α-Glycosylceramides Enhance the Antitumor Cytotoxicity of Hepatic Lymphocytes Obtained from Cancer Patients by Activating CD3$^-$CD56$^+$ NK Cells In Vitro

Soichiro Ishihara,*† Mie Nieda,* Joji Kitayama,† Takuya Osada,† Toshio Yabe,* Akiko Kikuchi,* Yasuhiko Koezuka,‡ Steven A. Porcelli,§ Kenji Tadokoro,* Hirokazu Nagawa,† and Takeo Juji*

α-Glycosylceramides, such as α-galactosylceramide and α-glucosylceramide, induce antitumor immunity in various murine cancer models. In the murine hepatic metastasis model, Vα14 TCR$^+$NK1.1$^+$ T cells, which accumulate preferentially in the liver, are considered to play a key role in the induction of antitumor immunity by α-glycosylceramides. We recently reported that Vα24 TCR$^+$ NKT cells, the human homologues of murine Vα14 TCR$^+$NK1.1$^+$cells, are rarely seen among freshly isolated human hepatic lymphocytes. Therefore, it is important to examine whether α-glycosylceramides also enhance the antitumor cytotoxicity of human hepatic lymphocytes, as they have been shown to do in murine systems, to determine the usefulness of α-glycosylceramides in cancer immunotherapy in humans. Here, we show that α-glycosylceramides greatly enhance the cytotoxicity of human hepatic lymphocytes obtained from cancer patients against the tumor cell lines, K562 and Colo201, in vitro. The direct effector cells of the elicited cytotoxicity were CD3$^-$CD56$^+$ NK cells. Even though Vα24 TCR$^+$NKT cells proliferated remarkably in response to α-glycosylceramides, they did not contribute directly to the cytotoxicity. Our observations strongly suggest the potential usefulness of α-glycosylceramides for immunotherapy of liver cancer in humans based on their ability to activate CD3$^-$CD56$^+$ NK cells in the liver. ***The Journal of Immunology,*** **2000, 165: 1659–1664.**

α-Glycosylceramides (AGCs),[2] such as α-galactosylceramide (α-GalCer) and α-glucosylceramide (α-GlcCer), have been shown to be presented by CD1d molecules (1–8), which are nonpolymorphic cell surface glycoproteins structurally related to MHC class I molecules expressed on specific APCs such as dendritic cells (DCs) (7, 9, 10). Mouse invariant Vα14 TCR$^+$ NK1.1 (NKR-P1C)$^+$ T cells (Vα14 NKT cells) are specifically activated by AGCs in a Vα14 TCR-CD1d-restricted manner, as opposed to the conventional TCR-MHC-restricted manner (2–5, 10).

AGCs have been shown to induce antitumor immunity in various murine cancer models both in vitro and in vivo (4, 11–17). Nakagawa et al.(11) and Kobayashi et al. (6) demonstrated that the administration of α-GalCer in mice resulted in complete regression of established hepatic metastases in vivo and marked augmentation of the cytotoxicity of hepatic lymphocytes (HLs) against tumor cell lines in vitro. They suggested the effectiveness of AGCs in the immunotherapy of liver cancer in humans. They demonstrated that the main effectors among the HLs of the induced cytotoxicity were CD3$^-$NK1.1$^+$ NK cells. They also suggested that CTLs specific to tumor cells were also generated in vivo in response to α-GalCer, because mice cured of hepatic metastases treated with α-GalCer acquired tumor-specific immunity. Vα14 NKT cells, which accumulate preferentially in the liver (18, 19), are considered to play a key role in the induction of antitumor immunity by AGCs in the mouse hepatic metastasis model (4, 5, 9, 11, 16). However, the precise mechanism by which AGCs induce antitumor immunity remains to be elucidated.

In humans, invariant Vα24 TCR$^+$CD161 (NKR-P1A)$^+$ T cells (Vα24 NKT cells), the human homologues of murine Vα14 NKT cells, in peripheral blood have been shown to specifically proliferate in response to AGCs, again in a Vα24 TCR-CD1d-restricted manner (1, 2, 7, 10, 20–23). We previously reported that the phenotype of human HLs was quite different from that of mice in terms of NKT cells (24). Although CD161$^+$ T cells comprised >30% of HLs, the percentage of cells using Vα24 TCR was as low as that in peripheral blood T cells. Therefore, it is important to examine whether AGCs also induce antitumor cytotoxicity in human HLs, as they have been shown to do in murine systems, to determine the usefulness of AGCs for cancer immunotherapy in humans. In this study, we evaluated in vitro the effects of AGCs on the cytotoxicity of human HLs obtained from surgically resected specimens against the tumor cell lines, K562 and Colo201. We demonstrate that the cytotoxic activity of human HLs is enhanced in the presence of AGCs and that the direct effector cells of the observed cytotoxicity are CD3$^-$CD56$^+$ NK cells, and not Vα24 NKT cells.

Materials and Methods

Tissue specimens

Surgically resected human liver specimens were obtained at hepatectomy performed in four cases with malignancy, shown as follows: case 1, 55-year-old man, primary hepatocellular carcinoma (HCC); case 2, 78-year-old man, metastases from gastric adenocarcinoma; case 3, 69-year-old man, metastases from colonic adenocarcinoma; and case 4, 75-year-old

*Department of Research, Japanese Red Cross Central Blood Center, Tokyo, Japan; †Department of Surgery, Division of Surgical Oncology, University of Tokyo, Tokyo, Japan; ‡Pharmaceutical Research Laboratory, Kirin Brewery, Gunma, Japan; and §Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461

Received for publication November 8, 1999. Accepted for publication May 17, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Soichiro Ishihara, Department of Research, Japanese Red Cross Central Blood Center, 4-1-31 Hiroo, Shibuya-ku, Tokyo 150-0012, Japan. E-mail address: i-sochan@kt.rim.or.jp

[2] Abbreviations used in this paper: AGCs, α-glycosylceramides; α-GalCer, α-galactosylceramide; α-GlcCer, α-glucosylceramide; DCs, dendritic cells; HLs, hepatic lymphocytes; HCC, hepatocellular carcinoma; Vα14 NKT cells, Vα14 TCR$^+$NK1.1 (NKR-P1C)$^+$ T cells; Vα24 NKT cells, Vα24 TCR$^+$CD161 (NKR-P1A)$^+$ T cells.

0022-1767/00/$02.00

man, metastases from rectal adenocarcinoma. All the metastatic liver cancers (cases 2, 3. and 4) were metachronous metastases, and the patients had received adjuvant oral chemotherapy after curative surgery for the primary disease. Tissue specimens were obtained from apparently normal areas distant from the tumors in the resected specimens. Informed consent for the study was obtained from all the patients.

Separation of hepatic mononuclear cells

Hepatic mononuclear cells were separated from liver tissue specimens as described previously (24). Briefly, the tissue specimen was cut into small pieces with a scalpel, minced mechanically, and then incubated in AIM-V medium (Life Technologies, Rockville, MD) supplemented with 10% FCS (HyClone, Logan, UT), 0.5 mg/ml type IV collagenase (Sigma, Poole, U.K.), and 0.02 mg/ml DNase (Boehringer Mannheim, Mannheim, Germany) at 37°C in a shaking water bath for 30 min. After incubation, the digested tissue was passed through a metal mesh filter (100 μm diameter) to remove cell clumps and undissociated tissue. After two washings, the cell suspension was layered over Ficoll-Hypaque gradients and centrifuged at 650 $\times$ *g* at room temperature for 20 min, and mononuclear cells were recovered from the interface.

α-Glycosylceramides

Two types of AGCs, α-GlcCer and α-GalCer, were provided by Kirin Brewery (Gunma, Japan).

Culture of hepatic mononuclear cells

Hepatic mononuclear cells (2×10^6) were cultured in 24-well plates in 1 ml AIM-V medium supplemented with 10% FCS in the presence of 100 ng/ml α-GlcCer, α-GalCer, or vehicle (0.1% DMSO) alone. After 7 days of culture, the nonadherent lymphocytes were harvested, analyzed for the percentage of Vα24 TCR^+ T cells and assayed for their cytotoxicity against tumor cell lines. The concentration of AGCs used was optimized in our previous experiments (7).

Flow cytometry

The expression of Vα24 TCR, CD3, CD56, and CD161 among the HLs was determined by flow cytometry (Cytoron Absolute, Ortho Clinical Diagnostics, Raritan, NJ), gating the lymphocyte fraction according to the characteristic forward and right angle scatters. The mAbs specific for human Vα24 TCR (C15), CD3 (SK7), CD56 (MY31), CD161 (DX12), and the isotype-matched control mAbs used were purchased from Immunotech (Marseille, France) and Becton Dickinson (Oxford, U.K.).

Immunomagnetic cell separation

To identify the effector cell population, HLs cultured with α-GlcCer for 7 days were separated immunomagnetically using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer's instructions. To separate cells positive and negative for CD3 or CD56, HLs were incubated with anti-CD3 or CD56 mAb-coated magnetic beads, respectively, for 15 min at 4°C and passed through magnetic columns. Cells positive and negative for Vα24 TCR were separated using purified mAbs specific for human Vα24 TCR (C15) and anti-mouse IgG1 mAb-coated magnetic beads. The purity of each cell subpopulation was determined to be >80% by flow cytometry.

Cytotoxicity assay

The cytotoxicity of HLs against K562 (human erythroleukemia) and Colo201 (human colonic adenocarcinoma) cell lines was assayed by the standard 4-h ^{51}Cr-release assay as described previously (25). The K562 and Colo201 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The percent cytotoxicity was calculated as 100 $\times$ [(cpm of experimental release − cpm of spontaneous release)/(cpm of maximum release − cpm of spontaneous release)]. The cpm of spontaneous release was always <10% of the cpm of maximum release. All the assays were performed in triplicate.

Blocking of CD1d and Vα24 TCR

To examine the blocking effect of CD1d and Vα24 TCR on the proliferation of Vα24 TCR^+ T cells and on the cytotoxicity of HLs activated by AGCs, mAbs specific for human CD1d (CD1d 42.1, mouse IgG1, 10 and 20 μg/ml) (22, 23) or Vα24 TCR (C15, mouse IgG1, 10 μg/ml), or irrelevant control mAbs (anti-CD34 mAb, Immu 133, mouse IgG1, Immunotech, Marseille, France) were added to the culture medium, and after incubation at 37°C for 1 h, α-GlcCer (100ng/ml) or vehicle (0.1% DMSO) was added to the culture medium. HLs were harvested after 7 days of culture, analyzed for the percentage of Vα24 TCR^+ T cells and assayed for their cytotoxicity. The concentration of anti-CD1d mAbs used was determined in accordance with the previous reports of Exley et al. (22, 23) and Spada et al. (26). They showed that 0.67–20 μg/ml anti-CD1d mAbs had a sufficient blocking effect on CD1d-mediated Vα24 NKT cell activation. The concentration of anti-Vα24 TCR mAbs used in our study was optimized in preliminary experiments (data not shown).

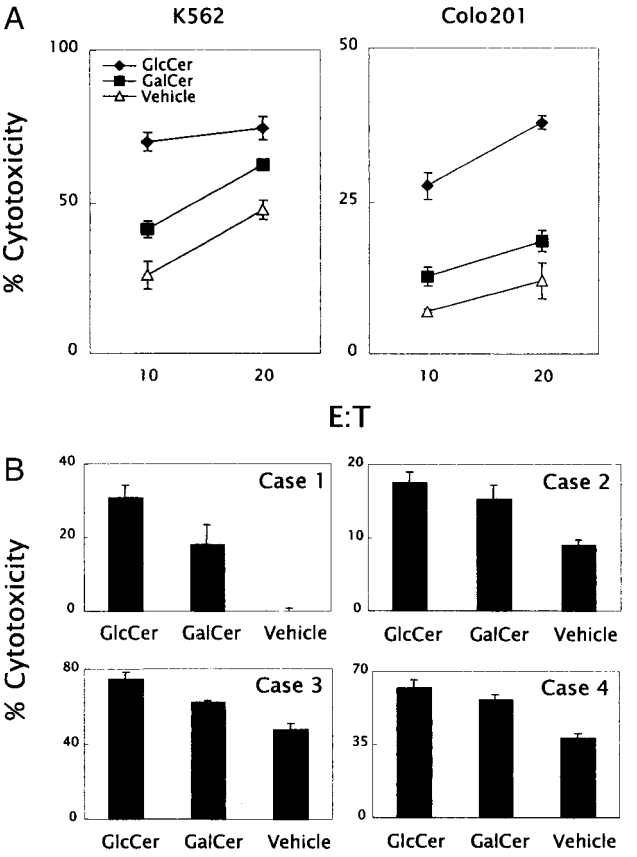

FIGURE 1. Antitumor cytotoxicity of hepatic lymphocytes enhanced by α-glycosylceramides. Hepatic lymphocytes, cultured for 7 days with α-GlcCer, α-GalCer, or vehicle (0.1% DMSO) alone, were assayed for their cytotoxicity against the K562 and Colo201 cell lines using the 4-h ^{51}Cr release assay. *A*, Results for hepatic lymphocytes from case 3. The E:T ratios are 20:1 and 10:1. *B*, Cytotoxicity against K562 cells at E:T 20:1. Data are the representative results of four independent experiments for case 1, two independent experiments for cases 2 and 4, and a single experiment for case 3. Mean values of triplicate samples with SD are shown.

Statistics

Values are expressed as mean ± SD. Student's *t* test was used, and *p* values <0.05 were considered statistically significant.

Results

Cytotoxicity of hepatic lymphocytes activated by AGCs

HLs cultured for 7 days with one of the two types of AGCs (α-GlcCer or α-GalCer) or vehicle alone were assayed for their cytotoxicity against tumor cell lines. Fig. 1*A* (case 3; targets, K562 and Colo201; E:T ratios, 20:1 and 10:1) and 1*B* (target, K562; E:T ratio, 20:1) show that both α-GlcCer and α-GalCer enhanced the antitumor cytotoxicity of HLs in all cases, although the level of spontaneous cytotoxicity of HLs cultured with vehicle alone varied among the cases (the mean cytotoxicity against K562 at E:T 20:1

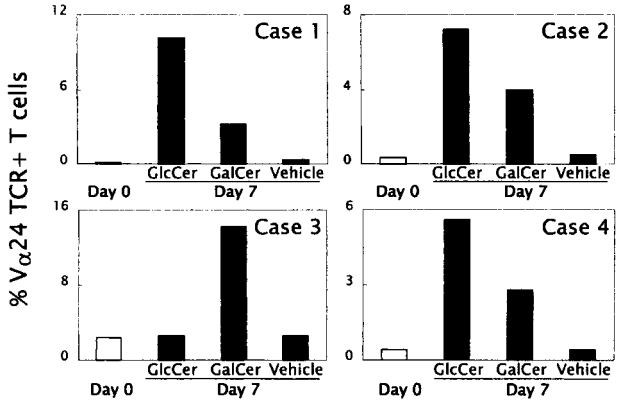

FIGURE 2. Frequency of Vα24 TCR$^+$ T cells in culture. The percentage of Vα24 TCR$^+$ cells in hepatic lymphocytes cultured with α-GlcCer, α-GalCer, or vehicle (0.1% DMSO) alone was determined by flow cytometry on days 0 and 7 of culture. Data are the representative results of five independent experiments for case 1 (mean ± SD of % Vα24 TCR$^+$ cells for the five experiments; GlcCer, 8.7 ± 1.4%, vs vehicle, 0.5 ± 0.3%; significant difference at $p < 0.01$), two independent experiments for cases 2 and 4, and a single experiment for case 3.

for the four cases: α-GlcCer, 46.5 ± 26.5% and α-GalCer, 38.0 ± 24.8%; significant difference at $p < 0.05$ vs vehicle, 18.1 ± 16.8%). HLs cultured with AGCs showed little cytotoxicity against autologous PHA blasts (data not shown).

Frequency of Vα24 TCR$^+$ T cells in culture

Fig. 2 shows the percentage of Vα24 TCR$^+$ T cells in HLs as determined by flow cytometry. Vα24 TCR$^+$ T cells were rarely detected among freshly isolated HLs (day 0). A marked proliferation of Vα24 TCR$^+$ T cells was observed among HLs cultured for 7 days in the presence of α-GlcCer or α-GalCer, but not in those cultured with vehicle alone. The majority of proliferating Vα24 TCR$^+$ T cells expressed CD161 (data not shown) thus represented Vα24 NKT cells. The level of proliferation of Vα24 TCR$^+$ T cells in response to α-GlcCer or α-GalCer varied among the HLs derived from the four cases examined. In the case of HLs from cases 1, 2 and 4, the effect of α-GlcCer on the proliferation of Vα24 TCR$^+$ T cells was stronger than that of α-GalCer. On the other hand, in the case of HLs from case 3, α-GlcCer failed to induce proliferation of Vα24 TCR$^+$ T cells, whereas α-GalCer induced significant proliferation of these cells. Expressions of other cell surface markers, CD3 and CD56, on HLs are shown in Table I.

Identification of the effector cell population

To identify the direct effector cells of the cytotoxicity induced by AGCs, we separated HLs cultured for 7 days with α-GlcCer into CD3$^+$ or CD3$^-$ cells, Vα24 TCR$^+$ or Vα24 TCR$^-$ cells, and CD56$^+$ or CD56$^-$ cells, and examined their cytotoxicity against the K562 and Colo201 cell lines at E:T 20:1. As shown in Fig. 3, CD3$^-$ cells showed significantly higher antitumor cytotoxicity than CD3$^+$ cells. In contrast, CD56$^-$ cells exhibited significantly lower antitumor cytotoxicity than CD56$^+$ cells. The mean cytotoxicities of each cell subpopulation against the K562 cell line (E:T 20:1) of the four cases were: CD3$^+$ cells, 6.3 ± 4.6%, vs CD3$^-$, 54.4 ± 18.9% (significant difference at $p < 0.01$); and CD56$^+$ cells, 67.4 ± 12.6%, vs CD56$^-$, 12.4 ± 4.6% (significant difference at $p < 0.01$). This suggests that CD3$^-$CD56$^+$ NK cells are the main effector cells of the induced antitumor cytotoxicity. As shown in the results for case 1 (Fig. 3*A*), Vα24 TCR$^+$ cells exhibited little cytotoxicity against the K562 and Colo201 cell lines. Although in the other cases, the cytotoxicity of Vα24 TCR$^+$ and Vα24 TCR$^-$ cells was not compared, the results suggest that the main effector cells are CD3$^-$ cells, and not Vα24 TCR$^+$ cells, which express CD3 (data not shown).

Table I. *Expressions of CD3 and CD56 on hepatic lymphocytes cultured with α-glycosylceramides*[a]

	CD3$^+$CD56$^-$	CD3$^-$CD56$^+$	CD3$^+$CD56$^+$	CD3$^-$CD56$^-$
Case 1				
Day 0	35.1	28.8	19.4	16.6
Day 7				
α-GlcCer	50.1	24.0	22.1	3.8
α-GalCer			ND	
Vehicle	48.0	20.2	24.9	6.9
Case 2				
Day 0	25.6	23.8	19.6	31.0
Day 7				
α-GlcCer	36.5	14.4	42.7	6.5
α-GalCer	34.8	15.6	41.9	7.8
Vehicle	34.1	13.3	43.1	9.4
Case 3				
Day 0	57.6	20.4	12.3	9.6
Day 7				
α-GlcCer	65.1	6.9	26.8	1.2
α-GalCer	69.2	8.2	21.0	1.6
Vehicle	64.1	6.8	26.7	2.5
Case 4				
Day 0	38.6	22.0	37.4	2.1
Day 7				
α-GlcCer	40.6	16.8	25.4	17.2
α-GalCer	50.3	15.9	25.6	8.9
Vehicle	44.6	13.1	27.8	14.6

[a] Hepatic lymphocytes were cultured with α-GlcCer, α-GalCer, or vehicle alone (0.1% DMSO) for 7 days. Expression of CD3 and CD56 was determined on days 0 and 7 of culture by dual-color flow cytometry. The percentages among lymphocytes are shown. Data are the representative results of three independent experiments for case 1, two independent experiments for cases 2 and 4, and a single experiment for case 3.

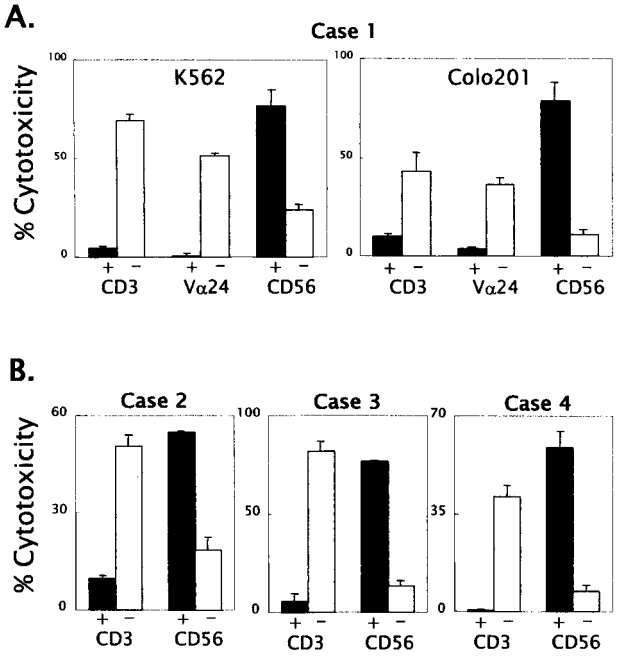

FIGURE 3. Identification of the direct effector cell population. Hepatic lymphocytes cultured with α-GlcCer for 7 days were separated into $CD3^+$ or $CD3^-$ cells, Vα24 TCR^+ or Vα24 TCR^- cells, and $CD56^+$ or $CD56^-$ cells by immunomagnetic bead separation, and their cytotoxicity against the K562 and Colo201 cell lines was examined. *A*, Results for hepatic lymphocytes from case 1. *B*, Cytotoxicity against the K562 cell line of $CD3^+$ and $CD3^-$ cells, and $CD56^+$ and $CD56^-$ cells for hepatic lymphocytes from cases 2, 3, and 4. The E: T ratio is 20:1. Data are expressed as mean values of triplicate samples with SD.

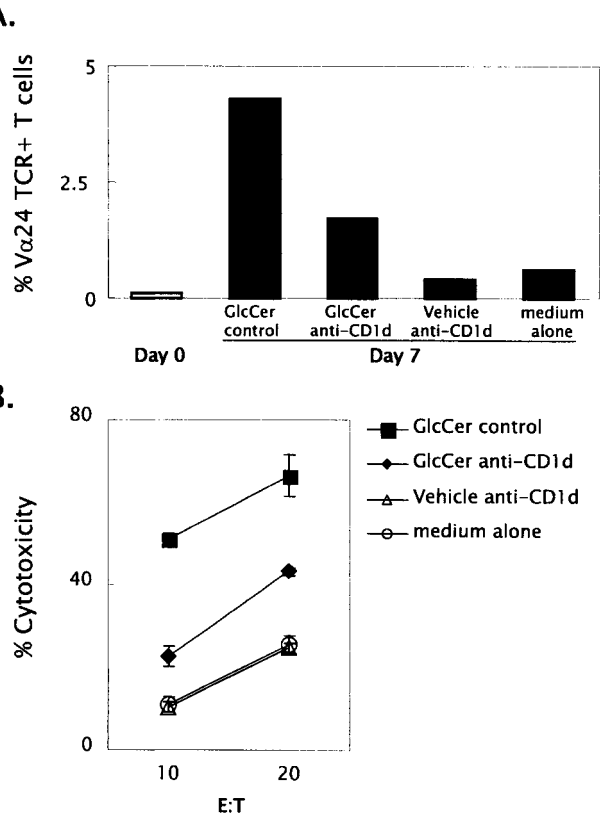

FIGURE 4. Blocking effects of anti-CD1d mAbs on the proliferation of Vα24 TCR^+ T cells (*A*) and cytotoxicity against K562 cells (*B*). Specific mAbs for CD1d (CD1d42.1, IgG1) or irrelevant control mAbs (anti-CD34 mAbs, IgG1) were added to the culture (20 μg/ml); then, after incubation for 1 h, α-GlcCer (100 ng/ml) or vehicle (0.1% DMSO) was added, or hepatic lymphocytes were cultured in the medium alone. The cells were harvested on day 7 and analyzed by flow cytometry and the 4-h ^{51}Cr release assay. Representative results for hepatic lymphocytes from case 1 are shown. We have also tested 10 μg/ml CD1d 42.1, resulting in similar partial inhibition (data not shown). Data are expressed as mean values of triplicate samples with SD. The experiments were repeated twice with similar results.

Effect of mAbs against CD1d and Vα24 TCR

To determine whether the proliferation of Vα24 TCR^+ T cells induced by AGCs presented on CD1d is a prerequisite for the induction of cytotoxicity of $CD3^-CD56^+$ NK cells, we examined the blocking effects of CD1d and Vα24 TCR by specific mAbs. HLs from case 1, cultured with α-GlcCer or vehicle alone in the presence of either anti-CD1d mAbs or anti-Vα24 TCR, or control mAbs for 7 days, were assayed for the percentage of Vα24 TCR^+ T cells in HLs and their cytotoxicity against the K562 cell line. As shown in Fig. 4, anti-CD1d mAbs partially impaired both the proliferation of Vα24 TCR^+ T cells (Fig. 4*A*) and the augmentation of cytotoxicity of HLs (Fig. 4*B*). On the other hand, whereas anti-Vα24 TCR mAbs completely inhibited the proliferation of Vα24 TCR^+ T cells (Fig. 5*A*), the cytotoxicity of HLs against the K562 cell line remained augmented regardless of the presence of anti-Vα24 TCR mAbs (Fig. 5*B*). In the blocking study of CD1d, we used the most specific mAbs available, at relatively higher concentrations than Exley et al. did (22, 23)**;** however, the blocking effect was still not complete. This might be due to the inherent ability of the mAbs, because Exley et al. (22, 23) and Spada et al. (26) also showed similar incomplete blocking effect on CD1d-Vα24 TCR interaction.

Discussion

We conducted this study to investigate whether HLs activated by AGCs exhibit cytotoxicity against tumor cells and whether the observed cytotoxicity of HLs is mediated by the activation or proliferation of Vα24 NKT cells. As we demonstrated in this study, Vα24 NKT cells among human HLs, which are rarely encountered among freshly isolated human HLs, proliferated significantly in response to AGCs. However, the direct effector cells of the elicited antitumor cytotoxicity were $CD3^-CD56^+$ NK cells. The proliferating Vα24 NKT cells did not exhibit any cytotoxicity against the K562 and Colo201 cell lines (Fig. 3), confirming our previous report that the Vα24 NKT cell lines established from PBMCs of healthy volunteers exhibit little cytotoxicity against the K562 cell line (7). Recently, Kawano et al. (27) showed that human Vα24 NKT cells, obtained from PBLs and activated by α-GalCer and IL-2, displayed potent cytotoxic activity against a variety of tumor cell lines, including K562, in vitro. The difference between their observations and ours, concerning the direct cytotoxic activity of Vα24 NKT cells against the K562 cell line, remains to be elucidated. The Vα24 NKT cell population used in their study contained 10% cells of an unknown phenotype, which might have contributed directly or indirectly to the cytotoxicity against the K562 cell line.

The blocking study using anti-CD1d mAbs indicates that the presentation of AGCs by CD1d-expressing APCs is necessary for the proliferation of Vα24 TCR^+ T cells and also for the induction of the cytotoxicity of $CD3^-CD56^+$ NK cells (Fig. 4). However, the blocking study using anti-Vα24 TCR mAbs indicates that the proliferation of Vα24 TCR^+ T cells is not necessary for the induction of antitumor cytotoxicity (Fig. 5). In addition, in the case

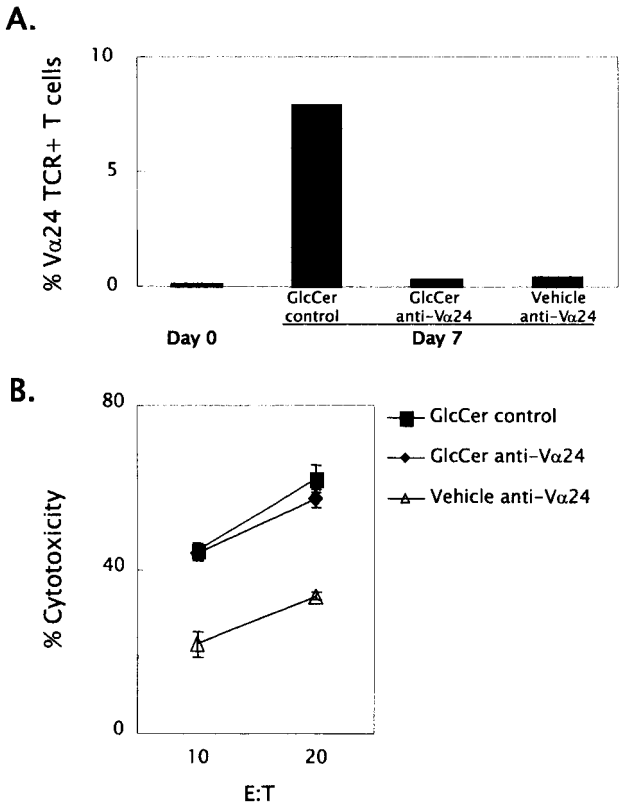

FIGURE 5. Blocking effects of anti-Vα24 TCR mAbs on the proliferation of Vα24 TCR$^+$ T cells (*A*) and cytotoxicity against K562 cells (*B*). Specific mAbs for Vα24 TCR (C15, IgG1) or irrelevant control mAbs (anti-CD34 mAbs, IgG1) were added to the culture (10 μg/ml); then, after incubation for 1 h, α-GlcCer (100 ng/ml) or vehicle (0.1% DMSO) was added. The cells were harvested on day 7 and analyzed by flow cytometry and the 4-h ^{51}Cr release assay. Representative results for hepatic lymphocytes from case 1 are shown. Data are expressed as a mean value of triplicate samples with SD. The experiments were repeated twice with similar results.

of HLs from case 3, unlike those from the other three cases, whereas α-GlcCer failed to induce the proliferation of Vα24 TCR$^+$ T cells when α-GalCer did, the cytotoxicity of HLs was induced to a significantly greater extent by α-GlcCer than by α-GalCer (Figs. 1 and 2). These observations strongly suggest that Vα24 NKT cells are not essential for the induction of the cytotoxicity of CD3$^-$CD56$^+$ NK cells by AGCs in this system.

It is postulated that an AGC by itself, or AGC-loaded APCs, directly activate NK cells without the help of other types of cells. We cultured CD3$^-$CD56$^+$ NK cells obtained from the peripheral blood of healthy volunteers with various AGCs in the presence or absence of CD1d-expressing DCs and found no apparent augmentation of the cytotoxicity (S. Ishihara, manuscript in preparation). These results could be due to functional differences between CD3$^-$CD56$^+$ NK cells and/or APCs expressing CD1d molecules in the liver and the peripheral blood. It also raises the possibility that AGCs presented by CD1d activate T cells using TCRs other than Vα24 TCR and that the T cells activate CD3$^-$CD56$^+$ NK cells through direct cell-to-cell interaction or cytokines.

Activated APCs such as DCs and monocytes produce or induce a variety of cytokines that augment the cytotoxicity of NK cells. In murine systems, Kitamura et al. (9) showed that DCs and Vα14 NKT cells produce substantial amounts of IL-12 and IFN-γ, respectively, through the engagement of Vα14 TCR and α-GalCer-loaded CD1d molecules on DCs. Because IL-12 is known to activate NK cells (28), we determined the content of IL-12 in a culture of HLs with AGCs by ELISA. Regardless of the presence or absence of AGCs, the IL-12 level was consistently low, and no apparent relationship between the IL-12 level and the magnitude of the induced cytotoxicity was observed (S. Ishihara, manuscript in preparation). Some other cytokines such as IL-15 and IL-18, which have recently been shown to activate NK cells (29–31), released by AGC-activated APCs including DCs or other specific APCs such as Kupffer cells in the liver, may be involved in the activation of CD3$^-$CD56$^+$ NK cells in our study. Although further investigations are necessary to clarify the underlying mechanism, AGCs have clearly been shown to augment the cytotoxicity of CD3$^-$CD56$^+$ NK cells in the liver.

Hata et al. (32) examined the NK activity of human HLs in various liver diseases and showed that the NK activity of HLs obtained from HCC patients was markedly impaired in comparison with that of HLs obtained from normal livers. However, as shown in this study, AGCs enhanced the cytotoxicity of HLs in all the cases, including the case with HCC. Therefore, AGCs can be expected to restore and enhance the impaired local NK activity in livers affected by cancer. Because human hepatocytes and vascular endothelial cells are known to express the CD1d molecule (33), it is possible that they function as APCs in vivo. Thus, AGCs can be presented more effectively in vivo in the liver than in the in vitro culture in this study. In addition, preclinical studies using mice, rats, and monkeys demonstrated no treatment-related adverse effects after AGCs administration, even at relatively high doses of 2200 μg/kg, for 28 days (34). Therefore, the concentration of AGCs used in this study could be realized in vivo without adverse effects, particularly when administered topically (i.e., via hepatic artery or portal vein). Our observations strongly indicate the potential usefulness of AGCs in the immunotherapy of liver cancer in humans, based on their ability to activate CD3$^-$CD56$^+$ NK cells in the liver.

Acknowledgments

We thank Drs. Haruhiko Shida and Kanako Ban (Department of Surgery, Tokyo Koseinenkin Hospital, Tokyo, Japan) for providing us with the surgical specimens. We are also grateful to Ms. Miki Okai for technical assistance.

References

1. Brossay, L., O. Naidenko, N. Burdin, J. Matsuda, T. Sakai, and M. Kronenberg. 1998. Structural requirements for galactosylceramide recognition by CD1- restricted NK T cells. *J. Immunol. 161:5124.*
2. Brossay, L., M. Chioda, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J. Exp. Med. 188:1521.*
3. Burdin, N., L. Brossay, Y. Koezuka, S. T. Smiley, M. J. Grusby, M. Gui, M. Taniguchi, K. Hayakawa, and M. Kronenberg. 1998. Selective ability of mouse CD1 to present glycolipids: α-galactosylceramide specifically stimulates Vα14$^+$ NK T lymphocytes. *J. Immunol. 161:3271.*
4. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of vα14 NKT cells by glycosylceramides. *Science 278:1626.*
5. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, H. Sato, E. Kondo, M. Harada, H. Koseki, T. Nakayama, et al. 1998. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Vα14 NKT cells. *Proc. Natl. Acad. Sci. USA 95:5690.*
6. Kawano, T., Y. Tanaka, E. Shimizu, Y. Kaneko, N. Kamata, H. Sato, H. Osada, S. Sekiya, T. Nakayama, and M. Taniguchi. 1999. A novel recognition motif of human NKT antigen receptor for a glycolipid ligand. *Int. Immunol. 11:881.*
7. Nieda, M., A. Nicol, Y. Koezuka, A. Kikuchi, T. Takahashi, H. Nakamura, H. Furukawa, T. Yabe, Y. Ishikawa, K. Tadokoro, and T. Juji. 1999. Activation of human Vα24NKT cells by α-glycosylceramide in a CD1d-restricted and Vα24TCR-mediated manner. *Hum. Immunol. 60:10.*
8. Sakai, T., O. V. Naidenko, H. Iijima, M. Kronenberg, and Y. Koezuka. 1999. Syntheses of biotinylated α-galactosylceramides and their effects on the immune system and CD1 molecules. *J. Med. Chem. 42:1836.*

9. Kitamura, H., K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, et al. 1999. The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med. 189:1121.*
10. Porcelli, S. A., B. W. Segelke, M. Sugita, I. A. Wilson, and M. B. Brenner. 1998. The CD1 family of lipid antigen-presenting molecules. *Immunol. Today 19:362.*
11. Nakagawa, R., K. Motoki, H. Ueno, R. Iijima, H. Nakamura, E. Kobayashi, A. Shimosaka, and Y. Koezuka. 1998. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an α-galactosylceramide, KRN7000. *Cancer Res. 58:1202.*
12. Kobayashi, E., K. Motoki, T. Uchida, H. Fukushima, and Y. Koezuka. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol. Res. 7:529.*
13. Kobayashi, E., K. Motoki, Y. Yamaguchi, T. Uchida, H. Fukushima, and Y. Koezuka. 1996. Enhancing effects of α-,β-monoglycosylceramides on natural killer cell activity. *Bioorg. Med. Chem. 4:615.*
14. Kobayashi, E., K. Motoki, T. Natori, T. Uchida, H. Fukushima, and Y. Koezuka. 1996. Enhancing effects of agelasphin-11 on natural killer cell activities of normal and tumor-bearing mice. *Biol. Pharm. Bull. 19:350.*
15. Yamaguchi, Y., K. Motoki, H. Ueno, K. Maeda, E. Kobayashi, H. Inoue, H. Fukushima, and Y. Koezuka. 1996. Enhancing effects of (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000) on antigen- presenting function of antigen-presenting cells and antimetastatic activity of KRN7000-pretreated antigen-presenting cells. *Oncol. Res. 8:399.*
16. Nakagawa, R., K. Motoki, H. Nakamura, H. Ueno, R. Iijima, A. Yamauchi, S. Tsuyuki, T. Inamoto, and Y. Koezuka. 1998. Antitumor activity of α-galactosylceramide, KRN7000, in mice with EL-4 hepatic metastasis and its cytokine production. *Oncol. Res. 10:561.*
17. Motoki, K., K. Maeda, H. Ueno, E. Kobayashi, T. Uchida, H. Fukushima, and Y. Koezuka. 1996. Antitumor activities of combined treatment with a novel immunomodulator, (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-2-(*N*- hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000), and radiotherapy in tumor-bearing mice. *Oncol. Res. 8:155.*
18. Ohteki, T., and H. R. MacDonald. 1994. Major histocompatibility complex class I related molecules control the development of $CD4^+8^-$ and $CD4^-8^-$ subsets of natural killer 1.1^+ T cell receptor-α/β^+ cells in the liver of mice. *J. Exp. Med. 180:699.*
19. Bendelac, A. 1995. Mouse $NK1^+$ T cells. *Curr. Opin. Immunol. 7:367.*
20. Dellabona, P., E. Padovan, G. Casorati, M. Brockhaus, and A. Lanzavecchia. 1994. An invariant Vα24-JαQ/Vβ11 T cell receptor is expressed in all individuals by clonally expanded $CD4^-8^-$ T cells. *J. Exp. Med. 180:1171.*
21. Porcelli, S., D. Gerdes, A. M. Fertig, and S. P. Balk. 1996. Human T cells expressing an invariant Vα24-JαQ TCR α are $CD4^-$ and heterogeneous with respect to TCR β expression. *Hum. Immunol. 48:63.*
22. Exley, M., J. Garcia, S. P. Balk, and S. Porcelli. 1997. Requirements for CD1d recognition by human invariant V$\alpha24^+$ $CD4^-CD8^-$ T cells. *J. Exp. Med. 186: 109.*
23. Exley, M., S. Porcelli, M. Furman, J. Garcia, and S. Balk. 1998. CD161 (NKR-P1A) costimulation of CD1d-dependent activation of human T cells expressing invariant Vα24 JαQ T cell receptor α chains. *J. Exp. Med. 188:867.*
24. Ishihara, S., M. Nieda, J. Kitayama, T. Osada, T. Yabe, Y. Ishikawa, H. Nagawa, T. Muto, and T. Juji. 1999. $CD8^+NKR^-P1A^+$ T cells preferentially accumulate in human liver. *Eur. J. Immunol. 29:2414.*
25. Furukawa, H., T. Yabe, K. Watanabe, R. Miyamoto, A. Miki, T. Akaza, K. Tadokoro, S. Tohma, T. Inoue, K. Yamamoto, et al. 1999. Tolerance of NK and LAK activity for HLA class I-deficient targets in a TAP1-deficient patient (bare lymphocyte syndrome type I). *Hum. Immunol. 60:32.*
26. Spada, F. M., Y. Koezuka, and S. A. Porcelli. 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J. Exp. Med. 188:1529.*
27. Kawano, T., T. Nakayama, N. Kamada, Y. Kaneko, M. Harada, N. Ogura, Y. Akutsu, S. Motohashi, T. Iizasa, H. Endo, et al. 1999. Antitumor cytotoxicity mediated by ligand-activated human Vα24 NKT cells. *Cancer Res. 59:5102.*
28. Kodama, T., K. Takeda, O. Shimozato, Y. Hayakawa, M. Atsuta, K. Kobayashi, M. Ito, H. Yagita, and K. Okumura. 1999. Perforin-dependent NK cell cytotoxicity is sufficient for anti-metastatic effect of IL-12. *Eur. J. Immunol. 29:1390.*
29. Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J. Exp. Med. 180:1395.*
30. Hyodo, Y., K. Matsui, N. Hayashi, H. Tsutsui, S. Kashiwamura, H. Yamauchi, K. Hiroishi, K. Takeda, Y. Tagawa, Y. Iwakura, et al. 1999. IL-18 up-regulates perforin-mediated NK activity without increasing perforin messenger RNA expression by binding to constitutively expressed IL-18 receptor. *J. Immunol. 162: 1662.*
31. Tomura, M., X. Y. Zhou, S. Maruo, H. J. Ahn, T. Hamaoka, H. Okamura, K. Nakanishi, T. Tanimoto, M. Kurimoto, and H. Fujiwara. 1998. A critical role for IL-18 in the proliferation and activation of NK1.1+ $CD3^-$ cells. *J. Immunol. 160:4738.*
32. Hata, K., D. H. Van Thiel, R. B. Herberman, and T. L. Whiteside. 1991. Natural killer activity of human liver-derived lymphocytes in various liver diseases. *Hepatology 14:495.*
33. Canchis, P. W., A. K. Bhan, S. B. Landau, L. Yang, S. P. Balk, and R. S. Blumberg. 1993. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. *Immunology 80:561.*
34. Sakai, T., and Y. Koezuka. 1998. Ceramide derivatives as therapeutic agents. *Exp. Opin. Ther. Patents 8:1673.*