Expression of Interleukin-9 in Nasal Natural Killer/T-Cell Lymphoma Cell Lines and Patients

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Abstract

Purpose: Nasal natural killer (NK)/T-cell lymphoma is associated with EBV and has distinct clinical and histologic features. However, little is known about its genetic features. In this study, we examined the genes expressed by SNK-6 and SNT-8 cells, which were established from nasal NK/T-cell lymphomas, and found that interleukin (IL)-9 was specifically expressed in these two cell lines.

Experimental Design: cDNA array was used to examine the genes expressed by SNK-6 and SNT-8 cells. Expression of IL-9 and IL-9 receptor was investigated by reverse transcription-PCR, ELISA, and flow cytometry. Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Immunohistologic staining and ELISA were used to examine IL-9 expression in biopsies and sera from patients, respectively.

Results: In cDNA array, expression of IL-9 mRNA was much higher in SNK-6 and SNT-8 cells than in NK-92 cells from non-nasal NK-cell lymphoma and peripheral blood mononuclear cells from healthy volunteers. Furthermore, IL-9 was specifically expressed by SNK-6 and SNT-8 cells but not by other NK-cell, NK-like T-cell, and T-cell lymphoma/leukemia cell lines. IL-9 receptor was also expressed on the surfaces of SNK-6 and SNT-8 cells. An IL-9-neutralizing antibody inhibited the growth of these two cell lines, whereas recombinant human IL-9 enhanced their growth. Most significantly, IL-9 was present in biopsies and sera from patients with this lymphoma.

Conclusions: These results suggest that IL-9 plays an important role in nasal NK/T-cell lymphoma possibly via an autocrine mechanism.

Nasal natural killer (NK)/T-cell lymphoma, previously called lethal midline granuloma (1), has distinct clinical and histologic features and is characterized by a poor prognosis and progressive necrotic lesions with tumor and inflammatory cell infiltrations in the nasal cavity, nasopharynx, and palate (2–5). Histologically, the features of this lymphoma include angiocentric and polymorphous lymphoreticular infiltrates, which are called polymorphic reticulosis (6, 7). This disease is more common in Asian countries than in the United States and Europe (3, 5, 8–16).

Recent reports suggest that this lymphoma may be derived from NK or γδT-cell lineages, both of which express the NK-cell marker, CD56 (3, 14, 15, 17–19). With respect to etiologic factors, we first showed the presence of EBV DNA, EBV oncogenic proteins, and the clonotypic EBV genome in this lymphoma, suggesting that EBV plays a role in its genesis (3, 13, 20). Histologic and etiologic features of this lymphoma are identified gradually. However, because it is relatively rare and it is often difficult to obtain a sufficient amount of tissues from necrotic lesions, little is known about its genetic features. To solve this problem, Nagata et al. (17) established recently two EBV-infected lymphoma cell lines from primary tumor lesions, SNK-6 and SNT-8. SNK-6 cells have a NK-cell phenotype [$CD3^-CD4^-CD8^-CD19^-CD56^+$ T-cell receptor (TCR) $\alpha\beta^-TCR\gamma\delta^-$] and unrearranged TCR genes, whereas SNT-8 cells have a γδT-cell phenotype ($CD3^+CD4^-CD8^-CD19^-CD56^+TCR\alpha\beta^-TCR\gamma\delta^+$) and rearrangements of the TCR β, γ, and δ chain genes. Therefore, these two cell lines, which were obtained from nasal NK/T-cell lymphomas, should allow analysis of this tumor's gene expression profile.

To determine which genes are expressed specifically in nasal NK/T-cell lymphoma, we compared the gene expression patterns among SNK-6, SNT-8, and NK-92 cells. NK-92 cells were established from a patient with non-nasal NK-cell lymphoma (non-Hodgkin's lymphoma with large granular lymphocytes; ref. 21). We also used peripheral blood mononuclear cells (PBMC) from healthy volunteers as a control. cDNA array analysis revealed that interleukin (IL)-9 was

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Table 1. Cell lines used in this study

Cell lines	Disease diagnosis	EBV	Reference
NK-cell lines ($CD3^-CD56^+TCR^-$)			
SNK-6	Nasal NK/T-cell lymphoma	+	17
NK-92	Non-Hodgkin's lymphoma with large granular lymphocytes	–	21
KHYG-1	Aggressive NK-cell leukemia	–	27
NK-like T-cell lines ($CD3^+CD56^+TCR^+$)			
SNT-8	Nasal NK/T-cell lymphoma	+	17
MTA	NK-like T-cell leukemia/lymphoma	–	28
DERL-2	Hepatosplenic γδT-cell lymphoma	–	30
T-cell lines			
Jurkat	Acute lymphoblastic leukemia	–	25
MOLT-4	Acute lymphoblastic leukemia	–	26
PEER	Acute lymphoblastic leukemia	–	29
Positive control for expression of IL-9			
HDLM-2	Hodgkin's disease	–	31
Positive control for expression of IL-9R			
MT-2	Adult T-cell leukemia	–	32
Negative control for expression of IL-9R			
PC-3	Prostate cancer	Not examined	33

strongly expressed in SNK-6 and SNT-8 cells. Furthermore, IL-9 has been reported to be expressed in some malignant lymphomas, such as Hodgkin's disease and large-cell anaplastic lymphoma (22, 23), and to act as an autocrine growth factor for Hodgkin and Reed-Sternberg cells (24).

In the present study, we found that IL-9 was specifically expressed in nasal NK/T-cell lymphoma cell lines and that it plays an important role as an autocrine growth factor. Moreover, we detected IL-9 expression in biopsies and sera from patients with this lymphoma. These results suggest that IL-9 is a crucial factor in the pathogenesis of nasal NK/T-cell lymphoma.

Materials and Methods

Patients. Three groups of patients were analyzed in the current study: 12 healthy patients; 17 patients who were diagnosed with nasal NK/T-cell lymphoma at the Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical College (Hokkaido, Japan) or Sapporo Medical University (Hokkaido, Japan) between 1976 and 2003; and 20 patients who were diagnosed between 2000 and 2003 at the Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical College with non-nasal NK/T-cell malignant lymphomas, including Hodgkin's disease and follicular, diffuse large B-cell, peripheral T-cell, anaplastic large-cell, angioimmunoblastic T-cell, Burkitt's, and marginal zone B-cell lymphomas.

Cell culture. The EBV-positive nasal NK/T-cell lymphoma cell lines SNK-6 and SNT-8 were established previously by Nagata et al. (17). NK-92 (21), Jurkat (25), and MOLT-4 cells (26) were purchased from the American Type Culture Collection (Manassas, VA). KHYG-1 (27), MTA (28), and PEER cells (29) were purchased from the Health Science Research Resources Bank (Osaka, Japan). DERL-2 (30) and HDLM-2 cells (31) were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). MT-2 is a human T-cell lymphoma virus type I (HTLV-I) – immortalized T-cell line (32). The PC-3 prostate cancer cell line (33) was purchased from the American Type Culture Collection. The features of the cell lines used in this study are listed in Table 1. SNK-6 and SNT-8 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated human serum and 700 units/mL recombinant human IL-2. NK-92 cells were cultured in α-MEM supplemented with 12.5% horse serum, 12.5% fetal bovine serum (FBS), and 200 units/mL recombinant human IL-2. KHYG-1 cells were cultured in RPMI 1640 supplemented with 10% FBS and 100 units/mL recombinant human IL-2. DERL-2 cells were cultured in RPMI 1640 supplemented with 20% FBS and 200 units/mL recombinant human IL-2. HDLM-2 cells were cultured in RPMI 1640 supplemented with 20% FBS. MTA, Jurkat, MOLT-4, PEER, MT-2, and PC-3 cells were cultured in RPMI 1640 supplemented with 10% FBS. All cell lines were incubated at 37°C in an atmosphere containing 5% CO_2. PBMCs from healthy volunteers were isolated by centrifugation using Ficoll-Hypaque (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

cDNA array analysis. Total RNA was extracted from SNK-6, SNT-8, and NK-92 cells and from PBMCs of healthy volunteers. ^{32}P-labeled cDNA probes were synthesized using the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA). Total RNA from the PBMCs of two healthy volunteers was mixed and used as a pooled sample. These probes were hybridized to the ATLAS Human Cancer 1.2 Array (Clontech) according to the manufacturer's protocols. The arrays were then exposed to a phosphorimaging screen at room temperature for 30 minutes and scanned using a BAS2000 phosphorimager (Fuji Photo Film, Tokyo, Japan). A grid was applied to the images of the hybridization spots, and the spot intensities were quantified using BAStation version 1.31 (Fuji Photo Film). Background signals were defined as the average of the hybridization signals produced by negative controls on the array. All hybridization signals were normalized by the mean of the internal control signals.

Reverse transcription-PCR analysis. Total RNA was extracted from all cell lines and from PBMCs of healthy volunteers using the SV Total RNA Isolation System (Promega, Madison, WI). The RNA was reverse transcribed for 60 minutes at 37°C using Moloney murine leukemia virus reverse transcriptase (GeneHunter, Nashville, TN) with oligo(dT) primers (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. The following primers were used for IL-9 (sense 5′-ATGCTTCTGGCCATGGTCCT-3′ and antisense 5′-TATCTTGCCTCTCATCCCTC-3′), IL-9 receptor α (IL-9Rα; sense 5′-ATGTGGTAGAGGAGGAGCGT-3′ and antisense 5′-TGAACAGGAGGTAGGTCGG-3′),

IL-2 receptor γ (IL-2Rγ; sense 5′-CCTCACTCTGCATTATTGGTACAAG-3′ and antisense 5′-TTCAGTAACAAGATCCTCTAGGT-3′), and β_2-microglobulin (sense 5′-TGTCTTTCAGCAAGGACTGG-3′ and antisense 5′-CCAGATTAACCACAACCATG-3′; Sigma Genosis Japan, Hokkaido, Japan). Hotstart PCR was done in a 10-μL reaction mixture containing 4.95 μL H_2O, 1 μL of 10× PCR buffer (containing 15 mmol/L $MgCl_2$), 1 μL of 2 mmol/L deoxynucleotide triphosphate mixture, 1 μL of 5 μmol/L sense primer, 1 μL of 5 μmol/L antisense primer, 0.05 μL of 5 units/mL AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1 μL of 100 ng/μL cDNA. The reaction was carried out as follows: initial denaturation at 94°C for 10 minutes followed by 30 (β_2-microglobulin), 35 (IL-9 and IL-2Rγ), or 40 (IL-9Rα) cycles of 1 minute at 94°C, 1 minute at 55°C (IL-9, IL-2Rγ, and IL-9Rα) or 57°C (β_2-microglobulin) and 1 minute at 72°C and a final elongation step of 5 minutes at 72°C. The PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

ELISA for interleukin-9. IL-9 protein in cell culture supernatants and sera was quantified using ELISA. Cell lines (5×10^5/mL) were cultured in 96-well round-bottomed plates, and supernatants of cell cultures were collected after 24, 48, and 72 hours. All sera were taken at diagnosis and frozen at −80°C. The goat anti-human IL-9 polyclonal antibody (R&D Systems, Minneapolis, MN) was coated directly onto 96-well ELISA plates (Nalge Nunc International, Tokyo, Japan) at a concentration of 2.0 μg/mL in coating buffer [100 mmol/L Na_2CO_3/$NaHCO_3$ (pH 9.5)] and incubated overnight at 4°C. The plates were blocked with blocking buffer (PBS containing 1% bovine serum albumin) for 1 hour at room temperature to eliminate nonspecific binding by the primary antibody. Next, the plates were washed with PBS-Tween (PBS containing 0.05% Tween 20). A standard curve was generated by serial dilution of recombinant human IL-9 (rhIL-9; R&D Systems). Cell culture supernatants or sera (100 μL) were incubated in the 96-well plates at room temperature for 2 hours. After incubation, the plates were washed with PBS-Tween. Biotinylated rabbit anti-human IL-9 polyclonal antibody (PeproTech, London, United Kingdom) was added to each well at a concentration of 1.2 μg/mL in PBS containing 1% bovine serum albumin and incubated at room temperature for 2 hours. The plates were then washed again, and 1:200 streptavidin-labeled horseradish peroxidase (R&D Systems) was added for 20 minutes. After washing again, 100-μL substrate solution (BD PharMingen, Bedford, MA) was added, and the plates were incubated for 20 minutes in the dark. The reaction was terminated by adding 50-μL stop solution (2 N H_2SO_4). The absorbance of each well was determined at 450 nm using a microplate reader. The lower limit of detection for the ELISA was 300 pg/mL. For cell culture supernatants, measurements were done in triplicate, and for sera, measurements were done in duplicate. The results correspond to mean ± SD.

Flow cytometric analysis. For flow cytometric analysis of surface molecules, cell lines were washed in cold PBS, centrifuged, and resuspended in an appropriate volume of fluorescence-activated cell sorting staining buffer (PBS containing 0.1% NaN_3 and 2% FBS). Cells were incubated with antibodies for 60 minutes in the dark at 4°C, and excess antibodies were removed by washing the cells twice in cold staining buffer. Detection of surface IL-9Rα was carried out using unconjugated mouse IgG1 (10 μg/mL; isotype control; R&D Systems) or unconjugated mouse anti-human IL-9Rα IgG1 (10 μg/mL; R&D Systems) followed by phycoerythrin-conjugated goat anti-mouse immunoglobulin antibody (1:100; DAKO, Glostrup, Denmark). Detection of surface IL-2Rγ was carried out using phycoerythrin-conjugated rat IgG2b-κ (10 μg/mL; isotype control; BD PharMingen) and phycoerythrin-conjugated rat anti-human IL-2Rγ IgG2b-κ (10 μg/mL; BD PharMingen). Fluorescence-activated cell sorting scanning and data analysis were carried out using the Becton Dickinson FACScan and accompanying CellQuest software (San Jose, CA) according to the manufacturer's protocols.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. SNK-6 and SNT-8 cells were cultured for 24 hours in RPMI 1640 with 0.1% FBS to wash out recombinant human IL-2 that was present in the culture medium. After washing twice, the cell lines (3×10^4/well) were cultured in 96-well round-bottomed plates for 72 or 96 hours in 100-μL RPMI 1640 containing 0.1% FBS and rhIL-9 (1, 10, 100, or 1,000 ng/mL; R&D Systems), rabbit anti-human IL-9 polyclonal antibody (0.01, 0.1, 1, or 10 μg/mL; PeproTech), or rabbit polyclonal antibody (0.01, 0.1, 1, or 10 μg/mL; isotype control; PeproTech). The number of viable cells was determined using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (20 μL) was added to each well, and after incubating the plates for 4 hours at 37°C in a 5% CO_2 atmosphere, the absorbance at 490 nm was measured using an ELISA plate reader. Results were expressed as the percentage of untreated controls. Measurements were done in duplicate, and experiments were repeated at least twice. The results correspond to mean ± SD.

Immunohistologic staining and* in situ *hybridization. Double staining for IL-9 and CD56 was done as follows. Formalin-fixed, paraffin-embedded specimens were obtained from pretreatment biopsy samples. The specimens were cut in 4-μm sections. The sections were deparaffinized in xylene and ethanol and then placed in 10 mmol/L citric acid buffer (pH 6.0). Antigen retrieval was carried out by microwave irradiation for 15 minutes at 750 W. The sections were then incubated with 3% hydrogen peroxide for 30 minutes overnight at 4°C with 1:400 rabbit anti-human IL-9 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 30 minutes at room temperature with EnVision+ peroxidase-labeled dextran polymer (DAKO). Immunoreactive IL-9 was visualized by immersing the slides in freshly prepared diaminobenzidine tetrahydrochloride substrate solution (DAKO) for 10 minutes. After washing out the substrate solution, the sections were incubated overnight at 4°C with 1:50 mouse anti-human CD56 monoclonal antibody (Novocastra, Newcastle, United Kingdom) followed by a 30-minute incubation at room temperature in EnVision/alkaline phosphatase–labeled dextran polymer (DAKO). Immunoreactive CD56 was visualized by immersing the slides in freshly prepared Fast Red substrate solution (DAKO) for 10 minutes. Finally, the sections were counterstained with Lillie-Mayer's hematoxylin and mounted on glass slides. A case was considered IL-9 positive if >30% of the CD56-positive cells were also IL-9 positive.

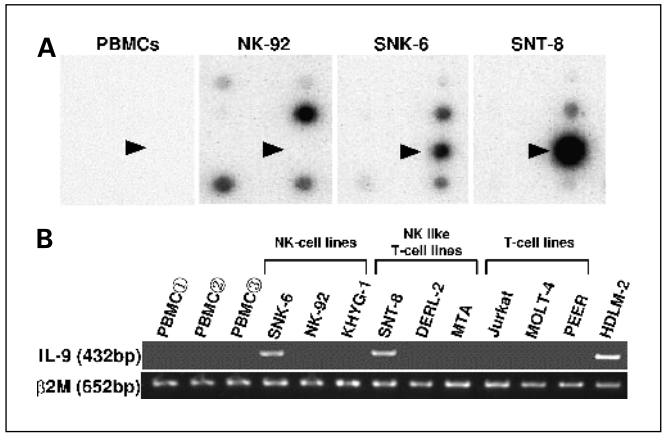

Fig. 1. Expression of IL-9 in the nasal NK/T-cell lymphoma cell lines, SNK-6 and SNT-8. *A,* cDNA array analysis of PBMCs from healthy volunteers and from SNK-6, SNT-8, and NK-92 cells. Expression of IL-9 (*arrowheads*) in SNK-6 cells was 3.88- and 3.57-fold higher than in PBMCs and NK-92 cells, respectively. Expression of IL-9 in SNT-8 cells was 19.3- and 29.3-fold higher than in PBMCs and NK-92 cells, respectively. *B,* RT-PCR analysis in PBMC from healthy volunteers and in NK-cell, NK-likeT-cell, orT-cell lymphoma/leukemia cell lines. Total RNA was reverse transcribed and analyzed by PCR for the expression of IL-9. SNK-6 and SNT-8 cells were positive for IL-9 mRNA, but PBMCs and other NK-cell, NK-likeT-cell, and T-cell lymphoma/leukemia cell lines were negative. As an internal control, β_2-microglobulin (*β2M*) cDNA was coamplified in each sample. In addition, HDLM-2 cells were used as a positive control for the expression of IL-9.

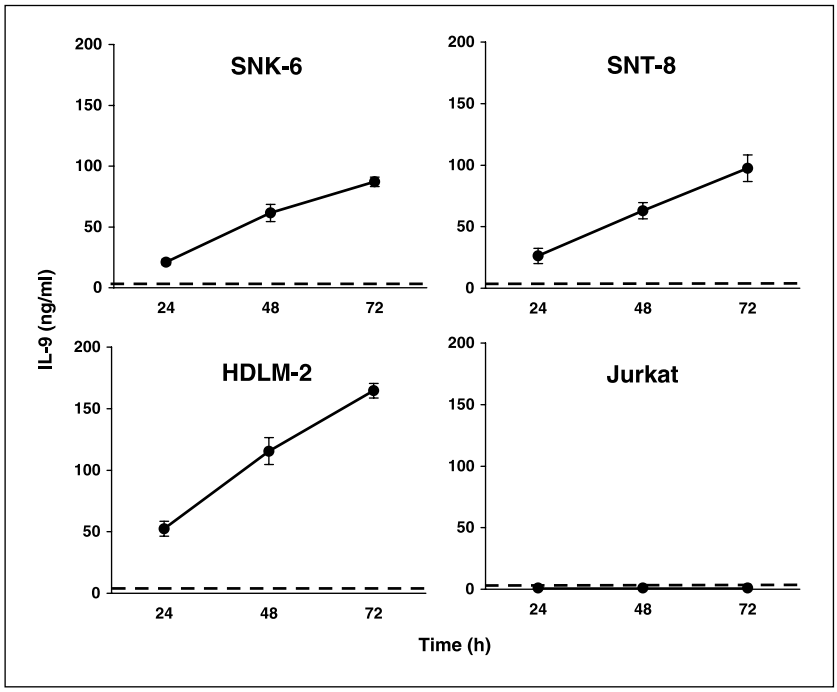

Fig. 2. Production of IL-9 by the nasal NK/T-cell lymphoma cell lines, SNK-6 and SNT-8. Supernatants from cell cultures (5×10^5/mL) in 96-well round-bottomed plates were collected after 24, 48, and 72 hours, and IL-9 production was assessed using ELISA. The amount of IL-9 produced by SNK-6 and SNT-8 cells increased in a time-dependent manner. NK-92, KHYG-1, DEAL-2, MTA, Jurkat, MOLT-4, and PEER cells did not produce detectable levels of IL-9. Representative results for Jurkat cells. HDLM-2 cells were used as a positive control for IL-9 production. Dotted lines, detection limit. Points, mean of triplicate determinations; bars, SD.

In situ hybridization for EBV-encoded small RNA (EBER) was described elsewhere (3). Briefly, EBER expression in tumor cells was assessed by *in situ* hybridization on formalin-fixed, paraffin-embedded tissue sections using a fluorescein-conjugated peptide nucleic acid probe for EBERs (DAKO) and a peptide nucleic acid *in situ* hybridization detection kit (DAKO) according to the manufacturer's instructions. Fuchsin (DAKO) was used as detection substrate.

Results

Nasal natural killer/T-cell lymphoma cell lines express and produce interleukin-9. To investigate the genes expressed in nasal NK/T-cell lymphoma, we used a cDNA microarray to compare the gene expression patterns in SNK-6 and SNT-8 cells (established from nasal NK/T-cell lymphoma patients; ref. 17), NK-92 cells (established from a patient with non-Hodgkin's lymphoma with large granular lymphocytes, a non-nasal NK-cell lymphoma; ref. 21), and PBMCs from healthy volunteers. IL-9 was expressed at much higher levels in SNK-6 and SNT-8 cells than NK-92 cells and PBMCs (Fig. 1A). Compared with PBMCs, expression was 3.88-fold higher in SNK-6 cells and 19.3-fold higher in SNT-8 cells; compared with NK-92 cells, expression was 3.57-fold higher in SNK-6 cells and 29.3-fold higher in SNT-8 cells.

To confirm that SNK-6 and SNT-8 cells specifically express IL-9 mRNA, we did reverse transcription-PCR (RT-PCR) analysis in PBMCs from three healthy volunteers, two NK-cell lymphoma/leukemia cell lines (NK-92 and KHYG-1; $CD3^-CD56^+TCR^-$), two NK-like T-cell lymphoma/leukemia cell lines (DERL-2 and MTA; $CD3^+CD56^+TCR^+$), and three T-cell leukemia cell lines (Jurkat, MOLT-4, and PEER; Table 1). We used all of the commercially available NK and NK-like T-cell lines and randomly selected three T-cell lines representing both TCRαβ and TCRγδ T-cell lines. Expression of IL-9 mRNA was detected in SNK-6 and SNT-8 cells. Except for the Hodgkin's lymphoma cell line, HDLM-2, which is known to express IL-9 (23, 24), IL-9 mRNA was not expressed in the non-nasal NK-cell, NK-like T-cell, or T-cell lymphoma/leukemia cells or in PBMCs from healthy volunteers (Fig. 1B). These results indicated that the nasal NK/T-cell lymphoma cell lines, SNK-6 and SNT-8, specifically expressed IL-9 mRNA.

We further examined the production and release of IL-9 protein into the cell culture supernatants. Expression was assessed after 24, 48, and 72 hours in culture by ELISA. As shown in Fig. 2, time-dependent accumulation of IL-9 was found in the cell culture supernatant from SNK-6 and SNT-8 cells. As reported previously (23, 24), IL-9 also accumulated in the cell culture supernatant from the Hodgkin's lymphoma cell line, HDLM-2. In contrast, even after 72 hours in culture, we did not detect IL-9 in the culture supernatants of non-nasal NK-cell, NK-like T-cell, or T-cell lymphoma/leukemia cell lines. These results are consistent with the RT-PCR findings shown in Fig. 1B and indicate that SNK-6 and SNT-8 cells expressed sufficient IL-9 mRNA to produce and release this cytokine.

Nasal natural killer/T-cell lymphoma cell lines express interleukin-9 receptor. We next investigated whether IL-9R is expressed in nasal NK/T-cell lymphoma cell lines. We first did RT-PCR analysis of IL-9Rα and IL-2Rγ mRNAs because they comprise IL-9R (34). We used the HTLV-I-immortalized T-cell line, MT-2, as a positive control for IL-9R because it is known to respond to IL-9 (35). We found that SNK-6, SNT-8, and MT-2 cells express both IL-9Rα and IL-2Rγ mRNAs (Fig. 3A). In contrast, PC-3 cells, an epithelial cell line from prostate adenocarcinoma cancer, expressed neither of the mRNAs. Flow cytometric analysis confirmed that IL-9Rα and IL-2Rγ are expressed on the surfaces of SNK-6, SNT-8, and MT-2 cells but not PC-3 cells (Fig. 3B).

Interleukin-9 is an autocrine growth factor for nasal natural killer/T-cell lymphoma cell lines. Our results showed that nasal NK/T-cell lymphoma cell lines express both IL-9 and IL-9R, suggesting that IL-9 can act as an autocrine growth factor for these cells. To determine the validity of this hypothesis, we examined the effect of IL-9-neutralizing antibodies on cell

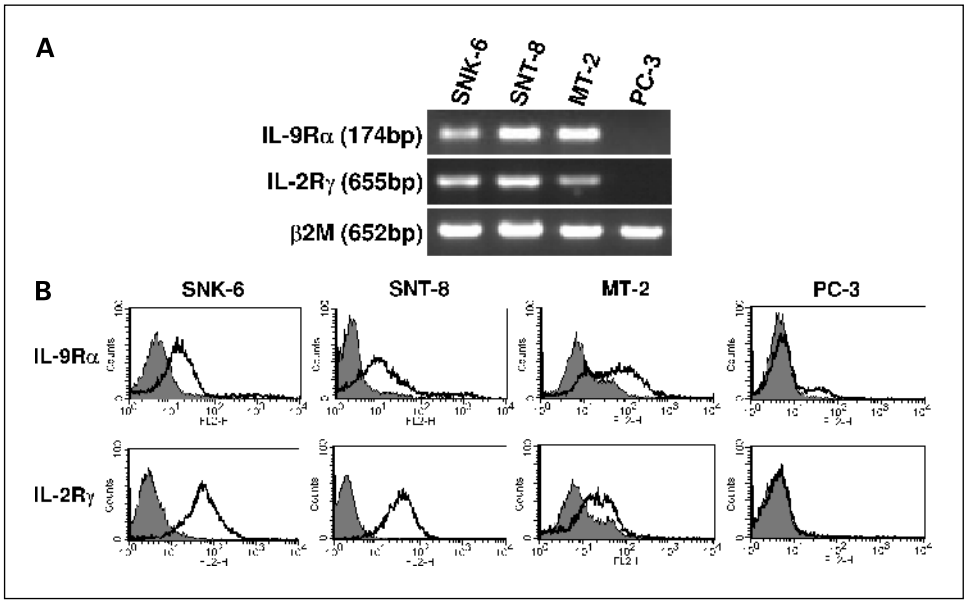

Fig. 3. Expression of IL-9R in the nasal NK/T-cell lymphoma cell lines, SNK-6 and SNT-8. *A,* RT-PCR analysis. Total RNA from SNK-6 and SNT-8 cells was reverse transcribed and analyzed by PCR for expression of IL-9Rα and IL-2Rγ. Both cell lines were positive for IL-9Rα and IL-2Rγ. As an internal control, β_2-microglobulin cDNA was coamplified in each sample. *B,* flow cytometric analysis of SNK-6 and SNT-8 cells for surface expression of IL-9R components. Cells were stained with an anti-IL-9Rα antibody followed by a phycoerythrin-conjugated secondary antibody or a phycoerythrin-conjugated anti-IL-2Rγ antibody (*thick lines*). Filled histograms, cells stained with isotype control antibodies. MT-2 and PC-3 cells were used as positive and negative controls, respectively, for the expression of IL-9R.

growth. SNK-6 and SNT-8 cells were cultured for 72 hours with medium alone, medium containing an IL-9-neutralizing antibody (0.01, 0.1, 1, or 10 μg/mL), or medium containing an isotype control antibody (0.01, 0.1, 1, and 10 μg/mL). Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. As shown in Fig. 4A, the IL-9-neutralizing antibody caused a dose-dependent reduction in the numbers of viable SNK-6 and SNT-8 cells. A significant change in viability was not observed in the cells cultured with the isotype control antibody. Furthermore, the growth of both cell lines was dose-dependently enhanced by a 96-hour incubation with rhIL-9 (1, 10, 100, and 1,000 ng/mL; Fig. 4B). These results indicated that IL-9 acts as an autocrine growth factor for SNK-6 and SNT-8 cells.

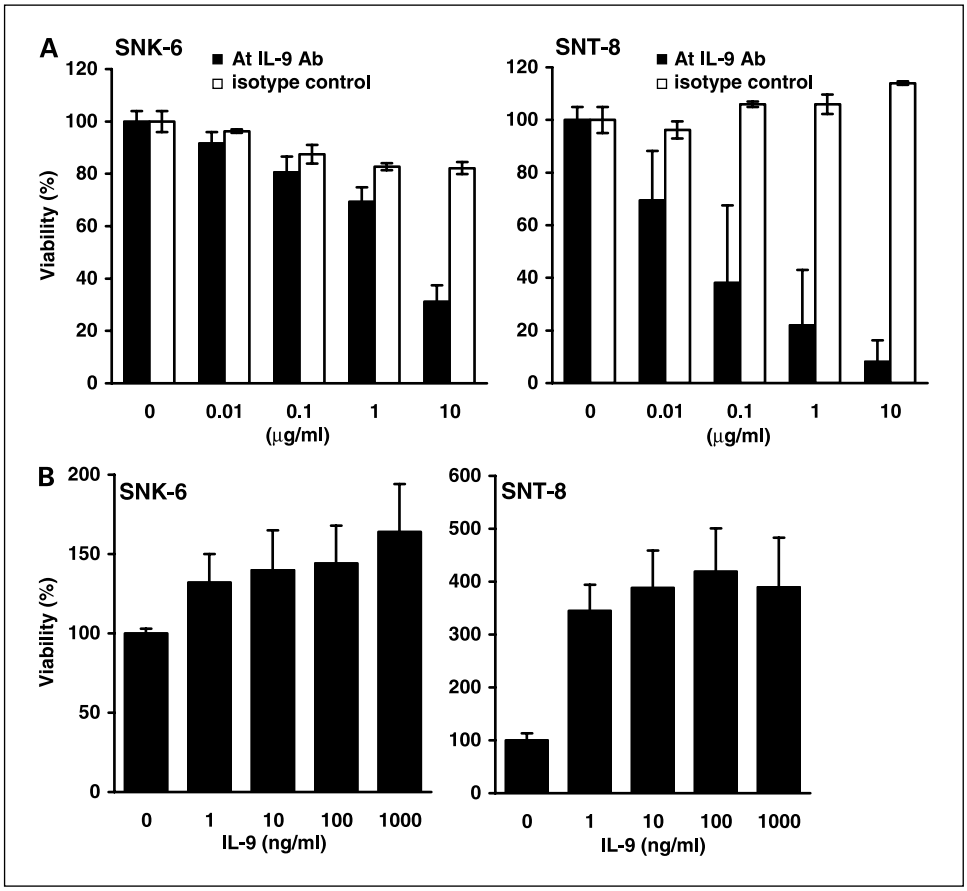

Fig. 4. Effect of anti-IL-9-neutralizing antibody and rhIL-9 on the viability of the nasal NK/T-cell lymphoma cell lines, SNK-6 and SNT-8. *A,* SNK-6 and SNT-8 cells (3×10^4/well) were cultured in 96-well round-bottomed plates for 72 hours in 100-μL RPMI 1640 containing 0.1% FBS and in the presence of various concentrations of anti-IL-9-neutralizing antibody (*black columns*) or isotype control antibody (*open columns*). *B,* SNK-6 and SNT-8 cells (3×10^4/well) were cultured in 96-well round-bottomed plates for 96 hours in 100-μL RPMI 1640 with 0.1% FBS in the presence of various doses of rhIL-9. The number of viable cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Percentage of untreated controls. All assessments were carried out in duplicate in at least two independent experiments. Columns, mean; bars, SD.

Interleukin-9 is expressed in tissues and sera from patients with nasal natural killer/T-cell lymphoma. Measuring levels of tumor makers, such as cytokines, allows evaluation of the progression of malignancies in a clinical setting. Thus, we did immunohistologic staining to analyze the expression of IL-9 protein in formalin-fixed, paraffin-embedded tissue sections from patients with nasal NK/T-cell lymphoma. Of 17 patients, it was possible to test 12. All biopsies tested showed that atypical lymphoid cells infiltrated the nasal mucosa (Fig. 5A). In addition, most neoplastic cells were positive for EBER (Fig. 5B). Because CD56 was expressed in all tested lymphoma patients, double staining for IL-9 and CD56 was done to confirm that tumor cells expressed IL-9. As shown in Fig. 5C, 8 of 12 patients were positive for IL-9.

Next, we measured the levels of IL-9 in sera from the patients with nasal NK/T-cell lymphoma, patients with non-nasal NK/T-cell malignant lymphoma, and healthy volunteers (Fig. 6). Sera from all patients were taken at diagnosis and analyzed with ELISA. Of 17 patients with nasal NK/T-cell lymphoma, it was possible to test 10. IL-9 was elevated in 4 of 10 sera from the patients with nasal NK/T-cell lymphoma. Of all patients with elevated serum level of IL-9, it was found that all biopsies that we could investigate were positive for IL-9 using immunohistologic staining. On the other hand, IL-9 was only detected in only 1 of 20 sera from the patients with non-nasal NK/T-cell malignant lymphoma. Twelve serum samples from healthy volunteers contained undetectable levels of IL-9.

Discussion

Nasal NK/T-cell lymphoma is a relatively rare disease and is characterized by progressive necrotic lesions that coexist with tumor, inflammatory, and normal tissue cells (2–5). Therefore, it is often difficult to obtain a sufficient amount of tissue from the necrotic lesions. Moreover, even if a sufficient amount of tissue is obtained, it is difficult to analyze the gene expression profile for an individual cell type because the biopsy samples contain a mixture of cell types. For these reasons, little is known about the genetic features in this lymphoma. Because the evaluation of gene expression is important for developing new diagnostics and therapeutic strategies, it is useful to examine tumor cells grown *in vitro*.

Recently, Nagata et al. (17) established two EBV-positive cell lines from primary nasal NK/T-cell lymphoma lesions, SNK-6 and SNT-8, which represent NK and γδT-cell lineages, respectively. Many previous studies using immunophenotypic and genotypic analysis have suggested that nasal NK/T-cell lymphoma may be derived from both NK and T-cell lineages (3, 14, 15, 17–19). NK-cell lymphomas are $CD56^{+}CD3^{-}$ do not show rearrangement of the TCR genes (15, 17, 18). In T-cell lymphomas, it is thought that γδT-cell is the predominant phenotype and that αβT-cell is a minor phenotype (3, 14, 17, 19). Moreover, like peripheral γδT-cell lymphomas (36–38), most nasal γδT-cell lymphomas express CD56. Because the phenotypes of SNK-6 and SNT-8 cells correspond with those reported for nasal NK/T-cell lymphoma, it is useful to compare their gene expression profiles with those of other lymphoma/leukemia cell lines to identify genes that are expressed specifically in nasal NK/T-cell lymphomas.

IL-9 is a multifunctional cytokine produced mainly by antigen-activated Th2 lymphocytes (34). IL-9 was initially described as a T-cell growth factor (39), but in humans, IL-9 is also thought to affect the differentiation of hematopoietic progenitors and B-cells (34) and to promote the proliferation of not only T-cell lines but also mast cell progenitors (40). Furthermore, IL-9 is thought to have a functional role in some malignant lymphomas, such as Hodgkin's disease and large-cell

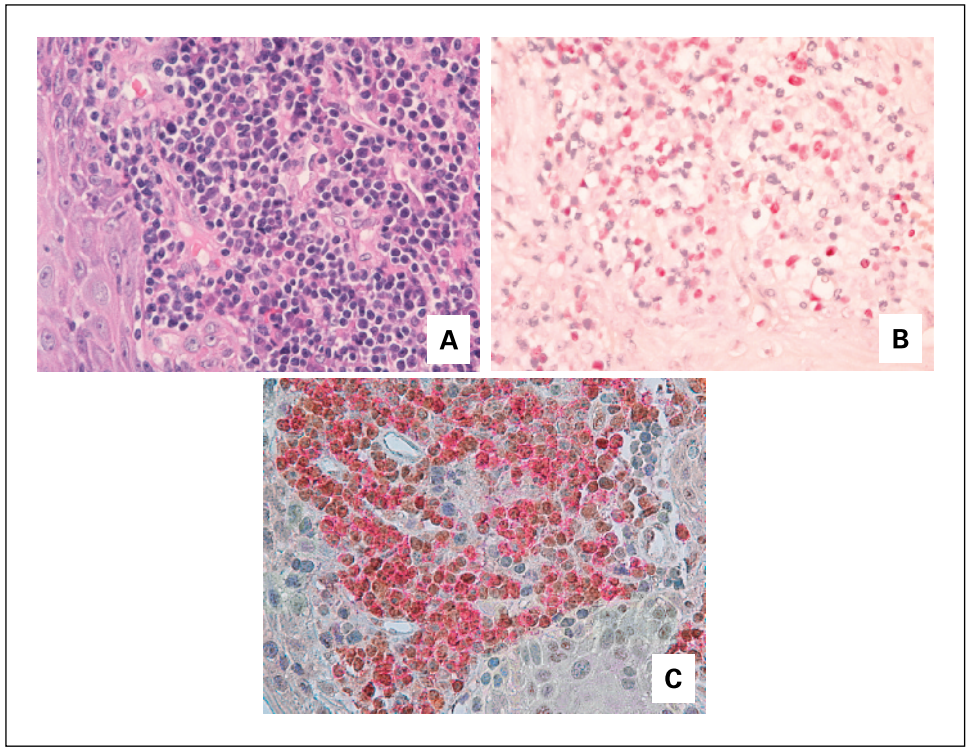

Fig. 5. Representative immunohistologic features and expression of IL-9 in a biopsy from a patient with nasal NK/T-cell lymphoma. *A,* the nasal mucosa is infiltrated by atypical lymphoid cells (H&E staining). *B, in situ* hybridization for EBER. Nuclei of EBER-positive cells are stained red. *C,* double staining for IL-9 and CD56. IL-9-positive cells are stained brown, and CD56-positive cells are stained red. Original magnification, ×400.

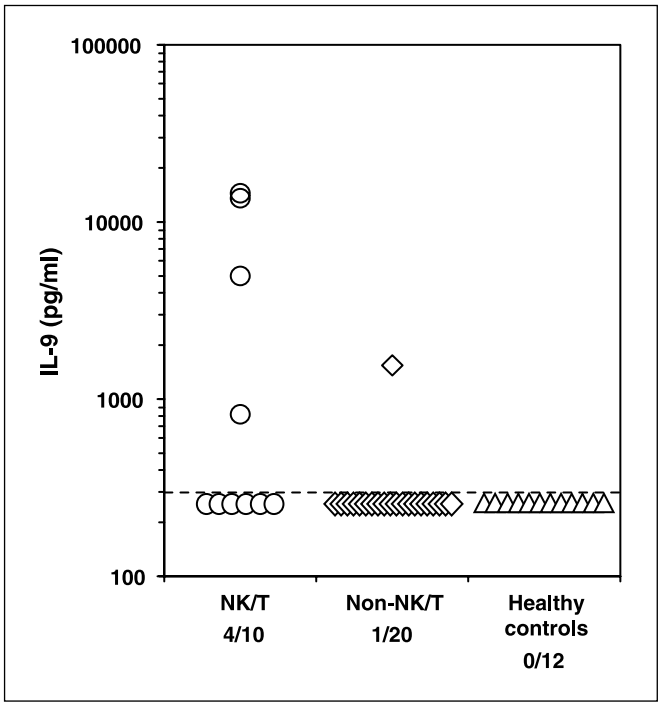

Fig. 6. Levels of IL-9 in sera. Sera from 10 patients with nasal NK/T-cell lymphoma (*NK/T*) and 20 patients with non-nasal NK/T-cell malignant lymphoma (*Non-NK/T*) were taken at diagnosis. The numbers of positive sera and the totals for each group are indicated. Dotted line, detection limit.

anaplastic lymphoma (22, 23), because it promotes oncogenesis (41) and acts as an autocrine growth factor for Hodgkin and Reed-Sternberg cells (24). However, the functional role of IL-9 in lymphomas derived from NK or NK-like T-cell lineages has not been reported previously.

In the present study, analysis of gene expression using cDNA array revealed that IL-9 mRNA is expressed at a much higher level in SNK-6 and SNT-8 cells than in PBMCs from healthy volunteers or NK-92 non-nasal NK-cell lymphoma cells. We further found specific expression of IL-9 mRNA and protein in SNK-6 and SNT-8 cells but not PBMCs from healthy volunteers or non-nasal NK-cell, NK-like T-cell, or T-cell lymphoma/leukemia cell lines. We also found that IL-9R was expressed on the cell surface of SNK-6 and SNT-8 cells.

The coexpression of IL-9 and IL-9R suggests that IL-9 might act as an autocrine growth factor for SNK-6 and SNT-8 cells. Therefore, we examined the effects of an IL-9-neutralizing antibody and exogenous rhIL-9 on cell growth. The antibody dose-dependently decreased the numbers of viable SNK-6 and SNT-8 cells, whereas rhIL-9 dose-dependently increased the cell numbers. These findings clearly show that IL-9 acts as an autocrine growth factor for SNK-6 and SNT-8 cells.

Recently, Yang et al. (35) used real-time quantitative RT-PCR to show that three of four EBV-positive nasal T-cell lymphoma biopsies expressed IL-9 mRNA. However, this method did not definitively prove that the tumor cells express IL-9 because the biopsies also contain inflammatory cells. Therefore, we examined coexpression of CD56 and IL-9 in nasal NK/T-cell lymphoma biopsies by immunohistologic staining. We found that tumor cells in these samples derived from $CD56^+$NK or γδT-cell lineages also express IL-9. Furthermore, we showed that IL-9 was present in sera from 4 of 10 NK/T-cell lymphoma patients. Recently, Fischer et al. (23) reported that high levels of IL-9 were present in 18 of 44 sera from patients with Hodgkin's disease but not in samples from healthy volunteers. These findings support the idea that IL-9 has an important role in tumor cell growth in patients with either nasal NK/T-cell lymphoma or Hodgkin's disease.

We found here that IL-9 was expressed and produced only in SNK-6 and SNT-8 cells, which are EBV positive, but that it is not expressed by EBV-negative NK and NK-like T-cell lines, including NK-92, KHYG-1, DERL-2, and MTA cells. These findings suggest that EBV may be associated with IL-9 expression and production in nasal NK/T-cell lymphomas. Concerning the induction of IL-9 by virus, the transcription of the IL-9 gene in HTLV-I-immortalized T-cells is induced by the HTLV-I Tax protein via activation of the nuclear factor-κB pathway (42–44). Recently, Yang et al. (35) succeeded in generating recombinant EBV-infected HTLV-I-expressing MT-2 cells. They showed that the expression of IL-9 was much higher in EBV-positive than EBV-negative MT-2 cells. They also reported that EBV-positive MT-2 cells grow faster due to increased production of IL-9 and that the elevated expression of IL-9 in these cells was induced by EBER through the pathway unassociated with nuclear factor-κB activated by HTLV-I Tax protein. Therefore, as in EBV-infected MT-2 cells, it is possible that EBV genes, especially EBER, induce IL-9 expression and production in nasal NK/T-cell lymphomas. Additional studies are needed to determine the role of IL-9 in EBV infection.

In conclusion, our results show that IL-9 is specifically expressed by nasal NK/T-cell lymphoma cell lines, acts as an autocrine growth factor for these cell lines, and is present in tissues and sera from patients with nasal NK/T-cell lymphoma. In addition, an IL-9-neutralizing antibody inhibited cell growth in nasal NK/T-cell lymphoma cell lines, suggesting that it acts as an autocrine growth factor for these tumors. These findings indicate that the IL-9 signaling pathway may be a new target for the treatment of nasal NK/T-cell lymphoma.

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