Impaired Proliferative Response of Vα24 NKT Cells from Cancer Patients Against α-Galactosylceramide[1]

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Human invariant Vα24$^+$ NKT cells are a relatively new subpopulation of lymphocytes. It has been reported that Vα24 NKT cells are significantly involved in some human diseases. We have evaluated the number and function of Vα24 NKT cells in both healthy volunteers and cancer patients. In this study we found that Vα24 NKT cells in unfractionated PBMCs obtained from cancer patients did not respond efficiently to α-galactosylceramide (α-GalCer) in vitro. Thus, their proportion after stimulation with α-GalCer was smaller than that found in healthy volunteers. However, the cancer patients' Vα24 NKT cells retained cytotoxic activity against malignant target cells, and they could efficiently proliferate to α-GalCer when fractionated by sorting. Furthermore, we found that addition of G-CSF to the culture could restore the low proliferative response of Vα24 NKT cells from cancer patients. These results suggest that some functions of NKT cells in cancer patients are impaired, and this observation carries significant implications for immunotherapy-based cancer treatments. ***The Journal of Immunology,*** **2002, 168: 6494–6499.**

Mouse invariant Vα14$^+$ NKT (Vα14 NKT) cells are a novel lymphoid lineage characterized by coexpression of the NK1.1 and a single, invariant TCR encoded by the Vα14 and Jα281 gene segments (1, 2). Although the physiological functions of NKT cells remain obscure, some studies have suggested that NKT cells play important roles in regulating various immune responses, such as autoimmune diseases, transplantation immunity, and rejection of malignant tumors (3–5). The murine Vα14 NKT cells recognize α-galactosylceramide (α-GalCer),[3] a glycolipid that can be presented by CD1d and that selectively activates NKT cells (6). The activated Vα14 NKT cells have been shown to display an NK-like cytotoxicity against various tumor cell lines. They have also inhibited tumor metastasis in certain experimental animal models (4, 7). Vα24 NKT (Vα24$^+$Vβ11$^+$ NKT) cells in humans are similar to murine Vα14 NKT cells. Their TCR is encoded by the Vα24 and JαQ gene segments. Human Vα24 NKT cells can be activated by α-GalCer in a CD1d-dependent fashion, as can murine Vα14 NKT cells (8–10). Vα24 NKT cells from humans display perforin-dependent antitumor cytotoxicity in vitro and in vivo after activation with α-GalCer (11). Therefore, this α-GalCer/CD1d-NKT cell system is expected to become a new and effective tool for use in cancer immunotherapy. It is worthwhile to examine further the properties of Vα24 NKT cells obtained from cancer patients. In this study we evaluated the proliferative response and cytotoxicity of Vα24 NKT cells in PBMCs obtained from cancer patients. We found that the proliferative response of Vα24 NKT cells in unfractionated PBMCs from cancer patients was significantly lower than that of healthy volunteers. We further analyzed the characteristics of the hyporesponsiveness and tried to restore it. Finally, we found that G-CSF can augment the proliferative response of cancer patients' Vα24 NKT cells. The clinical relevance of these findings is discussed.

Materials and Methods

Cell separation and in vitro cell culture

Human blood from healthy volunteers and from advanced cancer patients was collected after obtaining informed consent. Human PBMCs were isolated using density separation medium (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Little Chalfont, U.K.). Whole human PBMCs were cultivated in 24-well plates (1.0×10^6 cells/well) for 10 days in 1 ml RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Life Technologies, Gaithersburg, MD) and 100 μg/ml penicillin-streptomycin in the presence of 200 U/ml recombinant human IL-2 (BD Pharmingen, San Diego, CA) and 10 ng/ml α-GalCer (KRN7000; Kirin Brewery Co., Tokyo, Japan). To fractionate the PBMCs, Vα24 NKT and CD3$^-$ APCs were separated by FACSVantage (BD Biosciences, Mountain View, CA). Sorted Vα24 NKT (1.0×10^4 cells or 1.0×10^3 cells) and CD3$^-$ APCs ($0.5–1.0 \times 10^5$ cells) were cocultured for 10 days with same concentration of α-GalCer and IL-2 in the same medium in 96-well round-bottom plates. In some experiments total PBMCs were cocultured with cytokines or mAb for 10 days with α-GalCer and IL-2 in the RPMI 1640 medium. The cytokines were recombinant human GM-CSF (50 ng/ml; BD Pharmingen), recombinant human G-CSF (500 ng/ml; PeproTech, London, U.K.), recombinant human IL-12 (10 μg/ml; PeproTech), and recombinant human IFN-γ (25 pg/ml; BD Pharmingen). The Ab was monoclonal anti-human TGF-β (10 μg/ml; Genzyme, Miami, FL).

Flow cytometric analysis

In this study we defined Vα24 NKT cells as cells with both Vα24 and Vβ11 expression in the fraction of CD3$^+$ cells, as described by other groups (8–11). For detection of human Vα24 NKT cells, fresh human PBMCs (day 0) or whole cultured cells (day 10) were stained with PE-conjugated anti-TCR Vα24 Ab (C15), FITC-conjugated anti-TCR Vβ11 Ab (C21), and APC-conjugated anti-CD3 Ab (UCHT1) on ice for 30 min, washed twice, then fixed in PBS with 1% paraformaldehyde. All mAbs were purchased from Immunotech (Marseilles, France). Cells were acquired by FACSCalibur and were analyzed using CellQuest software (BD Biosciences).

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[3] Abbreviations used in this paper: α-GalCer, α-galactosylceramide.

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Cell-mediated cytotoxicity

Cytotoxic activity of Vα24 NKT cells was determined using a standard 4-h ^{51}Cr release assay. Vα24 NKT cells were sorted from 10-day cultured PBMCs with α-GalCer and IL-2 as described above by FACSVantage and used as effector cells. ^{51}Cr-labeled target cells, U937 (monocyte-like cell line, 3×10^3 cells/well), were incubated at 37°C for 4 h with the effector cells ($3.0–30 \times 10^3$ cells/well), giving E:T cell ratios between 1:1 and 10:1. ^{51}Cr release was determined using a gamma counter. The percentage of specific ^{51}Cr release was calculated as follows: (cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release) × 100. The ratio of spontaneous release to maximal release was <15% in all experiments.

RT-PCR for detection of expression of G-CSF receptor

Vα24 NKT cells from cancer patients and healthy volunteers were purified using FACS sorting. Total RNA from each sample was isolated using TRIzol reagent (Life Technologies) and phenol/chloroform. cDNA was synthesized using oligo(dT) primer (Amersham Pharmacia Biotech), which recognizes the poly(A) tail of mRNA, and 20 μl of those were made from 5 μg of each mRNA. Amplification of each cDNA (5 μl) was performed with a TaKaRa Extra *Taq* (Takara shuzo, Shiga, Japan) using specific primers for human G-CSF receptor and human β-actin as follows; human G-CSF receptor, 5′-AAG AGC CCC CTT ACC CAC TAC ACC ATC TT-3′ and 5′-TGC TGT GAG CTG GGT CTG GGA CAC TT-3′; and human β-actin, 5′-TCG TCG ACA ACG GCT CCG GCA TGT-3′ and 5′-CCA GCC AGG TCC AGA CGC AGG AT-3′. Thermal cycling of G-CSF receptor primers was performed as follows: denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min. β-Actin primers were annealed at 62°C, and all cycling was performed for 30 cycles. PCR products were analyzed by electrophoresis through 2% agarose gels and visualized under UV light after ethidium bromide staining.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U* test. Values of $p < 0.05$ were considered significant.

Results

Profiles of healthy volunteers and cancer patients, and sources of PBMCs

We collected PBMCs from 22 healthy volunteers (12 men and 10 women; median age, 47.68 years; range, 24–85 years) and 21 advanced cancer patients (12 men and 9 women; median age, 61.71 years; range, 47–80 years). We selected the cancer patients from the pretreatment (preoperation or prechemoradiotherapy) patients in our group of hospital in-patients. There was no significant difference in age or sex distribution between the two groups. The classification of cancer patients was as follows: advanced esophageal cancer, $n = 6$; advanced colorectal cancer, $n = 6$; advanced gastric cancer, $n = 4$; advanced gallbladder cancer, $n = 2$; and advanced pancreas, bile duct, and uterus cancer, $n = 1$ each (Table I). The nutritional status of the cancer patients was not significantly worse than that of healthy volunteers, as estimated by their body weights, performance status, and serum albumin levels (data not shown).

Proportion of Vα24 NKT cells in PBMCs freshly isolated and after a 10-day culture in the presence of α-GalCer and IL-2

The percentage of Vα24 NKT cells was evaluated by flow cytometric analysis. Before culturing, the proportions of Vα24 NKT cells in PBMCs were 0.58 ± 0.36% (mean ± SD) for healthy volunteers and 0.64 ± 0.31% for cancer patients (not a significant difference; Fig. 1*A*). A 10-day culture of PBMCs from healthy volunteers in the presence of α-GalCer and IL-2 resulted in a tremendous expansion of Vα24 NKT cells as described previously (8–11). After this 10-day culture, the proportion increased to 10.01 ± 15.26% (Fig. 1*B*). In contrast, Vα24 NKT cells from cancer patients expanded less significantly, to 1.67 ± 2.25% (Fig. 1*B*). Representative data are shown in Fig. 2. The percentage of Vα24 NKT cells from a healthy volunteer was 0.21% in freshly isolated PBMCs and 4.01% after the culture (Fig. 2, *upper panel*). However, those of a cancer patient were 0.72% before culture and 0.76% after culture (Fig. 2, *lower panel*). It was also observed that the total amounts of IFN-γ and IL-4 in the supernatants of culture medium from cancer patient-derived PBMCs were lower than those derived from healthy volunteers. The total amounts of IFN-γ and IL-4 were determined according to the impaired proliferative response of Vα24 NKT cells to α-GalCer (data not shown). This unresponsiveness of the Vα24 NKT cells from the cancer patients did not significantly vary by the diagnosis (Table I).

Proliferation of Vα24 NKT cells after sorting

A previous report by another group (11) has indicated that the Vα24 NKT cells from cancer patients could respond to α-GalCer as efficiently as those from healthy individuals. Thus, we evaluated the proliferative response of Vα24 NKT cells using similar methods. We prepared CD3$^-$ APC cells and Vα24 NKT cells from PBMCs by FACS sorting and cocultured 1×10^5 CD3$^-$ APC cells and 1×10^4 or 1×10^3 Vα24 NKT cells in the presence of α-GalCer and IL-2 in 96-well round-bottom plates. No significant difference was observed in the responses to α-GalCer of Vα24 NKT cells in PBMCs between those derived from healthy volunteers and those from cancer patients (Fig. 3). These results indicate that Vα24 NKT cells from cancer patients can proliferate in response to α-GalCer to the same degree as those from healthy volunteers when cultured after sorting.

Table I. *Profiles of cancer patients and proportion of Vα24 NKT cells before and after culture*[a]

Diagnosis	*n*	Age	Proportion of Vα24 NKT Cells (%)	
			Before culture	After culture
Esophageal cancer	6	66.67 ± 8.66	0.47 ± 0.16	2.38 ± 3.86
Colorectal cancer	6	61.67 ± 5.82	0.91 ± 0.32	1.66 ± 1.32
Gastric cancer	4	58.50 ± 5.97	0.57 ± 0.18	1.28 ± 0.47
Gallbladder cancer	2	57.00 ± 9.90	0.28 ± 0.16	0.73 ± 0.32
Uterus cancer	1	51	0.76	2.07
Bile duct cancer	1	47	0.56	0.5
Pancreas cancer	1	80	0.15	1.05
Healthy volunteers	22	47.68 ± 18.09	0.58 ± 0.36	10.01 ± 15.26

[a] Data presented as mean ± SD.

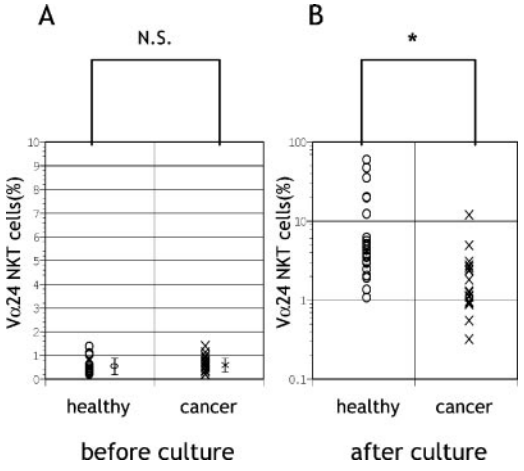

FIGURE 1. Proliferative response of Vα24 NKT cells to α-GalCer and IL-2. PBMCs from healthy volunteers (○) and cancer patients (×) were cultured for 10 days with α-GalCer (10 ng/ml) and IL-2 (200 U/ml). *A*, The proportion of Vα24 NKT cells in PBMCs before culture. N. S., not significant. *B*, The proportion of Vα24 NKT cells in PBMCs after culture. ∗, $p < 0.001$.

Restoration of low proliferative response of Vα24 NKT cells isolated from cancer patients

Next, we tried to restore the low proliferative response to α-GalCer in the Vα24 NKT cells from cancer patients. Cytokines or mAb were added to the culture medium. The cytokines were GM-CSF (50 ng/ml), G-CSF (500 ng/ml), IL-12 (10 μg/ml), IFN-γ (25 pg/ml), and anti-TGF-β (10 μg/ml). As indicated in Fig. 4*A*, neither the cytokines nor the mAb tested here (without G-CSF) affected the proliferative response. However, Vα24 NKT cells from cancer patients that were cultured with G-CSF had a proliferative response significantly different from that of Vα24 NKT cells cultured with α-GalCer and IL-2 alone. Representative data are shown in Fig. 4*B*. The percentage of Vα24 NKT cells from a gastric cancer patient was 0.06% in freshly isolated PBMCs (Fig. 4*B*, *left panel*) and 1.78% after the culture with α-GalCer (Fig. 4*B*, *upper right panel*). However, that after the culture with α-GalCer and G-CSF was 4.16% (Fig. 4*B*, *lower right panel*). We confirmed the expression of G-CSF receptor on Vα24 NKT cells using RT-PCR (Fig. 4*C*). Vα24 NKT cells from both healthy volunteers and cancer patients expressed mRNA of G-CSF.

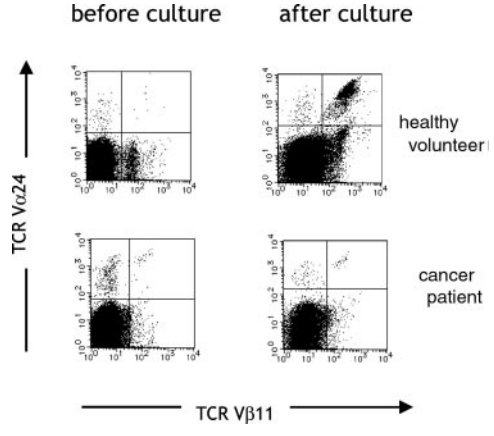

FIGURE 2. Representative flow cytometric profiles from one healthy volunteer (*upper panel*) and one cancer patient (*lower panel*). The frequency of Vα24 NKT cells before (*left panel*) and after (*right panel*) culture is shown. The healthy volunteer was a 34-year-old man, and the cancer patient was a 58-year-old woman with cancer of the rectum.

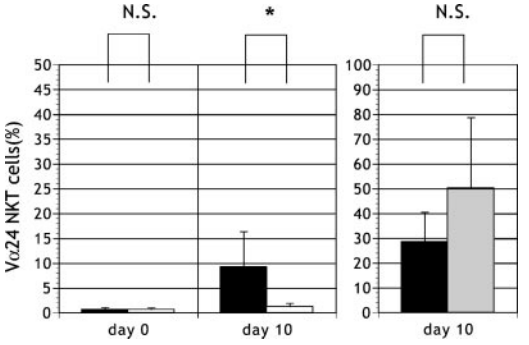

FIGURE 3. Coculture of sorted Vα24 NKT cells with $CD3^-$ APCs. Freshly isolated Vα24 NKT cells from healthy volunteers (■; $n = 10$) and cancer patients (▨; $n = 10$) were cultured for 10 days with $CD3^-$ cells as APCs in the presence of α-GalCer (10 ng/ml) and IL-2 (200 U/ml). In this method Vα24 NKT cells were well augmented, as suggested by previous reports (9) (*right panel*). The *left panel* shows unfractionated cultures on days 0 and 10. ∗, $p < 0.001$. N. S., not significant.

Cytotoxic activity of α-GalCer-activated Vα24 NKT cells

The cytotoxic activity of α-GalCer-activated Vα24 NKT cells was evaluated next. Vα24 NKT cells were collected from 10-day cultures of PBMCs with α-GalCer and IL-2 using FACS sorting. Vα24 NKT cells were used as an effector, and U937 cells were used as a target. No significant difference was found in the cytotoxicity of the α-GalCer-activated Vα24 NKT cells between those from healthy volunteers and those from cancer patients against U937 cells (Fig. 5). There was no significant difference in the cytotoxicity among the diagnosis of cancer of seven independent patients (data not shown). These results suggested that the Vα24 NKT cells from cancer patients retain cytotoxic activity, comparable to that of cells from healthy volunteers.

Discussion

We have demonstrated that Vα24 NKT cells derived from PBMCs of cancer patients show a much lower proliferative response to α-GalCer than those of healthy volunteers. In this study we assessed advanced cancer patients with operable conditions. It is well known that advanced cancer patients often have poor nourishment, which can cause immunological dysfunction (12). However, the patients assessed here were as well nourished as the healthy volunteers. Nutritional status was estimated by comparing body weight, performance status, and serum albumin levels, as described in *Results*. Thus, the impaired proliferative response of Vα24 NKT cells from these cancer patients cannot be attributed to a poor nutritional status.

Another group (11) has shown that Vα24 NKT cells in PBMCs from patients with malignant melanoma could respond well to α-GalCer, which caused their numbers to increase greatly. The proliferative response was similar in magnitude to that seen in healthy volunteers. Their cultivation method is to coculture enriched Vα24 NKT cells (1×10^5) and $CD3^-$ APCs (1×10^6) in the presence of α-GalCer (10 ng/ml) and recombinant human IL-2 (100 U/ml) (11). We also followed this cultivation procedure in this study (Fig. 3). In this method Vα24 NKT cells from cancer patients proliferated well after sorting (Fig. 3). Considering the difference between our primary (Fig. 1) and the other group's culture methods, it appears that $CD3^+$ cells (T cells) play a role in the low responsiveness of Vα24 NKT cells. This is in line with the

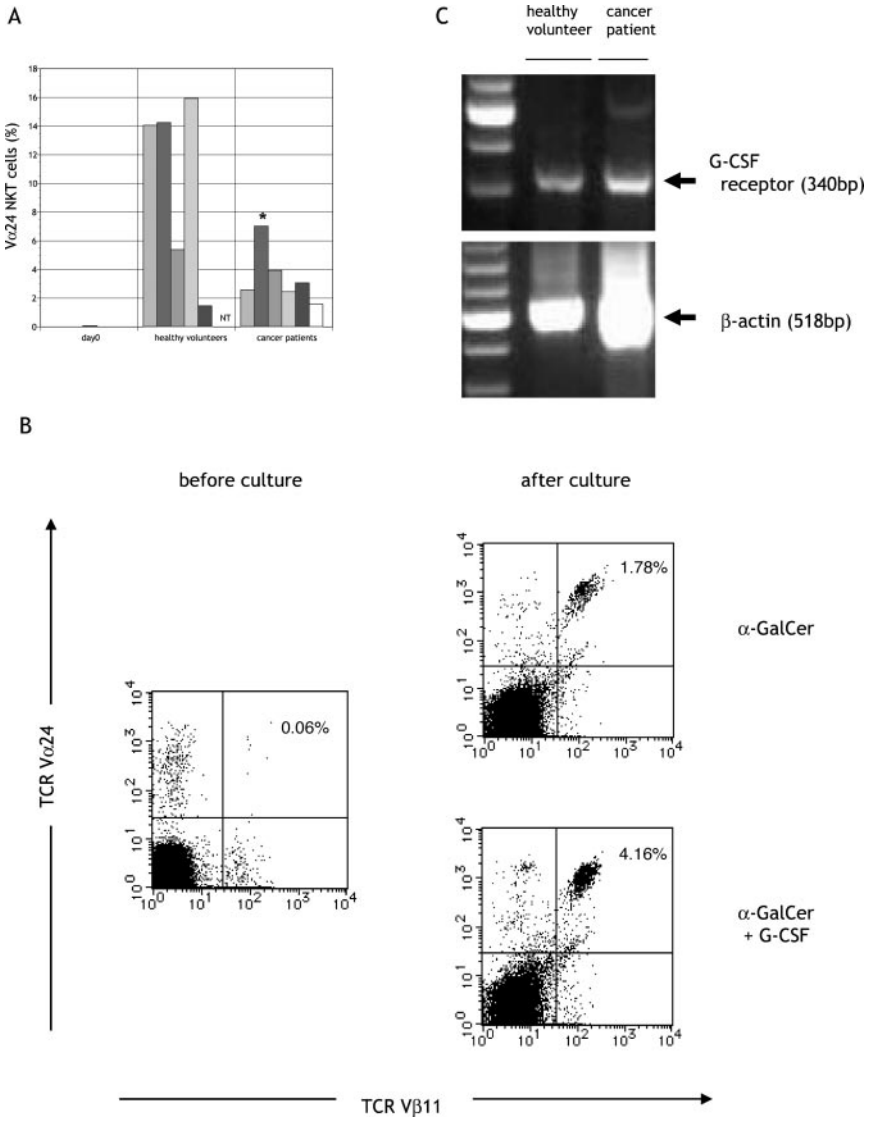

FIGURE 4. Restoration of low proliferative response of Vα24 NKT cells from cancer patients. *A*, Average percentages of Vα24 NKT cells from five independent patients are shown. In cancer patients only recombinant human G-CSF had augmentative activity. The PBMCs were cocultured in α-GalCer and IL-2 only (▨), with G-CSF (■), GM-CSF (▧), IFN-γ (▤), IL-12 (▩) and anti-TGF-β Ab (□). $*$, $p < 0.05$ compared with α-GalCer and IL-2 only. NT, not tested. ■, day 0. *B*, Representative flow cytometric profiles of PBMCs from a 47-year-old male gastric cancer patient. The frequencies of Vα24 NKT cells before (*left panel*) and after (*right panel*) the culture are shown. The PBMCs were cultured with IL-2 and α-GalCer only (*upper right panel*) or IL-2, α-GalCer, and G-CSF (*lower right panel*) for 10 days. *C*, RT-PCR of Vα24 NKT cells for detection of mRNA expression of G-CSF receptor.

proposal that T cells in cancer patients may produce some immunosuppressive factor (for example, TGF-β) that can modify other immune cells (13–15). Recently, Tahir et al. (16) reported that ex vivo expansion of Vα24 NKT cells from advanced prostate cancer patients with α-GalCer was significantly diminished compared with that of cells from healthy donors. A striking decrease in IFN-γ production of NKT cells was also demonstrated. These findings are essentially consistent with those observed in this study. It is also conceivable that some immunosuppressive factors in the serum of the tumor-bearing patients are involved in the impaired proliferative response of Vα24 NKT cells. To examine this possibility, we added heat-inactivated patients' serum to the culture instead of FCS (final concentration, 10%) and found no significant difference in comparison with healthy volunteers' serum or FCS (data not shown). However, because the exposure duration of lymphocytes to the serum is different between in vitro and in vivo conditions, this result could not completely exclude the possibility.

It has been demonstrated that $CD3^+CD56^+$ cells also have some features of NKT cells and anti-tumor activity (17), although CD56 is not usually expressed on Vα24 NKT cells (data not shown). Because $CD3^+CD56^+$ cells can be more easily obtained from PBMCs than Vα24 NKT cells, we also evaluated their response against α-GalCer and IL-2. The $CD3^+CD56^+$ cells from cancer patients variously expanded in the culture with α-GalCer and IL-2. However, there was no significant difference compared with cells from healthy volunteers (K. Yanagisawa and K. Seino, unpublished observations). Thus, the responses of Vα24 NKT and $CD3^+CD56^+$ cells to α-GalCer (and IL-2) are different.

In our next set of experiments we attempted to restore the low proliferative response of Vα24 NKT cells by culturing them in the presence of cytokines or mAb. IFN-γ, IL-12, G-CSF, GM-CSF, or mAb to TGF-β were added to the medium, because they have been implicated in NKT cell function as follows. IFN-γ is secreted from normal activated Vα24 NKT cells, and it is known to induce CD69 on NK cells, B cells, and CTLs (18). IL-12 is known to mediate anti-tumor responses, and it has been hypothesized to interact with NK cells (19), CTLs (20), and NKT cells (4). TGF-β is induced in the tumor-bearing state and is involved in immunosuppression (13–15). G-CSF and GM-CSF are cytokines produced by activated T cells, macrophages, endothelial cells, and stromal fibroblasts that act on bone marrow to increase the production of inflammatory leukocytes (21). GM-CSF was reported to be a critical molecule for the initiation of TCR gene rearrangement in Vα14 NKT cells during the development of these lymphocytes (22). It was reported that mRNA of the mouse G-CSF receptor was expressed in mouse NKT cells (23). They did not restore the low proliferative response of Vα24 NKT cells from cancer patients excluding G-CSF (Fig. 4, *A* and *B*). We also confirmed by RT-PCR that Vα24 NKT cells

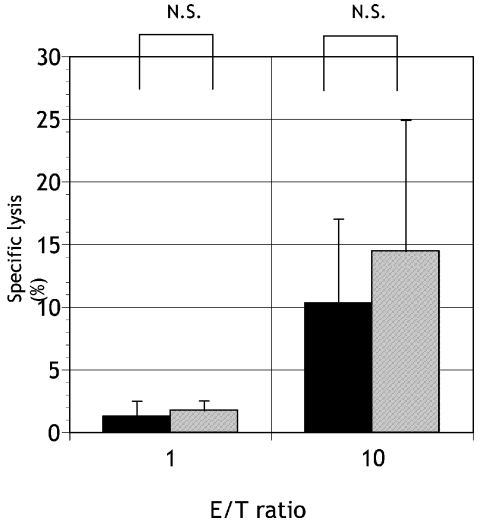

FIGURE 5. Antitumor cytotoxic activity of α-GalCer-activated Vα24 NKT cells from cancer patients. PBMCs were cultured with α-GalCer and IL-2 for 10 days, then Vα24 NKT cells were collected by sorting. The activated Vα24 NKT cells were used as effectors in the cytotoxicity assay. Cytotoxicity was measured by a standard ^{51}Cr release assay of U937 target cells (1.0×10*3*) at the indicated E:T cell ratio. PBMCs were obtained from healthy volunteers (■) and cancer patients (▨). Representative data are shown from experiments using Vα24 NKT cells from seven independent patients. N.S., not significant.

expressed G-CSF receptor (Fig. 4*C*). These results suggest that G-CSF blocks some inhibitory factors produced by T cells, or it stimulates the Vα24 NKT cells strongly in a collaborative fashion with α-GalCer. These results also suggest that administration of G-CSF with α-GalCer can induce efficient proliferation of Vα24 NKT cells in cancer patients in vivo, and this could constitute an effective approach to immunotherapy. Accordingly, it has been reported that NKT cells can be expanded in G-CSF transgenic mice (24). However, not only NKT cells, but also other cells, including granulocytes and monocytes, can express the receptor (25). Actually, in the G-CSF transgenic mice, both the number of granulocytes and macrophages increased as well as that of NKT cells (24). Therefore, the possibility that some APCs are the target of G-CSF cannot be excluded.

In this study we have also shown that Vα24 NKT cells from cancer patients and healthy volunteers displayed comparable antitumor cytotoxicity in vitro when activated with α-GalCer. It has been reported that NKT cells kill target cells using perforin (4, 6). In our experimental system the number of effector cells (Vα24 NKT cells) was compensated; thus, the cytotoxicity demonstrated in Fig. 5 represented the cytolytic activity of each effector cell. Therefore, our data indicate that Vα24 NKT cells respond specifically to α-GalCer with a lowered proliferative response while retaining their cytotoxicity.

An impaired proliferative response of Vα24 NKT cells from patients suffering from some autoimmune diseases has been also reported (26). Thus, the attenuated functions of Vα24 NKT cells may contribute generally to various pathological conditions in human diseases.

The mechanism of induction of the impaired proliferative response of Vα24 NKT cells from cancer patients has not been clarified yet, but we have suggested in this study that CD3$^+$ T cells play a role. However, several factors may be able to restore the proliferative response in these cells, as did G-CSF in this study. Administration of α-GalCer was expected to be a prophylaxis or treatment of cancer, because of its dramatic effect in mouse cancer models (4, 6, 7). However, a recent phase I study of α-GalCer in patients with solid tumors indicated that neither significant drug-related toxicity nor an anti-tumor effect was observed (27). Our present data serve as a warning that the administration of α-GalCer alone would result in insufficient therapeutic effects due to the low proliferative response observed, but the data strongly suggest that a combination therapy with reagents such as G-CSF might enhance the response to therapeutically significant levels. Further studies are needed to clarify the mechanisms and range of clinical applicability of the α-GalCer/CD1d-NKT system in cancer treatment.

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