Table 4 Univariate and multivariate analysis on progression

Factors	*Relative risk (95% confidence interval)*	*P-value*
Univariate		
Age		
per year	0.96 (0.92–1.00)	0.048[a]
Sex		
Male *vs* female	1.22 (0.50–2.95)	0.67
Performance status[b]		
2–4 *vs* 0–1	1.55 (1.02–2.33)	0.038[a]
Interval from diagnosis to transplant[c]		
Per year	0.88 (0.75–1.04)	0.14
Numbers of prior chemotherapy regimens[c]		
Per cycle	0.98 (0.85–1.14)	0.8
History of autologous transplant		
Yes *vs* no	1.52 (0.63–3.64)	0.35
History of radiation[d]		
Yes *vs* no	3.68 (1.51–8.95)	0.0041[a]
Clinical stage at transplant		
3–4 *vs* 1–2	1.28 (0.95–1.74)	0.11
Serum levels of LDH prior to transplant		
Elevated *vs* normal	1.95 (0.78–4.90)	0.15
Chemosensitivity		
Sensitive *vs* refractory	0.45 (0.19–1.07)	0.07
CNS involvement at transplant		
Yes *vs* no	7.27 (2.91–18.18)	<0.001[a]
Bone marrow involvement at transplant		
Yes *vs* no	0.49 (0.14–1.73)	0.27
Bulky disease at transplant[e]		
Yes *vs* no	3.13 (1.05–9.29)	0.040[a]
Histology		
Indolent	1	
Aggressive	4.15 (1.20–14.26)	0.024[a]
Highly aggressive	5.95 (1.22–29.10)	0.028[a]
Stem-cell sources		
Peripheral blood	1	
Bone marrow	0.64 (0.21–1.95)	0.44
Cord blood	1.28 (0.15–10.79)	0.82
Conditioning regimen		
Fludarabine and busulfan	1	
Fludarabine and cyclophosphamide	1.22 (0.27–5.4)	0.79
Fludarabine ane melphalan	3.19 (0.88–11.5)	0.077[f]
TBI based	4.02 (1.05–15.4	0.043[f]
Others	2.67 (0.61–11.7)	0.19
Methotrexate-containing GVHD prophylaxis		
Yes *vs* no	0.47 (0.18–1.21)	0.12
Grade II–IV acute GVHD		
II–IV/0–I	0.52 (0.19–1.45)	0.21
Multivariate		
History of radiation[d]		
Yes *vs* no	3.45 (1.12–10.0)	0.03[a]
CNS involvement at transplant		
Yes *vs* no	6.25 (2.08–20.0)	0.001[a]
Grade II to IV acute GVHD		
II–IV/0–I	0.28 (0.090–0.86)	0.026[a]

LDH = lactate dehydrogenase; CNS = central nervous system; GVHD = graft-versus-host disease.
[a]Statistically significant.
[b]Performance status was defined according to the Eastern Cooperative Oncology Group (ECOG) criteria.
[c]They were analyzed as a continuous variable.
[d]Any types of irradiation prior to RIST were included.
[e]When patients had at least one mass with its diameter longer than 10 cm, they were defined as cases with bulky disease.
[f]Flu/Mel and TBI entered a multivariate analysis and rejected in backward stepwise proportional-hazard modeling.

targeting minor histocompatibility antigens or tumor-specific antigens have been investigated.[36 38]

The risk of progression was significantly higher among patients with prior history of local radiation therapy (RT) than those who did not received RT (Table 4). RT is indicated when the patients have chemo-refractory disease, central nervous system involvement or bulky mass, which means that patients with a history of RT carry risk factors of poor outcomes.

The survival of patients with PS 0–1 was significantly longer than that with PS 2–4 (Table 5). PS is affected by age, infections, and aggressiveness of the diseases, and patients with poor PS carry the overlapping risk factors of poor outcomes. While RIST is considered feasible even for patients with worse PS than is conventional stem-cell transplantation, the present study showed that the poor PS is also a risk factor of poor RIST outcomes. The time from diagnosis to RIST also affected the outcomes; our univariate and multivariate analyses showed significant differences in PFS. The observations are comparable to the results by van Besien.[18]

While the present study provided novel information on RIST for advanced lymphoma, we need to take its limitations into consideration. It is a small-sized, retrospective study; unrecognized biases might have affected the results. However, it demonstrated that many patients with advanced lymphoma can survive after RIST. These observations provide a rationale for continuing our clinical trials on RIST for malignant lymphoma, focusing on minimizing toxicities, preventing GVHD, and controlling infectious complications. It is imperative to establish optimal preparative regimens and management of GVHD to enhance a GVL effect and to reduce TRM. Although the present study showed that patients with chemotherapy-resistant indolent lymphoma can achieve durable remission after RIST, we cannot yet conclude that RIST improves the prognosis. Despite progressive improvement of safety, the risk of significant TRM limits the widespread application of allo-SCT for malignant lymphoma. Without evidence of efficacy, most physicians considered this risk too high to justify studies of allo-SCT. Phase III clinical trials

Table 5 Univariate and multivariate analysis on progression-free survival

Factors	*Relative risk (95% confidence interval)*	*P-value*
Univariate		
Age	1.00 (0.97–1.03)	0.78
Sex		
Male *vs* female	0.80 (0.44–1.45)	0.47
Performance status[a]		
2–4 *vs* 0–1	1.99 (1.49–2.66)	<0.0001[b]
Interval from diagnosis to transplant[c]		
Per year	0.83 (0.73–0.94)	0.004[b]
Numbers of prior chemotherapy regimens[c]		
Per cycle	0.93 (0.82–1.06)	0.26
History of autologous transplant		
Yes *vs* no	1.69 (0.95–3.03)	0.077
History of radiation		
Yes *vs* no	1.57 (0.87–2.84)	0.14
Clinical stage at transplant		
3–4 *vs* 1–2	1.30 (0.99–1.72)	0.064
Serum levels of LDH prior to transplant		
Elevated *vs* normal	1.90 (1.04–3.49)	0.38
Chemosensitivity		
Sensitive *vs* refractory	0.35 (0.20–0.63)	0.0004[b]
CNS involvement at transplant		
Yes *vs* no	2.39 (1.11–5.15)	0.026[b]
Bone marrow involvement at transplant		
Yes *vs* no	0.91 (1.46–1.80)	0.79
Bulky disease at transplant		
Yes *vs* no	1.97 (0.83–4.66)	0.12
Histology		
Indolent	1	
Aggressive	3.04 (1.48–6.24)	0.0024[b]
Highly aggressive	1.25 (0.52–3.00)	0.62
Stem-cell sources		
Peripheral blood	1	
Bone marrow	1.47 (0.68–3.17)	0.32
Cord blood	0.66 (0.14–3.11)	0.6
Conditioning regimen		
Fludarabine and busulfan	1	
Fludarabine and cyclophosphamide	0.64 (0.29–1.37)	0.25
Fludarabine ane melphalan	0.80 (0.35–1.85)	0.6
TBI based	0.58 (0.22–1.52)	0.27
Others	0.87 (0.31–2.41)	0.79
Methotrexate-containing GVHD prophylaxis		
Yes *vs* no	0.33 (0.17–0.64)	0.0009[b]
Grade II–IV acute GVHD		
II–IV/0–I	0.89 (0.45–1.73)	0.72
Multivariate		
Performance status[a]		
2–4 *vs* 0–1	1.83 (1.32–2.53)	0.0003[b]
Interval from diagnosis to transplant[c]		
Per year	0.86 (0.74–0.99)	0.04[b]
Methotrexate-containing GVHD prophylaxis		
Yes *vs* no	0.26 (0.13–0.54)	0.0002[b]
Histology		
Indolent	1	
Aggressive	2.69 (1.17–6.15)	0.019[b]
Highly aggressive	1.89 (0.69–5.18)	0.21

LDH = lactate dehydrogenase; CNS = central nervous system; GVHD = graft-versus-host disease.
[a]Performance status was defined according to the Eastern Cooperative Oncology Group (ECOG) criteria.
[b]Statistically significant.
[c]They were analyzed as a continuous variable.

comparing RIST with standard chemotherapy are warranted. However, these trials are frequently problematic, considering that therapeutic approaches are different between transplant and chemotherapy, and that the standard therapies for some subtypes such as mantle cell and peripheral T-cell lymphoma are dismal. Registry multicenter data such as in this study will allow for a reasonable analysis of the role of RIST in advanced lymphoma.

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Appendix

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Biology of Blood and Marrow Transplantation 11:506-511 (2005)

1083-8791/05/1107-0003$30.00/0
doi:10.1016/j.bbmt.2005.03.005

Chest Computed Tomography of Late Invasive Aspergillosis after Allogeneic Hematopoietic Stem Cell Transplantation

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Received February 16, 2005; accepted March 26, 2005

ABSTRACT

Computed tomography (CT) is a powerful diagnostic tool for invasive aspergillosis (IA) after allogeneic stem cell transplantation (allo-SCT); however, little information is available concerning CT findings of late IA after allo-SCT. To characterize CT findings of late IA, we retrospectively examined medical records and high-resolution CT findings of 27 allo-SCT recipients with late IA. Either acute or chronic GVHD was diagnosed in 24 patients. All 27 patients were given corticosteroids at IA diagnosis. High-resolution CT findings included halo (n = 12), centrilobular nodules (n = 12), ill-defined consolidation (n = 13), ground-glass attenuation (n = 8), pleural effusion (n = 7), pleural-based consolidation (n = 4), and cavitation (n = 4). CT findings showing centrilobular nodules and either halo or cavitation were classified into bronchopneumonia type and angioinvasive type, respectively. Angioinvasive-type, bronchopneumonia-type, and combination-type IA were diagnosed in 11, 8, and 4 patients, respectively. CT findings were nonspecific in the other 4 patients. One bronchopneumonia-type case and 2 angioinvasive-type IA cases were subsequently diagnosed as combination type. Although there were no significant differences in patient characteristics between the 2 types of IA, bronchopneumonia-type IA had a poorer prognosis than angioinvasive IA ($P = .022$). Halo is a useful diagnostic marker in late IA as well as early IA, and late IA frequently manifests as bronchopneumonia.

KEY WORDS

Aspergillus • Halo • Bronchopneumonia • Invasive fungal infection • Centrilobular nodules

INTRODUCTION

Invasive aspergillosis (IA) is a significant complication of allogeneic stem cell transplantation (allo-SCT) [1]. It occurs in 4.5% to 15.1% of allo-SCT recipients [2-5]. Risk factors of IA include neutropenia, transplantation without laminar air flow equipment, transplantation from a matched unrelated donor, graft-versus-host disease (GVHD), use of corticosteroids, older age, and underlying diseases [2-5]. The survival of these patients depends on early diagnosis and prompt initiation of therapeutic measures; however, microbiological or histopathologic diagnosis is rarely established because blood cultures are rarely positive for *Aspergillus* species and because invasive procedures are required to obtain pathologic specimens. The difficulty in making an early diagnosis of IA is a critical problem.

Several new techniques have been developed for IA diagnosis. A double direct sandwich enzyme-linked immunosorbent assay (EIA; Platelia Aspergillus; Bio-Rad, Marnes la Coquette, France) for galactomannan

antigen has a 67% to 100% sensitivity and a 81% to 99% specificity for the diagnosis of IA [6]. High-resolution chest computed tomographic (CT) scanning is also beneficial for making an early diagnosis of IA [7,8]. The presence of a CT halo sign is highly indicative of IA in neutropenic patients [9]. The criteria of the European Organization for Research and Treatment of Cancer (EORTC)/Mycology Study Group (MSG) using these measures are widely used and validated for clinical diagnosis of IA [10].

There have been changes in the clinical presentations of IA. It shows a 2-peak pattern in the development of IA: first at the time of neutropenia in the early stage of transplantation and then several months after the transplantation. Recent trends show an increase in late IA, which has now come to account for the most cases of IA [11]. Much remains unknown regarding the clinical presentation of late IA. Most cases of late IA are not associated with neutropenia, and this indicates possible differences from early IA in terms of the mechanism of pathogenesis and clinical conditions. Chest CT is a powerful diagnostic tool for the diagnosis of IA during neutropenia, but there are few reports on late IA after allogeneic hematopoietic stem cell transplantation. To investigate the value of chest CT scans in the diagnosis of late IA, we reviewed chest CT findings in patients with late IA after allogeneic hematopoietic stem cell transplantation.

PATIENTS AND METHODS

Data Collection

We reviewed the medical records and chest CT findings of 27 patients who developed late IA after allo-SCT at the National Cancer Center Hospital, Toranomon Hospital, Tokyo Metropolitan Komagome Hospital, the University of Tokyo Hospital, Keio University Hospital, Hamanomachi Hospital, and Fukuoka University Hospital between January 1999 and November 2002. Data from participating centers were derived from questionnaires distributed to each center. Minimum data required for the inclusion of a patient in this study were age, history of IA before transplantation, underlying diseases, preparative regimens, GVHD prophylaxis, date of transplantation, date of last follow-up, disease status at last follow-up, onset of IA, treatment of IA, response to IA treatment, development of acute or chronic GVHD, date of acute or chronic GVHD, date of disease progression or death, and causes of death.

Diagnostic Criteria for Late IA

When patients who had achieved primary engraftment developed IA, it was defined as late IA. Even after engraftment, patients with neutropenia (<500/μL) or recurrence of underlying diseases were excluded from this study.

IA was categorized as proven, probable, or possible IA, according to the established EORTC/MSG criteria [10]. Briefly, proven IA was diagnosed when there was histologic evidence of tissue invasion by small and uniform dichotomously branching hyphae septate at regular intervals and/or culture findings positive for *Aspergillus* species from autopsy specimens, mostly the lung, and a compatible clinical presentation. Probable IA was defined as clinical findings compatible with IA and the positive findings in EIA for galactomannan antigen. CT findings were not incorporated into the diagnostic criteria of IA in this study. Aspergillus antigen positivity was defined as an optical density index of galactomannan higher than 1.5 in 2 consecutive blood samples by an EIA (Plateria Aspergillus EIA; Bio-Rad). Possible IA was not included in this study.

Management of Late IA

When patients developed an antibiotic-resistant fever, we performed chest CT and some blood tests, including EIA and (1-3)-β-D-glucan assay, for the early diagnosis of IA [12,13]. When the diagnosis of IA was confirmed or suspected, we generally initiated amphotericin B 1.5 mg/kg/d.

Review of High-Resolution CT Scan

CT was performed on commercially available helical or multidetector scanners. Helical techniques consisted of 10.0-mm collimation for individual scans of the entire lung (120 kilovolts [peak]; 100-150 mA) and reconstruction with a standard algorithm. Additional high-resolution CT images were obtained in all patients by using 1.0- to 2.0-mm collimation, a 15- to 20-cm field of view, 120 to 150 kilovolts (peak) and 150 to 200 mA per rotation, a 1.0-second gantry rotation, and a high-spatial-frequency reconstruction algorithm. Hard-copy images were photographed at window settings for lung (center, −700 to −650 Hounsfield Units [HU]; width, 1500 to 2000 HU) and mediastinum (center, 35 to 40 HU; width, 300 to 400 HU).

The CT images were assessed by 2 independent radiologists who did not know patients' characteristics or clinical courses. They assessed the presence of areas of ground-glass attenuation, consolidation, centrilobular nodules, ill-defined nodules, air bronchogram, cavitation, halo, and pleural effusion. When there was a discrepancy in CT diagnosis between the 2 radiologists, they discussed the case and made a final diagnosis.

CT findings were described on the basis of the recommendations of the Nomenclature Committee of the Fleischner Society [14]. Ground-glass attenuation

was defined as an area of hazy increased parenchymal attenuation without obscuration of the underlying vascular markings. Consolidation was defined as opacity obscuring the underlying vessels. Centrilobular nodules were defined when small nodular lesions located in the center of secondary pulmonary lobules were identified. Ill-defined nodules were defined when small nodular lesions were present, regardless of the secondary pulmonary lobules. Cavitation included a circumscribed enlarged air space with a wall of variable thickness. A halo sign consisted of a pulmonary mass or nodule surrounded by a zone or a halo of attenuation less than that at the center of the mass but greater than that of air in the surrounding uninvolved lung.

Definition

CT findings showing centrilobular nodules and either a halo sign or cavitation were classified into bronchopneumonia type and angioinvasive type, respectively. When both bronchopneumonia-type and angioinvasive-type findings presented in CT scans, they were classified into combination type.

A halo sign pathologically corresponds to a central fungal nodule surrounded by a rim of coagulative necrosis [15]. Cavitation is due to sloughed necrotic lung surrounded by a rim of air, indicating an immune response to the infection [15]. Centrilobular nodules are common signs of bronchiolar diseases, especially bronchopneumonia. They predominate in the centers of secondary pulmonary lobules, often in relation to centrilobular bronchioles or arteries. Pathologic examination of the centrilobular nodules shows an airway filled with exudate, which is also present in the surrounding alveoli.

Statistical Analysis

The primary objective of this study was to describe chest CT findings of late IA. The secondary objective was to investigate whether chest CT scans may be useful for making an early diagnosis of late IA.

Overall survival was determined by using the Kaplan-Meier method. Final follow-up was conducted in July 2004. The median follow-up of surviving patients was 16 months (range, 13.8-32.4 months). Surviving patients were censored on the last day of follow-up. The characteristics of patient groups (angioinvasive type versus bronchopneumonia type) were compared by using the Fisher exact test or Mann-Whitney U test. Differences between survival rates were calculated by using Wilcoxon log-rank analysis. P values $<.05$ were considered significant.

Table 1. *Backgrounds of Patients with Late Invasive Aspergillosis (n = 27)*

Variable	Data
Age, y, median (range)	50 (22–70)
Sex (male/female)	20/7
Underlying disease	
Acute myeloid leukemia	5
Acute lymphoblastic leukemia	9
Chronic myelogeneous leukemia	4
Myelodysplastic syndrome	3
Malignant lymphoma	5
Germ cell tumor	1
History of invasive aspergillosis before transplantation (yes/no)	2/25
Risk for transplantation* (low/high)	7/20
Stem cells (peripheral blood/bone marrow)	16/11
HLA (matched/mismatched)	25/2
Donor (related/unrelated)	19/8
Preparative regimen (reduced-intensity†/ myeloablative‡)	
GVHD prophylaxis	11/16
Cyclosporine alone	9
Cyclosporine and methotrexate	19
Tacrolimus and methotrexate	5
Cyclosporine and prednisolone	1

GVHD indicates graft-versus-host disease.

*We divided the risk of transplantation into 2 groups. The low-risk group was as follows: acute myeloid or lymphoid leukemia in first and second remission, chronic myelogeneous leukemia in chronic phase, and myelodysplastic syndrome/refractory anemia. The other patients were defined as having high-risk disease.

†Reduced-intensity preparative regimens comprised fludarabine/busulfan (n = 7), fludarabine/cyclophosphamide (n = 3), and fludarabine/melphalan (n = 1). Antithymocyte globulin was added in 4 patients.

‡Myeloablative preparative regimens comprised busulfan/cyclophosphamide (n = 8), cyclophosphamide/total body irradiation (n = 2), cytarabine/cyclophosphamide/total body irradiation (n = 4), and others (n = 2).

RESULTS

Clinical Characteristics of IA

Late IA was diagnosed in 27 patients. Patient characteristics are shown in Table 1. Clinical features at IA diagnosis are shown in Table 2. Either acute or chronic GVHD was diagnosed in 24 patients, and 27 patients were given corticosteroids.

Findings of Chest CT

A total of 111 chest CT scans were obtained during the follow-up of 27 patients (median, 3; range, 1-13). Chest CT findings at the establishment of IA diagnosis included halo (n = 12; Figure 1a), centrilobular nodules (n = 12; Figure 1b), ill-defined consolidation (n = 13), ground-glass attenuation (n = 8), pleural effusion (n = 7), pleural-based consolidation (n = 4), cavitation (n = 4), pneumothorax (n = 1), emphysema (n = 1), and air bronchogram (n = 4).

Angioinvasive-type, bronchopneumonia-type, and combination-type IA were diagnosed in 11, 8, and 4

Table 2. *Clinical Characteristics of Patients with Invasive Aspergillosis (n = 27)*

Variable	Data
Diagnosis of IA (proven/probable*)	4/23
Onset, day after transplantation, median (range)	104 (27-973)
Acute GVHD (0-I/II-IV)	3/12
Chronic GVHD (limited/extensive/none)	0/12/0
Use of corticosteroid (yes/no)	27/0
Dose of corticosteroid (<0.5/0.5-1.0/>1.0 mg/kg)	13/9/5
Number of neutrophils (/μL), median (range)	2250 (610-20 400)
Number of lymphocytes (/μL), median (range)	450 (20-10 100)
Performance status† (0-1/2-4)	6/21
Antifungal prophylaxis (fluconazole/none)	2/25
Treatment‡	
Deoxycholate amphotericin B	19
Liposomal amphotericin B	2
Itraconazole	5
None	1
Response to antifungal therapy§ (responded/no change/progression)	11/11/4
Mortality within 30 d of IA diagnosis	7

IA indicates invasive aspergillosis.

*All patients with probable IA were positive for circulating galactomannan antigens at least twice. Aspergillus species were cultured from bronchoalveolar lavage (n = 3) and sputum (n = 1).

†Performance status was defined according to the Eastern Cooperative Oncology Group criteria.

‡One patient had not received any anti-*Aspergillus* treatments because invasive pulmonary aspergillosis diagnosis was established after death.

§Twenty-six patients were given anti-*Aspergillus* treatments.

patients, respectively. CT findings were nonspecific in the other 4 patients. One of the 8 bronchopneumonia-type and 2 of the 11 angioinvasive-type aspergillosis cases were subsequently diagnosed as combination type on the basis of follow-up CT scans.

Comparison between Angioinvasive-Type and Bronchopneumonia-Type IA

Clinical characteristics were compared between angioinvasive-type (n = 11) and bronchopneumonia-type (n = 8) aspergillosis (Table 3). Except for survival after IA diagnosis, there were no significant differences in the patients' characteristics between the 2 types of IA.

Survival of IA

Twenty patients finally died, and IA was a primary cause of death in 9 patients. One-year overall survival after IA diagnosis was 28.1% (95% confidence interval, 10.7%-45.5%).

DISCUSSION

This study revealed that CT manifestations vary in late IA. A halo sign, which is specific for IA in neutropenic patients [9], was observed in 12 of 27 patients with late IA. These findings demonstrate that a CT halo is common in late IA as well as in early IA. Because posttransplantation pulmonary complications other than IA rarely manifest a halo, it will be a useful sign in making a diagnosis of late IA.

This study showed that the airways are common targets of late IA. Two types of IA involving the airways have been reported: bronchopneumonia and tracheobronchitis [16]. Although tracheobronchitis, also known as ulcerative and pseudomembranous tracheobronchitis, was not documented in this study, bronchopneumonia was common. These findings contrast with previous reports on early IA, in which aspergillus bronchopneumonia occurs in approximately 10% of cases of IA [16]. The present study, as well as previous reports [17-20], demonstrated that aspergillus bronchopneumonia results in peribronchial areas of consolidation and that its radiologic manifestations are nonspecific and indistinguishable from those caused by other microorganisms [21,22]. This study furthermore showed that nonneutropenic allo-SCT recipients occasionally develop airway aspergillosis. These findings were comparable to those from the study of Logan et al. [18], in which 5 of the 9 patients with airway aspergillosis were not neutropenic but were receiving immunosuppressive agents. We should recognize that late IA can manifest as bronchopneumonia. When patients develop antibiotic-resistant bronchopneumonia, we should be alert to the possibility of IA.

This study suggests a few possible pathologic explanations for late IA. First, a halo sign reflects an early pathologic change and may be seen during both early IA and an early phase of late IA. As IA progresses, angioinvasive type might change into bronchopneumonia type. Because we regularly obtain CT scans to make an early diagnosis of IA, we could have seen early changes of IA. Early detection of a halo sign leads to an early diagnosis and a prompt initiation of aggressive antifungal treatments and may

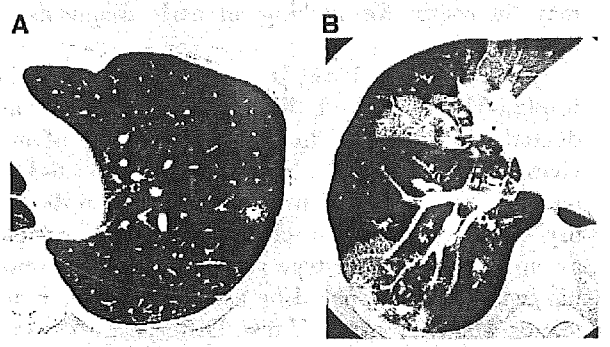

Figure 1. CT findings of late IA. a, CT image of angioinvasive-type late IA: a halo sign. b, CT image of bronchopneumonia-type late IA showing multiple centrilobular nodule opacities.

Table 3. *Comparison between Angioinvasive-Type and Bronchopneumonia-Type Invasive Aspergillosis*

Variable	Angioinvasive Type (n = 11)	Bronchopneumonia Type (n = 8)	P Value
Age before transplantation (y)	52 (22-70)	47.5 (23-60)	.39
Type of preparative regimen (myeloablative/reduced intensity)	5/6	7/1	.15
Posttransplantation variables			
Onset (day after transplantation)	82 (27-180)	84 (27-973)	.65
Neutrophils (/μL)	3400 (1200-8300)	2000 (900-20 400)	.38
Lymphocytes (/μL)	700 (200-3200)	440 (200-10 100)	.43
Cumulative dose of corticosteroid within 7 d of IA diagnosis (mg/kg)	4.4 (2.0-9.8)	2.9 (1.0-52.5)	.36
Acute GVHD (II-IV) and/or chronic GVHD (present/absent)	10/1	6/2	.55
Response to antifungal agents (responded/not responded)	5/6	4/4	.99
6-mo survival	73%	25%	.036*

Data are median (range) unless otherwise noted.
*Statistically significant.

be associated with a better prognosis of angioinvasive-type IA than that of bronchopneumonia-type IA. Second, it should be noted that angioinvasive-type and bronchopneumonia-type IA rarely coexisted in a single patient. One of 8 patients with bronchopneumonia-type and 2 of 12 patients with angioinvasive-type aspergillosis subsequently developed combination-type aspergillosis. These observations suggest that the 2 types of IA may have distinct etiologies. Whether the difference can be attributed to fungal or patient factors is unclear. The patient characteristics were not significantly different between the patients with angioinvasive-type IA and those with bronchopneumonia-type IA, whereas survivals were significantly different between bronchopneumonia-type and angioinvasive-type IA.

Immunity against *Aspergillus* species is mediated by 2 types of host responses: innate and adaptive [23]. The innate immunity includes pulmonary alveolar macrophages that ingest and kill the inhaled conidia and neutrophils that mediate hyphal damages. Recent studies have demonstrated that T-cell responses may have an important role in the host defense against IA in nonneutropenic patients [24]. GVHD and corticosteroids impair phagocytic functions of macrophages and T-cell responses [25]. The 2 types of late IA might reflect the differences in host immune responses to *Aspergillus* species in allo-SCT recipients. Third, the airway is the target of GVHD, and the airway mucosal damage by GVHD might also contribute to the susceptibility to bronchopneumonia. Further studies with animal models and clinical experiences are necessary to investigate the pathogenesis of late IA.

We believe that this study provides important information on late IA; however, it has limitations. First, this was a retrospective study that involved a small number of patients. It might have been influenced by unrecognized bias, and further large-scale studies are warranted. Second, only 3 patients had histopathologic evidence of IA, and we made an IA diagnosis according to the EORTC/MSG criteria [10]. We agree that considerable uncertainty and controversy exist regarding the best method for establishing an IA diagnosis; however, the EORTC/MSG criteria are the present consensus among researchers on invasive fungal infection in immunocompromised patients. Finally, some CT signs might have been attributable to or influenced by other etiologies, such as superimposed infection and allogeneic immune reaction, but postmortem examination in the 5 patients did not support this possibility.

In conclusion, this study demonstrates that halo and multiple centrilobular nodules are common CT signs in late IA. Halo is a useful diagnostic marker in late IA as well as early IA, and late IA frequently manifests as bronchopneumonia.

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Expansion of α-Galactosylceramide-Stimulated Vα24+ NKT Cells Cultured in the Absence of Animal Materials

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Summary: Vα24+ NKT is an innate lymphocyte with potential antitumor activity. Clinical applications of Vα24+ natural killer (NK) T cells, which are innate lymphocytes with potential antitumor activity, require their in vitro expansion. To avoid the potential dangers posed to patients by fetal bovine serum (FBS), the authors evaluated non-FBS culture conditions for the selective and efficient expansion of human Vα24+ NKT cells. Mononuclear cells (MNCs) and plasma from the peripheral blood of normal healthy donors were used before and after G-CSF mobilization. MNCs and plasma separated from apheresis products were also used. MNCs were cultured for 12 days in AIM-V medium containing α-galactosylceramide (α-GalCer) (100 ng/mL) and IL-2 (100 U/mL) supplemented with FBS, autologous plasma, or autologous serum. The cultured cells were collected and their surface markers, intracellular cytokines, and cytotoxicity were evaluated. The highest expansion ratio for Vα24+ NKT cells was obtained from G-CSF-mobilized MNCs cultured in medium containing 5% autologous plasma. Cultures containing MNCs and autologous plasma obtained before and after G-CSF mobilization had approximately 350-fold and 2,000-fold expansion ratios, respectively. These results suggest that G-CSF mobilization conferred a proliferative advantage to Vα24+ NKT cells by modifying the biology of cells and plasma factors. Expanded Vα24+ NKT cells retained their surface antigen expression and production of IFN-γ and exhibited CD1d-independent cytotoxicity against tumor cells. Vα24+ NKT cells can be efficiently expanded from G-CSF-mobilized peripheral blood MNCs in non-FBS culture conditions with α-GalCer and IL-2.

Key Words: NKT cells, G-CSF, α-galactosylceramide

(*J Immunother* 2005;28:314–321)

Received for publication August 13, 2004; accepted March 10, 2005.
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Supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare in Japan.
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Murine Vα14+ natural killer (NK) T cells express an extremely restricted T-cell receptor (TCR) consisting of a Vα14-Jα281 α chain paired mainly with a Vβ8.2 β chain. Human Vα24+ NKT cells are similar to murine Vα14+ NKT cells, as Vα24- NKT cells have an invariant Vα24-JαQ α chain preferentially paired with a Vβ11 chain.[1–3] α-Galactosylceramide (α-GalCer) is a specific ligand for human Vα24+ NKT cells and murine Vα14+ NKT cells.[4] Both types of NKT cells are activated by α-GalCer presented by CD1d. After stimulation with α-GalCer, Vα24- NKT cells exhibit CD1d-dependent cytotoxicity against various types of tumor cells.[5–7] Because CD1d is probably a class I molecule expressed mainly on antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells, it is speculated that NKT cells primarily interact with APCs.[6,8,9] NKT cells regulate innate tumor immunity by rapidly producing a large amount of IFN-γ and IL-4.[4,10]

The extremely low frequency of Vα24+ NKT cells in human peripheral blood[7,11,12] is an obstacle for their clinical application. To overcome this problem, the establishment of an effective in vitro expansion system for Vα24+ NKT cells by stimulation with α-GalCer has been explored. Significant expansion was reported in human Vα24+ Vβ11+ NKT cells cultured with a combination of IL-15, IL-7, IL-2, and α-GalCer.[13] Up to a 76-fold expansion of human Vα24+ Vβ11+ NKT cells was reported after culture with IL-7, IL-15, and α-GalCer-loaded monocyte-derived dendritic cells.[14] Alternative expansion methods use a combination of IL-2 and IL-15,[15] or α-GalCer and IL-2, with or without APCs.[16] Previously, we observed that Vα24+ NKT cells could be expanded 350-fold from human granulocyte-colony stimulating factor (G-CSF)–mobilized peripheral blood cells, upon stimulation with α-GalCer and IL-2 for 12 days.[17] However, in these culture systems, 10% fetal bovine serum (FBS) was used in the medium. To remove the potential risks related to FBS, we developed an efficient non-FBS expansion system for Vα24+ NKT cells.

MATERIALS AND METHODS

Cells, Plasma, and Serum Preparation

Peripheral blood and apheresis products were obtained from normal healthy individuals who were donating peripheral blood stem cells for allogeneic transplants. Written informed consent was obtained from the donors. This study was approved by our institution. Before and after G-CSF mobilization, samples were used immediately and cell fraction and

plasma were separated by centrifugation. The plasma and serum were obtained and cryopreserved at −80°C until use. Plasma and serum samples were heat-inactivated immediately before use. Mononuclear cells (MNCs) were isolated from peripheral blood and apheresis products by Ficoll-Hypaque (Immuno-Biologic Laboratories, Gunma, Japan) density gradient centrifugation. Apheresis plasma was also collected from the apheresis bags and used after heat inactivation.

G-CSF Procedure for Apheresis Donor

The apheresis was indicated for a healthy donor whose relative needed peripheral blood stem cell transplantation. This indication was checked by the clinical team of stem cell transplantation unit in our hospital. G-CSF was administered subcutaneously at a dosage of 300 μg/m^2 divided twice daily for 3 days just before the apheresis procedure. On the day of apheresis, one more dose of G-CSF was administered in the morning before apheresis.

Expansion of Vα24$^+$ NKT Cells

MNCs were cultured in six-well culture plates or culture flasks (Costar, Corning, NY) at 1.0×10^5 cells/mL in medium supplemented with 100 ng/mL α-GalCer (Kirin Brewery Co, Tokyo, Japan) and 100 U/mL recombinant human (rh) IL-2 (R&D Systems, Minneapolis, MN) for 12 to 14 days. The environment for the incubation was under 20% O_2 and 5% CO_2. Cells were cultured in AIM-V (Life Technologies, Rockville, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 10% recombinant human serum albumin (rHSA), 5% or 10% autologous plasma, or 5% or 10% autologous serum. The rHSA was kindly provided by Mitsubishi Welpharma Corporation (Osaka, Japan). Fresh IL-2 was added every 3 days.

Co-Culture and Expansion of Vα24$^+$ NKT Cells

To determine whether G-CSF mobilization conferred any benefits to plasma or cells for the expansion of Vα24$^+$ NKT cells, we tested the following culture conditions: (1) pre-G-CSF peripheral blood mononuclear cells (PBMCs) cultured in AIM-V with 5% pre-G-CSF plasma; (2) pre-G-CSF PBMCs cultured in AIM-V with 5% post-G-CSF plasma; (3) post-G-CSF PBMCs cultured in AIM-V with 5% pre-G-CSF plasma; and (4) post-G-CSF PBMCs cultured in AIM-V with 5% post-G-CSF plasma. After culturing cells with α-GalCer and IL-2 for 12 days, we quantified the expansion of Vα24$^+$ NKT cells.

Cell Surface Antigen Analysis

We used mouse anti-human mAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or peridinium chlorophyll (PerCP). CD3-PE, CD4-PerCP, CD8-PE, CD14-FITC, CD19-PE, CD25 (IL-2 receptor α chain)-FITC, and CD123 (IL-3 receptor)-PE mAbs were purchased from BD Biosciences (Mountain View, CA). Vα24-FITC, Vα24-PE, Vβ11-PE, CD124 (IL-4 receptor α chain)-PE, and CD127 (IL-7 receptor)-PE mAbs were purchased from Immunotech (Marseille, France). CD161-APC, CD114 (G-CSF receptor)-PE, and CD119 (IFN-γ recep-tor α chain)-PE mAbs were purchased from BD Pharmingen (San Diego, CA). PE-conjugated α-GalCer-CD1d tetramer was produced in our laboratory[18] and used to stain α-GalCer-loaded CD1d-reactive Vα24$^+$ NKT cells. Biotinylated anti-CD1d-mAb, which was originally produced by Dr. Steven A. Porcelli (Albert Einstein College of Medicine, Bronx, NY), was a kind gift from Kirin Brewery Co. The biotinylated mAb was detected using streptavidin-PerCP (BD Biosciences). For cell surface antigen staining, cells were incubated with mAbs for 30 minutes on ice. After staining, cells were washed twice and resuspended in PBS. Propidium iodide (Sigma-Aldrich, St. Louis, MO) staining preceded all experiments to remove dead cells. Data were acquired by flow cytometry (FACSCalibur; BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Vα24$^+$ NKT Cell Separation

After expansion of Vα24$^+$ NKT cells with α-GalCer and IL-2 in AIM-V with 5% autologous apheresis plasma for 12 days, cells were stained with Vα24-FITC for 20 minutes on ice and washed twice with 5 mM EDTA-PBS. After being incubated with anti-FITC microbeads (Miltenyi Biotec, Gladbach, Germany), Vα24$^+$ NKT cells were sorted by a magnetic cell separation system (Super MACS; Miltenyi Biotec), according to the manufacturer's protocol. After separation, the purity of isolated Vα24$^+$ NKT cells was determined to be more than 95% by flow cytometry. After the cells were re-cultured with 100 U/mL IL-2 for an additional 2 days, they were used for assays of cytotoxic activity against several tumor cell lines.

Intracellular Cytokine Assay

The intracellular cytokine production of cultured cells was measured by flow cytometry. Cells were activated with 10 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) for 4 hours at 37°C in 10 μg/mL Brefeldin A (Sigma-Aldrich) to prevent cytokine secretion. After activation, cells were stained with Vα24 antigens and permeabilized according to the manufacturer's protocol (BD Biosciences) for staining with IFN-γ-PE or IL-4-PE mAb (BD Biosciences). At least 30,000 gating events per sample were acquired by FACSCalibur, and the data were analyzed using CellQuest software.

Cytotoxicity Assay

The cytotoxicity of isolated Vα24$^+$ NKT cells against tumor cell lines was studied. The following cell lines were purchased from ATCC: Daudi (B-cell lymphoma), K562 (chronic myelogenous leukemia), and Jurkat (T-cell lymphoma).

Target cells were labeled with 50 μCi sodium [^{51}Cr] chromate (NEN Life Science Products, Inc, Boston, MA) per 5×10^5 cells for 1 hour, washed three times, and resuspended at 1×10^5 cells/mL in medium. Next, 100 mL of effector cells and 100 mL of ^{51}Cr-labeled target cells (1×10^4 cells/well) were added to 96-well round-bottomed plates (Nunc, Roskilde, Denmark) at effector-to-target (E/T) ratios of 10:1, 3:1, and 1:1. Plates were incubated for 4 hours at 37°C, and ^{51}Cr release from lysed target cells was measured by a gamma counter. The percentage of specific ^{51}Cr released in each well was analyzed using the following formula: specific lysis (%) = (test cpm − spontaneous cpm)/(total cpm − spontaneous cpm) × 100. "Test cpm" indicates the counts in experimental cultures of target cells and effector cells; "spontaneous cpm" indicates the counts in cultures containing only target cells and medium;

and "total cpm" indicates the counts obtained by adding 100 mL of 1 N HCl to target cells to lyse all cells. Data are expressed as the mean and standard deviation of triplicate cultures.

ELISA

Levels of IL-2, IL-3, IL-4, IL-7, IL-13, IL-15, IFN-γ, and G-CSF in pre-G-CSF peripheral blood plasma and apheresis plasma were measured by commercial ELISA kits according to the manufacturers' protocols. IL-12 levels were measured by OptEIA (BD Pharmingen), and the other cytokine levels were measured by Immunoassay ELISA kits (BioSource, Camarillo, CA).

Statistical Analysis

The Student *t* test was used to compare groups using the two-tailed method dealing with dependent samples. $P < 0.05$ was considered statistically significant. In multiple group analysis, we adapted Bonferroni adjustment to confirm the significance of *P* values.

RESULTS

Efficient Expansion of Vα24$^+$ NKT Cells in Autologous Plasma

In this study, Vα24$^+$ CD3$^+$ cells were defined as Vα24$^+$ NKT cells. In our preliminary experiments using anti-Vβ11 mAb, we found that expanded Vα24$^+$ NKT cells fully express Vβ11. To search for a suitable non-FBS medium for Vα24$^+$ NKT cell expansion, PBMCs were cultured in medium containing α-GalCer, IL-2, and 10% FBS, 10% rHSA, 5% autologous plasma or serum, or 10% autologous plasma or serum for 12 to 14 days. The percentage of cultured Vα24$^+$ NKT cells increased by 27-fold in 10% FBS, 2-fold in 10% rHSA, 342-fold in 10% autologous plasma, 382-fold in 5% autologous plasma, 315-fold in 10% autologous serum, and 355-fold in 5% autologous serum (n = 5). Representative flow cytometry data are shown in Figure 1. When cells were cultured in medium containing 10% FBS, the percentage of expanded Vα24$^+$ NKT cells was substantially lower than when autologous plasma or autologous serum was used to supplement medium. In medium containing rHSA, the Vα24$^+$ NKT cells were unable to proliferate, whereas CD3$^+$ T cells proliferated. There was no significant difference between Vα24$^+$ cell expansion in 5% or 10% autologous plasma or autologous serum. Additionally, 87% to 95% of Vα24$^+$ NKT cells reacted to the α-GalCer-CD1d tetramer after expansion in 5% autologous plasma. These results suggest that medium containing 5% autologous plasma is suitable for selective expansion of Vα24$^+$ NKT cells with α-GalCer and IL-2 in vitro.

G-CSF Mobilization Augmented Vα24$^+$ NKT Cell Expansion

To develop more efficient plasma-based culture conditions for Vα24$^+$ NKT cells, we collected PBMCs and plasma before and after G-CSF mobilization (n = 18) and compared their expansion efficiencies (Table 1). Vα24$^+$ NKT cells significantly expanded to 1,938 (±2,501)-fold in the post-G-CSF condition compared with 346 (±345)-fold in the pre-G-CSF condition ($P = 0.018$). Thus, the Vα24$^+$ NKT cell expansion was 5.6-times greater in the post-G-CSF condition than in the pre-G-CSF condition. As the total cell number including all cell populations was not significantly different between the two cultures, the addition of α-GalCer in the post-G-CSF condition appeared to selectively expand Vα24$^+$ NKT cells.

Characteristics of G-CSF-Mobilized PBMCs and Plasma

To elucidate the contributions of G-CSF-mobilized PBMCs and plasma to Vα24$^+$ NKT cell expansion, different combinations of PBMCs and plasma from pre- and post-GCSF peripheral blood were tested (n = 8) (Fig. 2). Post-G-CSF plasma enhanced Vα24$^+$ NKT cell expansion more than pre-G-CSF plasma. Likewise, more Vα24$^+$ NKT cell proliferation occurred in post-G-CSF PBMCs than in pre-G-CSF PBMCs. The most effective combination was post-G-CSF PBMCs and post-G-CSF plasma. Exogenous G-CSF did not enhance the effective expansion of NKT cells (data not shown). These results suggest that G-CSF mobilization indirectly contributed to both PBMCs and plasma for the expansion of Vα24$^+$ NKT cells.

G-CSF Did Not Increase the Percentage of Vα24$^+$ NKT Cells in Peripheral Blood

We compared the percentages of Vα24$^+$ NKT cells in peripheral blood before and after G-CSF mobilization (n = 10). The percentage of Vα24$^+$ NKT cells in peripheral blood was 0.128% (±0.034) and was reduced to 0.082% (±0.040) by G-CSF mobilization ($P < 0.001$), although the absolute number of Vα24$^+$ NKT cells was similar in pre- and post-G-CSF peripheral blood—4.32 (±2.97) counts/μL and 6.03 (±3.41) counts/μL ($P > 0.05$), respectively. This means that mobilized PBMCs contain a high proportion of monocyte, which resulted in decreasing the percentage of Vα24$^+$ NKT cells relatively. As the total number of Vα24$^+$ NKT cells in peripheral blood did not change, therefore the Vα24$^+$ NKT cells were not mobilized by G-CSF administration.

G-CSF-Induced Changes in Peripheral Blood Cytokine Concentrations

We measured cytokine concentrations in the plasma of pre-G-CSF and apheresis products (n = 6) (Fig. 3). The level of G-CSF increased dramatically after G-CSF administration. There were significant differences between the levels of three cytokines (IL-3, IL-7 and IL-13) between apheresis products and pre-G-CSF plasma. The levels of other cytokines, such as IL-2, IL-12, IL-15, and IFN-γ, which enhance Vα24$^+$ NKT cell function, were not changed by G-CSF mobilization. The concentrations of IL-4 were below the detection limit.

Expression of Cytokine Receptors on T Cells and NKT Cells After G-CSF Mobilization

We evaluated the expression of cytokine receptors for IL-2, IL-3, IL-4, IL-7, G-CSF, and IFN-γ on CD3$^+$ T cells and Vα24$^+$ NKT cells in pre- and post-G-CSF PBMCs (n = 5) (Fig. 4). The expression levels of IL-3, IL-7, and IL-4 receptor (which has IL-13 common receptor[19]) on CD3$^+$ T cells and Vα24$^+$ NKT cells were not affected by G-CSF mobilization, although the corresponding cytokine levels (IL-3, IL-7, and

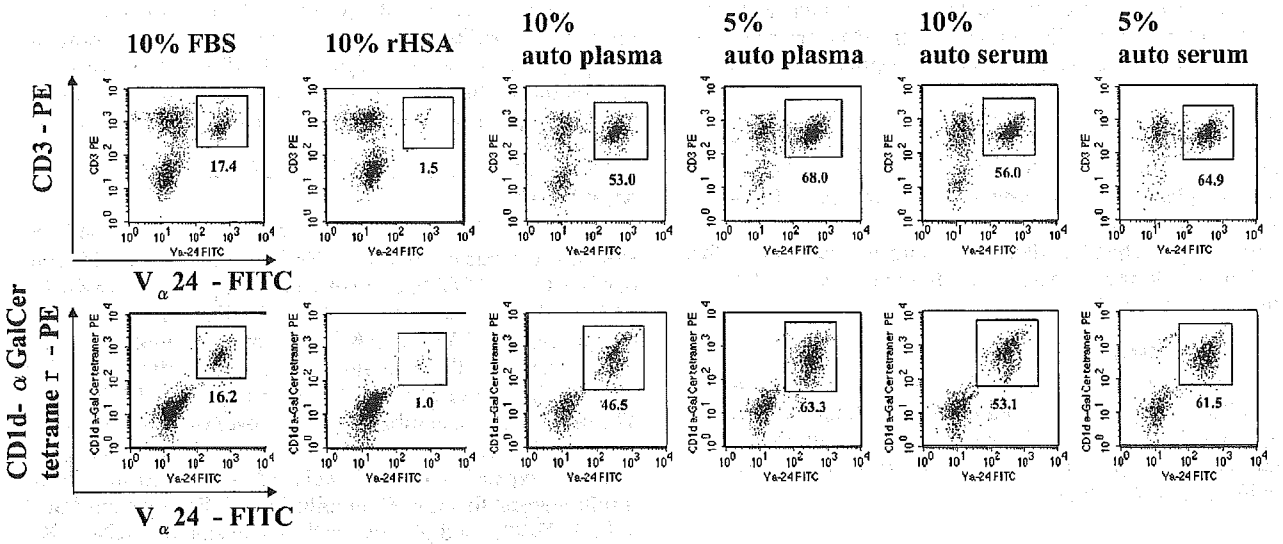


FIGURE 1. Differences in Vα24+ NKT cell expansion according to the type of supplemented protein. PBMCs from normal healthy donors were cultured for 12 to 14 days with α-GalCer and IL-2 in the presence of 10% FBS, 10% rHSA, 5% or 10% autologous plasma, or 5% or 10% autologous serum. Vα24+ CD3+ cells were defined as Vα24+ NKT cells. Vα24+ CD1d-α-GalCer tetramer-positive NKT cells were also stained, and the percentage of the gated population is shown. These flow cytometry results are representative of five independent experiments.

IL-13) were increased by G-CSF mobilization. The IL-7 receptor was expressed on most Vα24+ NKT cells, although some CD3+ T cells showed downregulation of the IL-7 receptor after G-CSF mobilization. There was no obvious tendency for G-CSF mobilization to enhance the expression level of the G-CSF receptor or the α chain of the IL-2 receptor on both CD3+ T cells and Vα24+ NKT cells. Interestingly, only the α chain of the IFN-γ receptor increased after G-CSF mobilization with a significant difference ($P = 0.009$), and this increase occurred on Vα24+ NKT cells but not on CD3+ T cells.

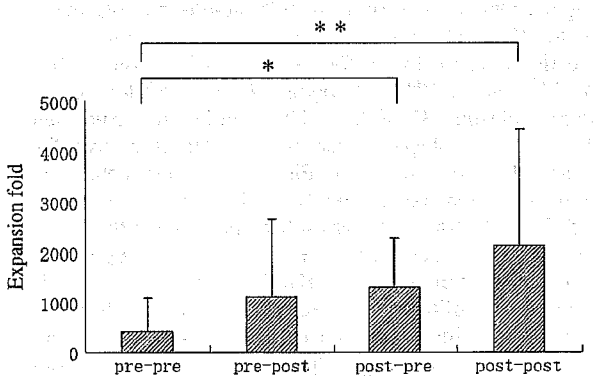
Cell Populations

Table 2 shows mean values and standard deviations for the cell kinetics of apheresis MNCs cultured with autologous apheresis plasma (n = 11). The apheresis procedure did not affect the percentage of Vα24+ NKT cells. On day 0, Vα24+ NKT cells represented only 0.10% (± 0.06) of apheresis MNCs, and the CD4+ to CD8+ T-cell ratio was more than 1.0. Monocytes accounted for approximately 30% of MNCs at day 0, which was substantially higher than the percentage of monocytes (2.7–7.9%) in pre-G-CSF PBMCs. When stimulated with α-GalCer, Vα24+ NKT cells propagated linearly until day 14. CD8+ T cells expanded to become the predominant T-cell population, changing the CD4+ to CD8+ T-cell ratio to less than 1.0. B cells and monocytes almost completely disappeared by day 14 (2.33% and 0.16%, respectively).

TABLE 1. Comparison of Expansion Efficiencies

	Vα24+ NKT Cells			Whole Cells
	Day 0 (%)	Day 12–14 (%)	Expansion Fold	Day 12–14 (expansion fold)
Pre-G-CSF	0.19	10.45 ± 8.53	×345.96 ± 345	×5.33
Post-G-CSF	0.11	21.97 ± 11.70*	×1,938.11 ± 2,501*	×4.62

*$P < 0.05$.



* P<0.05 both in t-test and Bonferoni adjustment,
** P<0.05 in t-test but not Bonferoni adjustment

FIGURE 2. Differences in Vα24+ NKT cell expansion influenced by a combination of PBMCs and plasma. The expansion of Vα24+ NKT cells was analyzed in several co-culture combinations of PBMCs and 5% plasma before and after G-CSF mobilization. Cells were cultured for 14 days in the presence of α-GalCer and IL-2. Values are the mean and standard deviation of the Vα24+ NKT cell expansion fold. Samples were obtained from the same donor (n = 8), and the following co-culture conditions were examined: (1) pre-G-CSF PBMCs and pre-G-CSF plasma (pre-pre); (2) pre-G-CSF PBMCs and post-G-CSF plasma (pre-post); (3) post-G-CSF PBMCs and pre-G-CSF plasma (post-pre); and (4) post-G-CSF PBMCs and post-G-CSF plasma (post-post). *$P < 0.05$. P values were determined using the Student t test and Bonferroni adjustment.

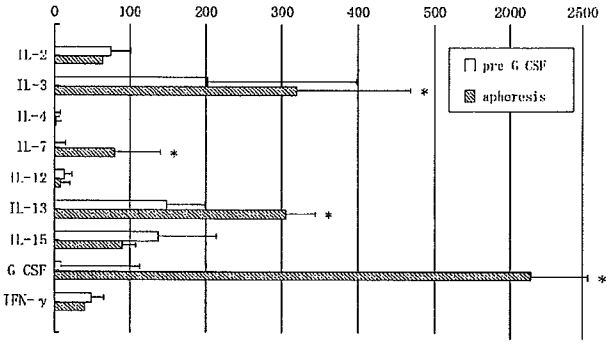


FIGURE 3. Cytokine levels in plasma. Cytokine levels in peripheral blood were measured by ELISA before G-CSF mobilization and in apheresis products from the same normal healthy donors (n = 6). IL-2 levels are plotted in U/mL; all other cytokine levels are plotted in pg/mL. Results are shown as mean values with standard deviations. *$P < 0.05$ vs. pre-G-CSF peripheral blood and apheresis product.

NK cells were also remarkably reduced after day 7, although they grew rapidly in the first 7 days of culture.

Cytokine Production

We measured IFN-γ and IL-4 production in apheresis MNCs (n = 10) that were cultured with or without α-GalCer for 14 days. Representative flow cytometry data are shown in Figure 5. The percentage of IFN-γ-producing MNCs was 58.7 ± 13.9% when cultured with α-GalCer and 44.8 ± 15.6% when cultured without α-GalCer. The percentage of IL-4-producing MNCs was 8.6 ± 8.5% when cultured with α-GalCer and 5.0 ± 2.9% when cultured without α-GalCer. When cultured with α-GalCer, 75.7 ± 12.2% of Vα24$^+$ NKT cells produced IFN-γ and 16.2 ± 10.5% produced IL-4. In the comparison of IFN-γ and IL-4 produced by Vα24$^+$ NKT cells, IFN-γ was significantly dominant ($P = 0.023$).

Cytotoxicity Assays

Three tumor cell lines were used as target cells in the cytotoxic assay. CD1d expression on the target tumor cells was evaluated using CD1d mAb. CD1d was expressed on 87% of Jurkat cells and 13% of Daudi cells. K-562 did not express CD1d. Vα24$^+$ NKT cells purified from MNCs stimulated with α-GalCer mediated strong cytotoxic effects against all of these hematologic cell lines (Fig. 6). The cytotoxicities were unrelated to CD1d expression on the target cells.

DISCUSSION

NKT cells help regulate a variety of immune responses, including the immune responses associated with autoimmune diseases,[20] including inflammatory bowel disease,[21] graft-versus-host disease,[22] and tumor rejection.[23] Two main strategies have been devised to use the specific ligand for NKT cells, α-GalCer, in therapeutic settings: the in vivo use of α-GalCer to enhance an immune response and the ex vivo use of α-GalCer to expand NKT cells for adoptive transfer. When the former approach was tested in patients with various solid tumors,[24] there were short-term elevations in IL-12 and GM-CSF levels and NK cell activity, and a slight elevation in serum IFN-γ and IL-4 levels occurred in some patients. Interestingly, the NKT cells disappeared from peripheral blood within 24 hours of α-GalCer injection. Although no adverse events were associated with this approach, no therapeutic benefits were apparent either. In murine models, high doses of α-GalCer showed significant liver toxicity.[25]

Nieda et al[16] studied the alternative approach of the infusion of α-GalCer-pulsed dendritic cells. They reported a transient decrease in the number of Vα24$^+$ Vβ11$^+$ NKT cells in the peripheral blood within 48 hours of the infusion. This transient decrease was followed by significant increases in Vα24$^+$ Vβ11$^+$ NKT cells and the serum levels of IFN-γ and IL-12, in addition to the activation of NK cells and T cells. No significant adverse events were reported in a clinical trial of this approach.[26]

The clinical use of Vα24$^+$ NKT cells requires the development of a highly effective expansion method for Vα24$^+$ NKT cells ex vivo. Previous reports of ex vivo cell expansion for clinical applications have focused on T cells,[27,28] NK cells,[29] or dendritic cells[30] rather than NKT cells. A few reports have found that the expansion of human NKT cells from steady-state peripheral blood cells or cord blood cells can be mediated by α-GalCer and several cytokines.[13–16] However, the expansion ratios of these NKT cells were limited. Our previous study showed that G-CSF-mobilized peripheral blood cells, whether from normal donors or cancer patients, had a significantly higher expansion potential for Vα24$^+$ NKT cells in a combination culture of α-GalCer and IL-2.[17] These results provide a realistic rationale for performing adoptive transfer of α-GalCer-expanded Vα24$^+$ NKT cells in combination with high-dose chemotherapy and G-CSF treatment or in combination with autologous or allogeneic hematopoietic stem cell transplantation including G-CSF mobilization. Nevertheless, these approaches are seriously limited by the use of FBS, and the development of a non-FBS culture system is critical.

In the present study, we tested a culture system that uses autologous plasma for the expansion of Vα24$^+$ NKT cells in the presence of α-GalCer and IL-2. We also evaluated the sustained usefulness of G-CSF-mobilized specimens. We found that autologous serum and autologous plasma had greater capacities to expand Vα24$^+$ NKT cells than did FBS and rHSA. Indeed, there was no significant difference between Vα24$^+$ NKT cell expansion in 5% or 10% autologous plasma or autologous serum. However, the percentage of Vα24$^+$ cells in culture medium was the highest and 87% to 95% of Vα24$^+$ NKT cells reacted to the α-GalCer-CD1d tetramer after expansion in 5% autologous plasma. Additionally, plasma can easily be obtained in the process of PBMC preparation from peripheral blood samples and in the process of apheresis. Thus, we selected plasma as a medium supplement. We also found that G-CSF-mobilized PBMCs and G-CSF-mobilized plasma, which were used instead of steady-state PBMCs and plasma, yielded the highest expansion ratio for Vα24$^+$ NKT cells. When we comparatively analyzed cells and plasma before and after G-CSF mobilization, we found that both G-CSF-mobilized PBMCs and G-CSF-mobilized plasma had the capability to support expansion of Vα24$^+$ NKT cells (see

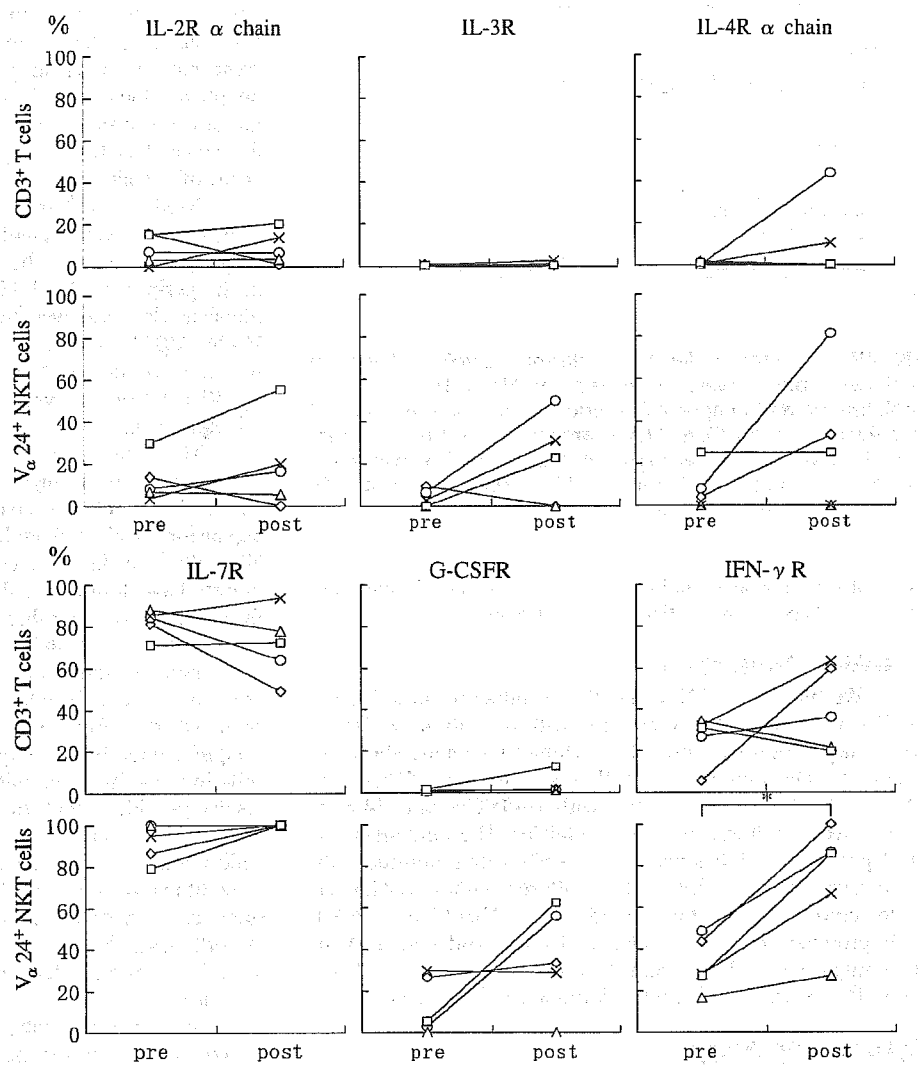


FIGURE 4. Cytokine receptor expression. Changes in the cytokine receptor expression of $CD3^+$ T cells and $V\alpha24^+$ NKT cells in peripheral blood before and after G-CSF mobilization are shown as five independent experiments. Peripheral blood before and after G-CSF-mobilization was obtained from the same healthy donors. Figure symbols indicate individual donors. $^*P < 0.05$.

Fig. 2). In the clinical setting, we plan to use mobilized PBMCs and apheresis product derived from cancer patients in the autologous setting or derived from a healthy donor in the allogeneic setting. The clinical application of ex vivo expanded NKT cells has a possibility of wide modification, including combination therapy with stem cell transplantation.

TABLE 2. Cell Kinetics of Apheresis MNCs Cultural with Autologous Apheresis Plasma

Cell Population	Day 0	Day 7	Day 14
$V\alpha24^+$ $CD3^+$ (NKT)	0.10 ± 0.06	12.90 ± 15.15	21.77 ± 21.68
$CD3^-$ $CD161^+$ (NK)	3.41 ± 2.08	26.03 ± 15.47	8.79 ± 6.85
$CD161^-$ $V\alpha24^-$ $CD4^+$ (CD4 T)	18.57 ± 7.53	18.07 ± 7.02	16.91 ± 12.28
$CD161^-$ $V\alpha24^-$ $CD8^+$ (CD8 T)	12.42 ± 3.42	26.71 ± 12.28	23.69 ± 12.20
$CD19^+$ (B cell)	7.40 ± 4.30	5.62 ± 3.27	2.33 ± 2.06
$CD14^+$ (monocyte)	29.39 ± 15.58	0.93 ± 1.12	0.16 ± 0.16

Data are given as percentages ± SD.

Contrary to our expectations, our flow cytometry data revealed that the percentage of $V\alpha24^+$ NKT cells in vivo decreased after G-CSF mobilization. As the absolute number of $V\alpha24^+$ NKT cells did not change by G-CSF mobilization, the decreased percentage of it was caused by the increment of other cell populations after G-CSF mobilization. That means that G-CSF does not mobilize $V\alpha24^+$ NKT cells directly. Also, ex vivo supplementation of G-CSF did not enhance the expansion of $V\alpha24^+$ NKT cells (data not shown), which suggests an indirect contribution of G-CSF in the expansion of NKT cells, contrary to a previous report.[31] On the other hand, the post-G-CSF PBMCs (see Table 2) and apheresis products contained a high percentage of monocytes, which include APCs capable of presenting α-GalCer. This observation indicates that the number of CD1d-expressing PBMCs also increased after G-CSF mobilization and might be one factor responsible for the significant expansion of $V\alpha24^+$ NKT cells in post-G-CSF

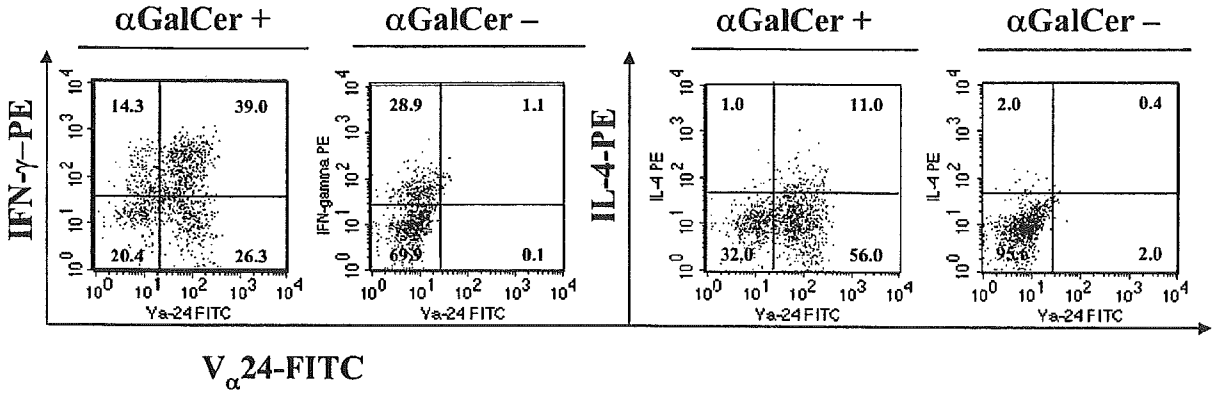


FIGURE 5. Intracellular cytokines in cultured Vα24⁺ NKT cells. Intracellular IFN-γ and IL-4 were stained in whole cells after culture with or without α-GalCer. Cells were activated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours. Representative data from 1 of 10 independent experiments are presented. $*P < 0.05$, difference between the production of IFN-γ and IL-4, Student *t* test.

PBMCs. We previously reported that cell-to-cell contact with CD14⁺ cells was needed for the expansion of NKT cells.[17]

The plasma collected after G-CSF mobilization also had an enhanced capacity for Vα24⁺ NKT cell expansion. IL-2, IL-7, IL-12, IL-15, IL-18, and IFN-γ directly induce proliferation and activation of NKT cells.[13,14,32,33] However, none of these cytokines, with the exception of IL-7, was increased in the plasma of G-CSF-mobilized peripheral blood. When Vα24⁺ NKT cells were cultured with α-GalCer and increased levels of cytokines (IL-3, IL-7, IL-13, and G-CSF) in medium containing pre-G-CSF plasma, the expansion efficiency of Vα24⁺ NKT cells was not enhanced to the level achieved with post-G-CSF plasma (data not shown). These results suggest that IL-3, IL-7, IL-13, and G-CSF do not directly contribute to the proliferation of Vα24⁺ NKT cells. The identification of these unknown factors in post-G-CSF plasma, which promote the proliferation of Vα24⁺ NKT cells, would increase the effectiveness of Vα24⁺ NKT cell expansion.

To determine whether the characteristics of cells were changed by G-CSF mobilization, we evaluated the expression of several cytokine receptors on CD3⁺ T cells and Vα24⁺ NKT cells isolated from peripheral blood before and after G-CSF mobilization. A significant increase was observed in the expression of the IFN-γ receptor α chain on Vα24⁺ NKT cells after G-CSF mobilization ($P = 0.009$). This increased α-chain expression may be partially responsible for the proliferative advantage of Vα24⁺ NKT cells after G-CSF mobilization. However, the variability of response between individuals is essential issue, especially in the evaluation of receptor intensity. This variation suggests that the ex vivo expansion of NKT cells is controlled by mutual change, which exists in cellular and humoral factor.

We found that expanded Vα24⁺ NKT cells predominantly produced IFN-γ. The expanded Vα24⁺ NKT cells exhibited augmented cytotoxicity against CD1d⁺ tumor cell lines (Daudi and Jurkat) as well as CD1d⁻ tumor cell line (K562). In CD1d-blocking experiments, we found that expanded Vα24⁺ NKT cells mediated cytotoxic activity against CD1d-blocked Jurkat cells that was comparable to the cytotoxic activity against CD1d-unblocked Jurkat cells (data not shown). Thus, the expanded Vα24⁺ NKT cells yielded lytic activity against tumor cells in a CD1d-independent manner. Although the mechanism of CD1d-related cytotoxicity mediated by Vα24⁺ NKT cells has not been clarified, other recent studies of NKT cells suggest that CD1d expression on the target tumor cells is not essential for cytotoxicity.[34–36] The Vα24⁺ NKT cells

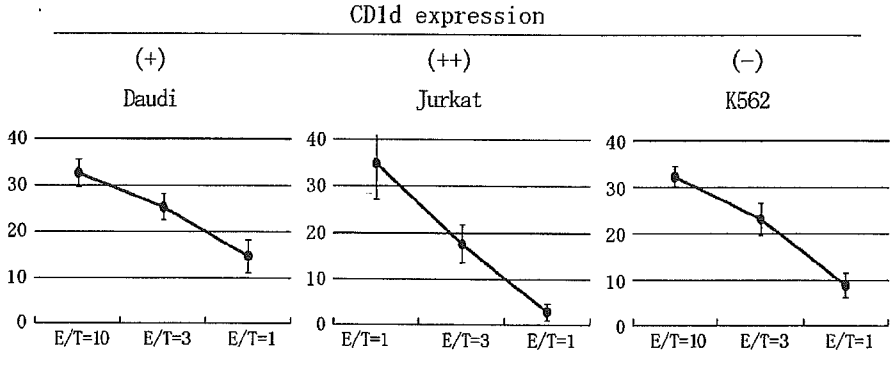


FIGURE 6. Cytotoxicity of purified Vα24⁺ NKT cells after culture. Vα24⁺ NKT cell-mediated cytotoxicity against tumor cells was measured with effector-to-target ratios of 10:1, 3:1, and 1:1. Cell lines were classified into the following four groups based on the expression level of CD1d: (-), 0–3%; (±), 3–10%; (+), 10–60%; (++), 60–100%. Cytotoxicity was evaluated with ^{51}Cr release assays. The means and standard deviations of triplicate culture are shown in representative result of four independent experiments.

obtained in our culture system appear to be T_H1-type NKT cells that have strong antitumor activity through direct and indirect mechanisms.

In the present study, we developed an effective method for $V\alpha24^+$ NKT cell expansion through the use of G-CSF-mobilized peripheral blood. We also featured the possible clinical applications of $V\alpha24^+$ NKT cells in adoptive immunotherapy, both in autologous and allogeneous settings. Further research needed to achieve this goal is underway.

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Immunology Letters
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Cytokine production and migration of in vitro-expanded NK1.1$^-$ invariant Vα14 natural killer T (Vα14i NKT) cells using α-galactosylceramide and IL-2

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Received 31 March 2005; received in revised form 25 May 2005; accepted 26 May 2005
Available online 20 June 2005

Abstract

Mouse natural killer T cells with invariant Vα14 rearrangement (Vα14i NKT cells) can rapidly produce both Th1 and Th2 cytokines and regulate various immune responses, such as autoimmunity and tumor immunity. In this study, we describe the phenotypical and functional characterization of in vitro-expanded mouse Vα14i NKT cells from spleen using a combination of α-galactosylceramide (α-GalCer) and IL-2. The expanded Vα14i NKT cells retained the memory/activated (CD44$^+$CD69$^+$CD62L$^-$) and CD4$^+$ or CD4$^-$8$^-$ double negative phenotypes but modulated or lost the classical NKT cell marker, NK1.1. The expanded Vα14i NKT cells continuously released IL-4 and IFNγ and induced NK cell IFNγ production in vitro. Furthermore, the expanded Vα14i NKT cells migrated into the liver and spleen after adoptive transfer into lymphopenic SCID mice, and they were able to rapidly produce IL-4 and IFNγ after α-GalCer injection. Our findings suggest that the intrinsic characteristics of the cytokine secretion of Vα14i NKT cells were equivalent to that of in vitro-expanded Vα14i NKT cells. In vitro-expanded Vα14i NKT cells are considered to be useful for NKT cell defect-related diseases, such as autoimmunity and cancer.

Keywords: CD1d; α-Galactosylceramide; NKT cell; Cytokine

1. Introduction

Mouse natural killer T cells with an invariant Vα14-Jα18 TCR rearrangement (Vα14i NKT cells) preferentially associate with Vβ8.2, Vβ7 or Vβ2 TCR, recognize glycolipid antigens in the context of the non-classical MHC class I molecule CD1d [1–3], and respond strongly to a synthetic glycolipid, α-galactosylceramide (α-GalCer) [1–3]. The Vα14i NKT cells display a memory or activated phenotype (CD44^{high}CD62L$^-$CD69$^+$) and express NK cell markers, such as NK1.1 [1–3]. A feature of Vα14i NKT cells is the rapid secretion of several immunoregulatory cytokines, such as Th1 cytokines (IFNγ) and Th2 cytokines (IL-4 and IL-10) [1–3]. Cytokine production by Vα14i NKT cells plays an important role in various immune responses, including autoimmunity and tumor-immunity.

Vα14i NKT cells have antitumor activities [4,5]. Vα14i NKT cells exhibit direct cytotoxicity against various tumor cell lines [6] and rapidly produce IFNγ to induce NK cell activation [7,8]. Furthermore, the administration of α-GalCer, a specific ligand for Vα14i NKT cells, prevents tumor metastasis [9,10], and the antimetastatic activity of α-GalCer is mediated by sequential production of IFNγ by Vα14i NKT cells and NK cells [11,12].

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doi:10.1016/j.imlet.2005.05.007

In humans, NKT cells with invariant Vα24 chains paired with Vβ11 chains (Vα24i NKT cells) are the counterpart to mouse Vα14i NKT cells, and they also respond to α-GalCer and rapidly secrete IFNγ and IL-4 [1–3]. Several investigators have reported the robust expansion of human Vα24i NKT cells from peripheral blood mononuclear cells (PBMCs) using α-GalCer plus a combination of cytokines, such as IL-2, IL-7 and IL-15, in vitro [13–18]. α-GalCer-loaded dendritic cells and IL-2 also can induce the expansion of $V\alpha24^+$ NKT cells from PBMCs [15,18]. After culture with α-GalCer and cytokines, the expanded human Vα24i NKT cells retain the ability to produce IFNγ and IL-4 [15,19], and they exhibit cytotoxic activity against tumor cell lines [20,21]. Recent studies have revealed significant reductions in numbers of Vα24i NKT cells and deficiencies in their proliferative responses and IFNγ production of Vα24i NKT cells in some patients with advanced cancer [22,23]. Therefore, adoptive transfer of in vitro-expanded Vα24i NKT cells is expected to induce antitumor activities in cancer patients with reduced circulating Vα24i NKT cell numbers.

It has been reported that mouseVα14i NKT cells also respond to α-GalCer by proliferating in vitro [1–3]. However, little is known regarding the function and phenotype of in vitro-expanded Vα14i NKT cells because hitherto there was no appropriate marker to identify these cells. In this study, we used CD1d/α-GalCer tetramer to monitor Vα14i NKT cells. We demonstrate that splenic Vα14i NKT cells can be expanded up to 8-fold after 4 days of culture with α-GalCer and IL-2. In vitro-expanded Vα14i NKT cells migrate to the liver and spleen of recipient mice and produce IFNγ and IL-4 in vivo after administration of α-GalCer.

2. Material and methods

2.1. Mice

Female C57BL/6N mice, BALB/cAnN mice and C.B-17/Icr SCID mice were purchased from Charles River Japan Inc. (Kanagawa, Japan). All mice were used at 8–12 weeks of age and maintained in our facilities. Animal studies were performed according to guidelines from the animal experimental ethics committee.

2.2. Cell culture

Spleen, liver, thymus and bone marrow mononuclear cells were prepared as previously described [24]. Mononuclear cells (1×10^6 cells/ml) were cultured in 7 ml of RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 8% fetal calf serum (JRH Biosciences, Lenexa, KS), 5×10^{-5} M 2-mercaptoethanol (Sigma–Aldrich, Saint Louis, MO), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in 25 cm^2 culture flask (Greiner Bio-One GmbH, Germany). α-GalCer (50 ng/ml; Pharmaceutical Research Laboratory, Kirin Brewery, Gunma, Japan) and/or recombinant human IL-2 (100 IU/ml; Takeda Chemical Ind. Ltd., Osaka, Japan) were added to the culture. On day 4, non- and semi-adherent cells were harvested, spun down and resuspended in the medium. The cells (5×10^5 cells/ml) were cultured in 7 ml of the medium in the presence of α-GalCer and/or IL-2 for an additional 2 days.

2.3. Flow cytometry

The surface phenotype of cells was determined by multicolor flow cytometry as previously described [24]. Before staining cells with mAb, cells were pre-incubated with anti-CD16/32 (clone 2.4G2) to block non-specific FcRγ binding. The following antibodies were used in this study: FITC- or PE-conjugated anti-CD3 (clone 145-2C11) or anti-CD24 (clone M1/69), PE-conjugated anti-NK1.1 (clone PK136), anti-CD69 (clone H1.2F3), or anti-IL-2Rβ (clone TM-β1), and APC-conjugated mouse CD1d tetramers loaded with α-GalCer (CD1d/α-GalCer tetramers). Stained cells were analyzed using a FACSCalibur equipped with CELLQuest software (BD Biosciences, San Jose, CA). Dead cells were excluded by propidium iodide staining and electronic gating. The data were processed with Flow Jo software (Tree Star, San Carlos, CA). For intracellular staining, cells were stimulated for 2 h with 25 ng/ml PMA and 1 μg/ml ionomycin. The cells were then washed and incubated with anti-CD16/32, stained with PE-conjugated α-GalCer/CD1d tetramers, permeabilized with Cytofix/Cytoperm (BD PharMingen), and stained with APC-conjugated anti-IL-4 (clone 11B11), IFNγ (clone XMG1.2), or rat IgG1 isotype control (clone R3-34). The stained cells were analyzed using a FACSCalibur. All mAbs were purchased from BD PharMingen. PE- or APC-conjugated CD1d/α-GalCer tetramers were prepared in a baculovirus expression system as previously described [25]. Mouse CD1d/β2-microglobulin expression vector was provided by Dr. M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA).

2.4. Cytokine levels

Culture supernatants were collected following 1, 4, or 6 days of culture and stored at −20 °C before analysis. IFNγ and IL-4 concentrations in culture supernatants were determined by ELISA kits (OptEIA ELISA set; BD PharMingen).

2.5. Adoptive transfer of in vitro-expanded Vα14i NKT cells

Spleen cells from BALB/cAnN mice were culture in the presence of α-GalCer and IL-2 for 4 days. The cultured spleen cells containing expanded Vα14i NKT cells (2×10^7) were transferred i.v. to C.B-17/Icr SCID mice. The recipient mice were killed on day 7 after cell transfer, and the percentage of CD1d/α-GalCer $tetramer^+$ cells in the spleen and liver were