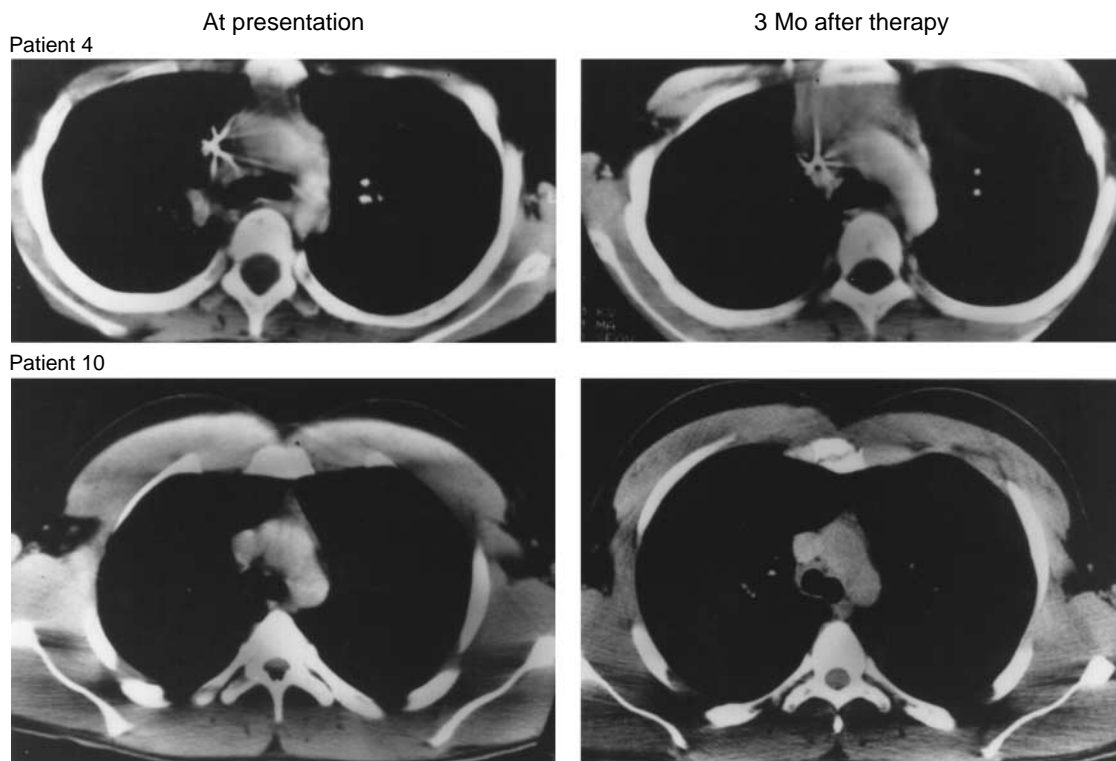


Figure 4. CT Scanning of the Thymus before and after Chemotherapy.

The mediastinum is shown at presentation (left-hand panels) and three months after the completion of chemotherapy (right-hand panels). For an 11-year-old patient (Patient 4, upper panels), the calculated thymic volumes were 4.83 cm³ at presentation and 61.8 cm³ three months after chemotherapy. For a 19-year-old patient (Patient 10, lower panels), the volumes were 4.65 cm³ at presentation and 1.31 cm³ three months after chemotherapy.

of CD4+ T lymphocytes in HIV infection and other clinical settings. It is known that HIV can infect thymocytes and thymic epithelium,^{7,8} and some evidence suggests that thymus-dependent pathways may be deficient even in young patients with HIV infection.²³ However, studies of the regeneration of CD45RA+CD4+ T lymphocytes and thymic imaging in the HIV-infected child in this series suggest that a thymic-dependent pathway of CD4+ T-lymphocyte reconstitution did function despite HIV infection. Furthermore, there is preliminary evidence that in children receiving antiviral dideoxynucleotides to treat HIV infection there is a more sustained increase in the CD4+ T-lymphocyte count than in adults similarly treated.²⁴ These data suggest that thymopoiesis may indeed be important for the regeneration of CD4+ T lymphocytes in the setting of HIV infection.

Our work has implications for new therapies being undertaken in HIV infection and bone marrow transplantation. For example, for gene therapy using genes for resistance to HIV to succeed,²⁵⁻²⁷ it is critical that the targeted cell be a progenitor of long-lived CD4+ T lymphocytes. Placing these genes into prethymic stem cells would be logical in patients with intact thymopoietic pathways. If, however, the thymic pathway has been compromised by either advancing age or progressive disease, targeting prethymic bone marrow

progenitors may not result in sufficient progeny to yield a beneficial outcome. Similarly, the use of highly purified populations of bone marrow stem cells to reconstitute marrow function in transplant recipients²⁸ may result in varying degrees of immune reconstitution, depending on the age of the patient. The studies described here may provide a means of evaluating and interpreting the results of such therapeutic strategies and of studying T-cell regeneration in a variety of clinical settings.

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