Human Tumor Antigen-specific T Lymphocytes and Interleukin-2-activated Natural Killer Cells: Comparisons of Antitumor Effects in Vitro and in Vivo

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ABSTRACT

Human antitumor effector cells include class I major histocompatibility complex (MHC)-restricted T cells and non-MHC-restricted natural killer (NK) cells. These two types of effector cells have not been directly compared for the ability to eliminate tumor cell targets. Here, we compare in vitro and in vivo antitumor functions of two human T-cell lines specific for a shared tumor antigen to the antitumor functions of A-NK cells, a subset of IL-2-activated NK cells. Human squamous cell carcinoma of the head and neck cell lines cultured in suspensions or as spheroids or tumor xenografts established in nude mice were used to evaluate antitumor functions of IL-2-activated and expanded T and NK effector cells in various assays, both in vitro and in vivo. Both tumor cell targets, PCI-13 and OSC-19, expressed class I and II MHC antigens after IFN-γ pretreatment, gave rise to tumors upon injection into immunosuppressed nude mice, and were resistant to lysis by resting NK cells but sensitive to lysis mediated by A-NK cells or HLA-A2-restricted T-cell lines specific for a shared squamous cell carcinoma of the head and neck antigen. No significant differences were observed in the ability of A-NK cells or tumor-specific T cells to bind to tumor cell monolayers or to enter into spheroids. However, A-NK cells mediated significantly higher killing than tumor-specific CD8+ T cells in 4-h 51Cr-release assays (a measure of cell membrane damage and necrosis), 1-h [3H]thymidine-release assays (a measure of DNA fragmentation and apoptosis), and in terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assays (a measure of apoptosis). In contrast, CD8+ T cells were consistently more effective than A-NK cells in inducing growth inhibition of tumor cells in 24-h MTT assays. In the presence of tumor-specific antibodies, A-NK cell binding, entry into spheroids, and infiltration into tumor in vivo were significantly increased. In vivo perilesional delivery of effector cells to mice with established tumors indicated that human A-NK cells exert antitumor effects as potent as those of tumor-specific T cells. However, in contrast to tumor-specific T cells, A-NK cells are readily available for cancer therapy, expand rapidly in culture without prior sensitization, and can be armed with antitumor antibodies to increase localization of effector cells to the tumor.

INTRODUCTION

Human peripheral blood NK3 cells are able to eliminate certain NK-sensitive tumor cell targets but are largely ineffective in lysing most fresh or cultured human tumor cells, which are considered to be NK resistant (1, 2). In contrast to resting NK cells found in the peripheral blood of healthy individuals, those NK cells that are exposed to IL-2 in vitro or in vivo rapidly acquire the capability to kill a broad spectrum of cellular targets, including fresh tumor cells and tumor cell lines (1, 2). The ability of NK cells to generate lymphokine-activated killer cell activity is directly related to constitutive expression of IL-2Rβ on the surface of a considerable proportion of these cells (3). Thus, in the presence of IL-2, NK cells rapidly up-regulate expression of IL-2Rα and acquire phenotypic and functional characteristics of activated lymphocytes (4). This ability of NK cells to rapidly respond to IL-2 distinguishes them from other lymphoid cells, and it may be biologically significant for their role in innate immunity, and especially for effective response against infectious agents and metastasizing cells (4).

We have observed that in the presence of 22 nm IL-2, a small subset of human NK cells selectively acquires a property of adherence to plastic surfaces (5). The process of IL-2-induced adherence is dependent on rapid up-regulation of expression and function of cellular adhesion molecules. It can be exploited for...
separation from peripheral blood or lymphoid tissues of a subset of
NK cells named A-NK cells (5). These cells do not remain
adherent to plastic but detach during culture and proliferate
vigorously in the presence of IL-2. A-NK cells have been
extensively characterized in our laboratory and found to mediate
high levels of antitumor activity both in vitro and in vivo after
adoptive transfer into nude mice with established tumors (6–8)
or into patients with cancer (9, 10).

A-NK cells have been shown to possess a number of
features that appear to qualify them as excellent antitumor
effector cells. They can be generated in substantial numbers
from PBLs of normal donors as well as patients with malignan-
cies; they are highly mobile in tissue and preferentially localize
to metastases; they are able to secrete an array of cytokines and
growth factors; and they are non-MHC-restricted and require no
prior sensitization for killing of a broad range of tumor targets
(8, 11, 12). On the basis of these properties, A-NK cells have
been used for adoptive transfer in Phase I clinical trials at our
institute (9, 10).

In contrast to A-NK cells, CTLs are MHC class I-restricted
effector cells. They use TCR for recognition of nonapeptides
presented by class I molecules on the surface of a tumor cell or
an antigen-presenting cell (13). Thus far, it has not been possible
to directly compare A-NK cells with MHC class I-restricted
CTLs for antitumor functions in humans. Both an autologous
tumor cell target and a well-characterized CTL line or clone are
necessary for such a comparison, and these are not readily
available in humans. We have previously established CTL lines
specific for a human SCCHN and have shown that such lines
lyse the autologous tumor and MHC class I-identical allogeneic
SCCHN cell lines (14). Using these allogeneic but tumor-spe-
cific CTL lines maintained in our laboratory and allogeneic
A-NK cells generated from peripheral blood of healthy donors,
has been possible to compare antitumor effects of these cell
cultures in vitro and in vivo and to demonstrate that non-
MHC restricted A-NK cells are as effective as (if not more
effective than) tumor antigen-specific CTLs in mediating anti-
tumor activities.

MATERIALS AND METHODS

Cell Lines. SCCHN cell lines, the PCI series, were es-
ablished in our laboratory from fresh tumor biopsy specimens,
as described previously (15). A human oral carcinoma cell line
able to form lymph node metastases in nude mice, OSC-19, was
generously provided by Dr. Kawashiri et al. (16). The cell lines
were maintained in DMEM or Eagle’s essential medium (OSCI-
9) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100
IU/ml penicillin, and 100 μg/ml streptomycin (all from Life
Technologies, Inc., Grand Island, NY). For comparison, K562
(chronic myelogenous leukemia) and Daudi (B-cell lymphoma)
cell lines were also used. These cells were maintained in RPMI
1640 supplemented with 10% FBS, L-glutamine, and antibiotics.

Abs. Human/mouse cM Abs SF-25, 323/A3, and U36,
which are reactive with antigens expressed on human SCCHN
cells, were obtained from Dr. Guus van Dongen (Free Univer-
sity of Amsterdam, Amsterdam, The Netherlands). These
cM Abs were genetically engineered and consisted of a variable
region of murine (ab')2 and constant regions of human k light
and y-1 heavy chains (17). The antigens recognized by cMAb
SF-25 or 323/A3 were highly expressed on PCI-13 cells disso-
ciated by trypsinization, as confirmed by flow cytometry,
whereas expression of the antigen recognized by cMAb U36
was diminished by trypsinization but recovered after an over-
night culture. MAbs used for staining of target cells also in-
cluded anti-MHC class I (prepared by Dr. A. DeLeo from 4B95
(W6/32) hybridoma obtained from American Type Culture Col-
lection), anti-MHC class II (Becton Dickinson, San Jose, CA)
and anti-ICAM-1 (Becton Dickinson).

Other MAbs used for staining of effector cells included a
series of labeled MAbs to surface antigens on human lympho-
cytes: anti-CD3, anti-CD8, anti-CD4, anti-CD56, anti-CD16,
and anti-class II MHC. All MAb s were purchased from Becton
Dickinson.

Preparation of Single-Cell Suspensions of Tumor Cells
for Cytotoxicity or Flow Cytometry. To prepare single-cell
suspensions of adherent tumor cell lines, confluent cultures
were trypsinized with 0.05% (w/v) trypsin in 0.02% (w/v) EDTA
solution (Life Technologies, Inc.) for 5 min at 37°C. The cells
were washed in medium, resuspended in fresh medium,
and checked for viability using a trypan blue dye.

Preparation of Effector Cells. PBMCs were obtained
from venous blood of normal volunteers by ficoll-hypaque
centrifugation. CD3+CD56+ NK cells were purified by a neg-
ative immunoselection technique from the monocyte-depleted
PBMCs, as described earlier (7, 8). A-NK cells were generated
by a two-step method of initial adherence to plastic and subse-
quent proliferation in the presence of 6000 IU/ml of IL-2, as
described by us previously (7, 8). Prior to their use in experi-
ments described below, A-NK cells were phenotyped and tested
for effector cell function in 4-h 31Cr-release assays against K562
and Daudi cell targets (18).

CTLs were established from PBLs of a patient with SC-
CHN, as described previously (15). The CD3+CD8+CD4+
T-cell line was maintained in the presence of IL-2 and IL-4 for
10–12 weeks with repeated sensitization on autologous tumor
cell monolayers (15). Phenotypic and functional characteristics
of the line have been described previously (15). The CD8+ and
CD4+ T-cell lines were derived from the original bulk T-cell
line by negative selection with Ab-coated magnetic beads, using
anti-CD8 MAbs to remove CD8+ T cells or anti-CD4 MAbs to
remove CD4+ T cells from the cultured T-cell line, as described
by us earlier (19). Although the CD8+ CTL line lysed autolo-
gous SCCHN targets, it also lysed HLA-A2-matched allogeneic
SCCHN lines, including PCI-13 and OSC-19. This lysis
was blocked with anti-CD3, anti-CD8, anti-TCR α/β, and anti-
class-I MHC Abs. The CD4+ T-cell line grew in culture for 4–6
weeks. CD4+ T cells were found to be HLA-DR4 restricted,
and they produced IL-2 and IFN-γ in response to stimulation
with autologous tumor or DR-matched allogeneic SCCHN (19).

Flow Cytometry for Detection of Surface Antigens.
Expression of surface molecules on effector or target cells
was studied by two-color flow cytometry performed on a FACScan
flow cytometer (20). FITC- or phycoerythrin-labeled MAbs
were used for direct staining, and fluorescein-conjugated sec-
ondary Ab, goat F(ab')2, antimonouse IgG, or goat F(ab')2 anti-
human IgG (both from Caltag, San Francisco, CA) was used for
indirect staining of MHC class I and tumor antigens, respec-
tively. As negative controls, PBS alone or the appropriate iso-
type Abs (Becton Dickinson) were always included. All Abs
were pretitred to determine their optimal dilution for staining.
Prior to flow cytometry, cells were washed in PBS, 0.1% (w/v)
NaCl buffer and fixed in 2% (w/v) of paraformaldehyde in PBS.

**Cytotoxicity Assays.** Four-h \(^{51}\)Cr-release assays were
performed at four different E:T ratios as described previously
(18).

An MTT colorimetric assay was performed as described by
us previously (21). The \([\text{H}]\)TDrd release assay was performed
exactly as described previously by Matzinger (22).

**TUNEL Assay.** Effector cells were coincubated with
target cell monolayers cultured overnight in chamber slides for
1 h at 37°C. The slides were then washed several times to
remove effector cells, air dried, and fixed with 4% (w/v)
paraformaldehyde for 30 min at room temperature. The cells
were then permeabilized with 0.1% (v/v) Triton X-100 in 0.1%
(w/v) sodium citrate for 2 min on ice, washed twice in PBS and
once in cold ethanol, and dried in air. The TUNEL reaction was
performed in a moist chamber for 1 h at 37°C by incubating
the cells in the following solution: 0.3 mmol of Cy3-DUTP
(Biological Detection Systems, Pittsburgh, PA), 3 mmol of
dATP, 2 \(\mu\)l of 25 mm CaCl\(_2\), 25 units of TdT (Boehringer
Mannheim), and TdT buffer (30 nm Tris, pH 7.2, 140 mm
sodium cacodylate) in a total reaction volume of 50 \(\mu\)l. As a
positive control, monolayers were treated with DNase I (25
\(\mu\)g/ml for 10 min at room temperature) before staining to induce
DNA strand breaks. In negative controls, TdT was omitted. Cell
analysis was performed in an Olympus fluorescent microscope.

**Preparation of Spheroids.** Spheroids of PCI-13 cells
were prepared as described by us previously (8). Briefly, wells
of a 96-well U-bottomed culture plate were coated with 0.5%
(w/v) agarose in PBS and dried overnight. PCI-13 cells (2000–
5000) were suspended in DMEM with 10% (v/v) FBS and
incubated at 37°C in an atmosphere of 5% CO\(_2\) in air. As early
as the next day, clusters of cells were seen in each well; the
clusters developed into spheroids over the course of the next
10–14 days in culture. The medium was changed twice a week.
Only wells containing single well-formed spheroids of approx-
imately the same size were selected for experiments.

**Labeling of Effector Cells.** Cultured effector cells were
adjusted to a concentration of 5–10 \(\times\) 10\(^6\)/ml in serum-free
RPMI 1640 in glass tubes. A lipophilic-fluorescent dye, DiO
(green fluorescence) or Dil (orange fluorescence), was added to
the tubes at a range of concentrations of 20–40 \(\mu\)g/ml. This
concentration was determined in a series of preliminary exper-
iments to have no harmful effects on the functions of the cells
and to give adequate staining for performing fluorescence mi-
croscopy. After incubation at room temperature for 5 min, the
reaction was neutralized by the addition of an aliquot of FBS,
and the cells were centrifuged and washed twice with medium
containing 10% FBS. Cytotoxicity and ability of labeled effector
cells to proliferate in the presence of 100 units of IL-2 were
compared with those of unlabeled effector cells.

In some experiments, the effector cells were radiolabeled
with 100 \(\mu\)Ci of \(^{51}\)Cr (specific activity 5 \(\mu\)Ci/mmol; New
England Nuclear, Boston, MA) by incubating cell pellets with
an aliquot of sodium chromate for 40 min at 37°C. The cells
were washed three times with medium before use.

**Fluorescence-labeled Effector Cell Binding and Entry
into PCI-13 Spheroids.** Patterns of binding and migration of
fluorescence-labeled effector cells into spheroids were exam-
ined by confocal microscopy. PCI-13 spheroids were washed
with RPMI 1640 containing 10% FBS and coincubated with
labeled effector cells (1 \(\times\) 10\(^6\)) in individual wells for 2 h at
37°C. After incubation, the spheroids were washed with EDTA
(2.5 mm) in PBS and then with PBS alone to remove effector
cells bound to the spheroid surface. The spheroids were fixed
with 1% (w/v) paraformaldehyde in PBS and placed in the dark
at 4°C until confocal microscopy could be performed. Molecular
Dynamics Multiprobe 2001 laser confocal microscope was
used to record effector cell localization in the spheroids as
described previously (8).

**\(^{51}\)Cr-Labeled Effector Cell Binding to Monolayers
and Entry into PCI-13 Spheroids.** The spheroids were incubated
with \(^{51}\)Cr-labeled effector cells (for A-NK cells in the presence
or absence of cMAbs) for 2 h, and the unbound effector cells
were washed extensively as described above. Twelve spheroids,
each in an individual well of a microplate, were used for each
experimental condition. Every spheroid was harvested into a
separate tube, and radioactivity incorporated in the spheroid was
measured using a gamma counter.

In some cases, binding of \(^{51}\)Cr-labeled effector cells to
monolayers was evaluated in the presence or absence of cMAbs.
To prepare effector cells for these experiments, purified resting
NK cells were incubated in the presence of 6000 IU of IL-2 and
\(^{51}\)Cr (see above) for 30 min at 37°C. The cells were washed
several times in medium and adjusted to a concentration of 1 \(\times\)
10\(^6\)/ml. Confluent PCI-13 monolayers were prepared in wells of
96-well plates by culturing 5000 tumor cells/well for 3 days.
The monolayers were incubated with cMAbs (1 \(\mu\)g/ml) for 30
min, at which time \(^{51}\)Cr-labeled effector cells (1 \(\times\) 10\(^6\)) were
added. The effector cells were allowed to adhere to the mono-
layers for periods of time ranging from 15 min to 3 h. The
monolayers were extensively washed with medium to remove
unattached effector cells and then lysed using Triton X-100. The
lysates were harvested from each well for measurements of
radioactivity in a gamma counter.

**In Vivo Entry of Effector Cells into SCCHN Cells.** Nude mice
(BALB/c females obtained from Taconic Farms through a
contract with NIH) were splenectomized and pretreated with
cyclophosphamide (200 mg/kg) and with Ab to asialo-GM1
(0.2 mg/mouse; Biomedical Diagnostics, Edgewood, NY) as
described previously (6). Using PCI-13 cells, s.c. tumors were
established by injecting 10 \(\times\) 10\(^6\) cells/mouse in the
dorsal region. Three days later, when the tumors were
palpable, 5 \(\times\) 10\(^6\) Dil-labeled effector cells alone or effector
cells and cMAbs were injected in 0.1-ml aliquots in the peri-
ural region. Twenty-four h later, the animals were sacrificed.
Histology was determined on H&E-stained sections, and
fluorescence microscopy with filters for rhodamine (580 \(\mu\)m in
wavelength) was used to evaluate localization of labeled effec-
tor cells in the tumor stroma and parenchyma.

To establish an in vivo model of OSC-19 (16), nude mice
were immunosuppressed as described above, and 4 \(\times\) 10\(^5\)
OSC-19 cells were injected at the floor of the oral cavity by an
external route. Tumors grew rapidly in this location, and by day 21, animals developed lymph node metastases. Mice with 7-day established OSC-19 tumors were treated by delivery of 10 × 10⁶ human A-NK cells or CD8⁺ CTLs plus 6000 IU of IL-2/mouse. Effector cells were injected s.c., without disturbing the tumor capsule, every other day for three injections and IL-2 was given twice daily for a week. Animals were weighed and sacrificed on day 21. Tissues were harvested for histology. H&E sections were examined microscopically, and cervical lymph nodes were scored for the presence of metastases. Analysis of the areas of lymph nodes involved by metastases was performed using morphometry with a Leica Quantimed 500C system.

Statistical Analyses. Differences between experimental and control samples or between groups were determined using a nonparametric Mann-Whitney test. P < 0.05 was considered to denote significant differences.

RESULTS

A-NK cells were routinely obtained from human purified NK cells by selection of plastic-adherent cells and their subsequent expansion in the presence of 22 nm of IL-2 (8). On average, a 14-day culture of A-NK cells yields 45 × 10⁷ CD3⁻CD56⁺ cells, starting with 1 × 10⁷ purified NK cells (n = 31). A-NK cells can be maintained in culture for up to 21 days, but then their viability declines. The bulk T-cell line was established and maintained in the presence of IL-2 and IL-4 from cryopreserved PBLs of a patient with squamous cell carcinoma of the tongue. In addition to lysing the autologous SCCHN targets, the CD8⁺ T-cell line derived from the bulk culture selectively lysed those SCCHN lines that shared HLA antigens with the autologous tumor (14, 15, 23). Of the two SCCHN cell lines selected for the experiments reported here, PCI-13 shared HLA-A2, DR4, and DQ2,3 with the autologous tumor; the OSC-19 cell line shared HLA-A2 only. Whereas unlimited numbers of allogeneic A-NK cells were available for the experiments described below, the CTL line, especially when separated into CD8⁺ and CD4⁺ T cells, had a limited life span in culture and thus was available for only some of the experiments. Although the CD8⁺ T-cell line was allogeneic to PCI-13 as well as OSC-19 targets, it recognized a shared SCCHN antigen on these tumor cells, based on the specificity experiments reported previously (14, 15) and in more recent studies performed with OSC-19.⁴

Characteristics of Tumor Cell Targets. Cell surface expression on tumor cell targets of molecules that may be important for interactions with effector cells was first evaluated. PCI-13 and OSC-19 cell lines grow as tightly adherent monolayers. For flow cytometry or cytotoxicity studies, tumor cells in suspension were necessary. Therefore, SCCHN cell monolayers were dissociated by mild trypsinization or treatment with a Sigma dissociating solution, as described in “Materials and Methods.” This treatment was shown not to alter expression of cell surface antigens that are usually detectable on these cells. The data in Table 1 show that PCI-13 cells abundantly express the antigen defined by cMab SF-25 (24) and, less abundantly, MHC class I molecules on the cell surface. Both MHC class II antigens and ICAM-1 are weakly expressed on the surface of PCI-13 targets. However, after incubation with 1000 units/ml of IFN-γ for 72 h, expression of MHC class I and class II antigens, as well as of ICAM-1, was increased in PCI-13 targets. For comparison, expression of the same surface antigens on K562 and Daudi targets (commonly used in cytotoxicity assays as NK-sensitive and NK-resistant tumor targets, respectively) is also shown in Table 1. OSC-19 cell line expressed MHC class I and class II antigens as well as ICAM-1, but incubations in the presence of IFN-γ for 72 h significantly up-regulated expression of all three molecules on the tumor cell surface, as shown in Fig. 1.

To determine sensitivity of the two SCCHN targets to allogeneic A-NK or bulk CTLs, 4-h ⁵¹Cr-release assays were performed. In previous experiments, we determined that these SCCHN targets were consistently resistant to lysis by nonactivated human PBMCs or freshly purified NK cells in 4-h cytotoxicity assays (Ref. 19 and unpublished data). However, as shown in Table 2, A-NK cells were quite effective in lysing both SCCHN targets, although less so than in lysing K562 targets. In the presence of cMab SF-25, A-NK cell lytic activity against PCI-13 was mildly increased (not a significant difference), but this increment was much greater when nonactivated NK cells were used as effector cells, as reported by us previously (20). In contrast, the CD8⁺ CTL line did not lyse K562 or Daudi targets but was cytotoxic for the SCCHN targets sharing HLA-A2, in agreement with our previous reports indicating that the CTLs recognize a shared SCCHN antigen on these targets (14, 15, 23). The CD4⁺ T-cell line did not mediate lysis by perforin release against PCI-13 targets or OSC-19 targets and showed only a variably low level of lysis against K562 targets. The data shown in Table 2 indicate that PCI-13 and OSC-19 targets tested in 4-h ⁵¹Cr-release assays appear to be almost equally sensitive to lysis by non-MHC-restricted allogeneic A-NK cells in the presence or absence of antitumor MAbs and to lysis by CD8⁺ T cells, which are allogeneic but tumor antigen specific and MHC class I restricted. Similar results were consistently obtained in cytotoxicity assays repeated 3 or 4 times over a period of several


Table 1 Expression of surface molecules on tumor cells targets

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Target</th>
<th>PCI-13</th>
<th>PCI-13 + IFNγ</th>
<th>K562</th>
<th>Daudi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (isotype)</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>MHC class I</td>
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<td>352</td>
<td>9</td>
<td>22</td>
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<tr>
<td>MHC class II</td>
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<td>83</td>
<td>8</td>
<td>946</td>
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<tr>
<td>ICAM-1</td>
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<td>108</td>
<td>29</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>SF-25</td>
<td>2527</td>
<td>ND³</td>
<td>284</td>
<td>796</td>
<td></td>
</tr>
</tbody>
</table>

³ Suspensions of PCI-13 cells were stained with the appropriate MAbs and examined by flow cytometry as described in “Materials and Methods.” Tumor cells were incubated with 1000 units/ml of IFN-γ for 72 h prior to flow cytometry. The data are expressed as mean fluorescence intensity. Shown is one representative experiment of 3–5 performed.

³ ND, not determined.
months with these SCCHN targets harvested at different passages in culture.

**Mechanisms of Tumor Target Cell Lysis by A-NK Cells or CTLs.** Secretory and nonsecretory types of killing are now thought to be mediated by immune effector cells (25, 26). Three different types of cytotoxicity assays were performed to determine whether A-NK cells used different mechanisms of lysis than CTLs, when PCI-13 cells served as targets. As shown in Table 3, A-NK cells generated from peripheral blood of normal volunteers were more effective in 4-h $^{51}$Cr-release assays (a measure of perforin-mediated necrotic killing) than either the CD8$^+$ or CD4$^+$ T-cell lines. In addition to inducing perforin-mediated lysis, A-NK cells were also able to induce DNA fragmentation following 1 h of incubation with PCI-13 targets, as measured by $[^3H]$TdR-release (JAM) assays (22). The ability A-NK cells to induce DNA fragmentation in PCI-13 targets was confirmed in the Tdt-based assay (TUNEL), as shown in Fig. 2. Although the T-cell lines were also able to induce DNA fragmentation in PCI-13 monolayers after 1 h of coincubation, A-NK cells appeared to be significantly more effective, as shown by $[^3H]$TdR-release (Table 3) or TUNEL assay (data not shown).

Interestingly, the CD8$^+$ T cells were found to consistently mediate particularly high levels of killing, when compared with other effector cells, in 24-h MTT assays performed with PCI-13 monolayers (Table 3). This 24-h assay measures a sum of secretory and nonsecretory lytic events induced by coincubation of effector cells with tumor targets. A-NK cells showed particularly strong lytic activity against PCI-13 cells in 4-h $^{51}$Cr-release assays, whereas CTLs were best able to induce cell death during a prolonged interaction with these tumor targets. These experiments suggest that A-NK cells and CTLs might preferentially use different lytic pathways in elimination of tumor cell targets. In all of these experiments, resting NK cells, used as controls, showed no cytotoxicity in 4-h $^{51}$Cr-release assays but were able to mediate killing of PCI-13 targets by a nonsecretory killing pathway, as reported by us previously (25). Overall, our data suggest that both A-NK cells and CTLs kill PCI-13 targets in vitro by more than one mechanism. These mechanisms may, in part, overlap, and whereas perforin-mediated rapid cell death appears to play a major role in 4-h assays, a combination of
necrotic and apoptotic mechanisms appears to be involved in killing by A-NK cells or CTLs of tumor targets coincubated with effector cells for a longer time period (e.g., 24 h).

**Entry and Binding of Effector Cells to PCI-13 Spheroids.** PCI-13 cells were found to be capable of forming spheroids *in vitro* (8). We took advantage of this property of the PCI-13 cell line to investigate the process of entry of A-NK cells or CTLs into these multicellular formations, resembling actual tumors. Although spheroids are not vascularized, they nevertheless offer a model in which binding, migration, and interactions of effector cells with tumor cells in tissue can be examined in a greater detail. In the initial experiments, A-NK cells or CTLs were labeled with ¹¹⁵⁷Cr and coincubated with PCI-13 spheroids for 2 h. We have shown previously that functions of A-NK cells were not impaired by labeling with ¹¹⁵⁷Cr (8). As shown in Figs. 3 and 4, A-NK cells and CTLs were comparable in their ability to enter into PCI-13 spheroids. However, A-NK cells armed *in vitro* with cMAb SF-25 or U36 (20) had a significantly increased capacity (*P* < 0.05) to enter into PCI-13 spheroids. To determine whether the observed improved entry resulted from better binding of armed A-NK cells, we next incubated the effector cells with PCI-13 monolayers for 30 min. Binding of IL-2-activated purified ¹¹⁵⁷Cr-labeled NK cells to PCI-13 monolayers was considerably increased in the presence of cMAbs reactive with antigens present on PCI-13 targets (Fig. 5).

To further study the process of penetration and migration of A-NK cells or CTLs into PCI-13 spheroids, these effector cells were labeled with a lipophilic dye, DiO or Dil (27). As the dye incorporated into the cellular membrane may be potentially reactive with antigens present on PCI-13 targets (Fig. 5).

**Peritumoral Injections of A-NK Cells in Vivo.** We have previously established a xenograft model of SCCHN in immunosuppressed nude mice (6). S.c.-injected PCI-13 cells (10⁶) form palpable tumor nodules in these mice 3–6 days later (6). Here, we used 3-day established tumors to evaluate the ability of human A-NK cells, which were either unarmed or armed with cMAb SF-25, to enter into the tumor tissue. The A-NK cells were labeled with the DiI dye and delivered to the tumor site by multiple peritumoral injections (6). The animals were sacrificed 24 h later, and localization of the labeled cells in tissue was determined in cryostat tissue sections examined in a fluorescent microscope equipped with the appropriate filters. In Fig. 3, C and D, it can be seen that unarmed A-NK cells largely localized to the tumor stroma and did not enter in a substantial number into the tumor parenchyma. In contrast, A-NK cells transferred with cMAb SF-25 were found throughout the tumor parenchyma. These results indicate that the presence of the tumor-specific Ab enhances the ability of A-NK cells to enter the tumor tissue *in vivo*, similar to its *in vitro* effects reported above.

To compare *in vivo* effectiveness of A-NK cells and CTLs, we established a lymph node metastasis model of human SCCHN in nude mice by injecting 4 × 10⁶ OSC-19 cells into the floor of the mouth by the extraoral route. Following perilesional administration of human A-NK cells or the CD8⁺ CTLs...
Fig. 3 Entry of A-NK cells (A) or CTLs (B) into PCI-13 spheroids. Following 2 h of incubation of spheroids (obtained by culturing 3 × 10^5 tumor cells for 2 weeks) with 1 × 10^5 effector cells labeled with Dil, as described in “Materials and Methods,” and extensive washing, confocal microscopy was performed with optical cuts through the spheroids every 10 μm. Labeled effector cells can be seen penetrating through the surface and entering the spheroids. C and D, sections of PCI-13 tumors that were established in nude mice and injected perilesionally with Dil-labeled A-NK cells (10 × 10^6 cells) in the presence or absence of cMAb SF-25. Twenty-four h later, the mice were sacrificed, and cryostat sections of the tumors were examined in a fluorescence microscope using rhodamine filters. C, representative section of the tumor injected with A-NK cells in the absence of cMAb. Note that the labeled cells are mostly localized to the tumor stroma (ST). D, a representative section of the tumor injected with A-NK cells in the presence of cMAb. Note that labeled A-NK cells are localized to the tumor parenchyma. ×100.

line to nude mice with 7-day tumors growing at this site, we evaluated three separate parameters to judge the effectiveness of therapy: a proportion of lymph nodes with metastases, mean percent area of metastases, and body weight of treated versus control animals. Because the tumor grows rapidly in the oral cavity, restricting access of food to esophagus, a loss of weight is an excellent measure of tumor progression. The results presented in Table 4 show that mice treated with either CTLs or A-NK cells had significantly fewer lymph nodes involved by metastases and that metastases occupied a significantly smaller area of lymph nodes, as determined by morphometry. In addition, total body weights of treated mice were significantly higher than those of tumor-bearing untreated controls (Table 4). There were no significant differences observed between mice treated with A-NK or CD8+ T cells and those treated with IL-2.

DISCUSSION

The role of immune cells in surveillance against cancer and antitumor responses has been intensively debated for a number of years (28). The immunosurveillance theory originally formulated by Drs. F. M. Burnet (29) and Lewis Thomas (30) and later modified to reflect more recent developments in immunology (31) proposes that effector functions mediated by the immune system play a crucial role in the process of tumor development and progression. However, it has been difficult to dissect the involvement and contribution to this process of various subsets of human immune cells, specifically, those mediating the tumor-specific memory response from those responsible for innate or natural immunity. On the basis of extensive work in preclinical animal models of tumor growth or metastasis, it has been suggested that NK cells participate early in the course of immune response to cancer and are particularly effective in eliminating blood-borne metastases (32). In contrast, T and B lymphocytes, mediating antigen-specific cellular responses and Ab synthesis, respectively, are thought to be the main antitumor effector cells responsible for specific long-lasting immunity to the tumor (33). The ultimate test of this hypothesis, at the therapeutic level, has been very difficult to implement in humans, mainly because pure subsets of tumor-specific CTLs or helper T cells or of purified NK cells have not been available for use in therapeutic models or human clinical trials. Tumor-infiltrating lymphocytes, which expand in the presence of IL-2 and which were previously thought to represent tumor-specific T cells, are now known to be mixtures of activated lymphocytes,
Antitumor Effects of Human A-NK Cells and CTLs

which may, at best, be enriched in autotumor-specific T cells (34). Similarly, lymphokine-activated killer cell cultures are heterogeneous populations of activated, non-MHC-restricted effector cells (NK as well as T cells; Ref. 35). Thus, in the absence of purified subsets of human tumor-specific T cells and paucity of purified human NK cells, it has not been possible to directly compare these cell subsets for antitumor efficacy in vivo.

The development in our laboratory of methodology for purification and culture of human A-NK cells and the availability, at the same time, of both human SCCHN cell lines and T-cell lines specific for this tumor provided us with a unique opportunity for comparing antitumor functions of these subsets of effector cells in vitro and in vivo. The comparison has had some limitations. Allogeneic A-NK cells as well as allogeneic HLA-A2-restricted CTLs were used because autologous effector cells of both types could not be generated from patients with SCCHN who donated specimens for establishment of the autologous tumor cell lines. Although such tumor cell lines can be established from about 30% of patients with SCCHN (36), tumor-specific CTL lines can only rarely be generated and maintained (15), and the patients whose CTL and tumor lines are available often do not survive to provide additional material for immunological studies. Nevertheless, using A-NK cells and allogeneic but tumor-specific and HLA-A2-matched CTLs, we were able to perform in vitro comparisons and in vivo studies in the animal model of SCCHN established in nude mice (6, 8) to assess antitumor potential of these effector cells. Of the two SCCHN cell lines used in these studies, PCI-13 was found to express class I and II MHC antigens, especially after in vitro pretreatment with IFN-γ (Table 1), as well as a number of carcinoma-associated antigens recognized by a set of cMAbs we had obtained. The line readily formed spheroids in vitro and grew as a s.c. nodular mass in immunosuppressed nude mice. It was found to be sensitive to lysis by a subset of IL-2-A-NK cells and by allogeneic HLA-A2-restricted CD8+ T-cell lines previously shown to recognize shared SCCHN antigens presented by the class I MHC molecules or class II MHC molecules, respectively (14, 15, 19). The second SCCHN cell line, OSC-19, also dramatically up-regulated class I and II antigens during incubation in the presence of IFN-γ, and it was sensitive to lysis by the HLA-A2-restricted, SCCHN-specific CD8+ T-cell line that we generated. The A-NK cell subset of non-MHC-restricted effector cells has been extensively evaluated in our laboratory and was found to have potent antitumor activities, including the ability to lyse a wide variety of tumor targets, to abundantly produce cytokines (which, individually or in combination, are capable of mediating antitumor effects), and to enter and localize to tumor metastases (5, 8, 11).

Having assembled the necessary components, we were in a unique position to be able to compare allogeneic, SCCHN cross-reactive CTLs with allogeneic A-NK cells for capabilities to lyse various human tumor targets and to enter into PCI-13 spheroids. The PCI-13 targets were found to be sensitive in vitro to both types of effector cells, but somewhat to our surprise, A-NK cells were more effective than CTLs in killing PCI-13 targets in short-term 51Cr-release assays. Both types of effector cells were comparable in binding to PCI-13 monolayers and entering into spheroids. We expected the CTLs to have an advantage, based on their ability to specifically interact with the...
necessary for perforin-mediated lysis (37), the reason for this disparity is not clear. However, it is likely that both A-NK cells and CTLs are capable of mediating lysis by more than one mechanism (25, 26) and that they can preferentially use these mechanisms, depending on the properties of the target or conditions of the assay. Although CTLs have been often viewed as superior effector cells, A-NK cells may contain a greater variety of cytotoxic molecules than do T cells, including soluble factors present in their supernatants (23) and thus could have a more rapid or greater effect on tumor cells. In support of this hypothesis, A-NK cells were found to be able to more readily induce DNA fragmentation in PCI-13 targets than CTLs, as measured in the [3H]Tdr release and TUNEL assays. Although CTLs and A-NK cells entered PCI-13 spheroids with comparable efficiency, the possibility of arming of A-NK cells with SCCHN-reactive cMAbs or just having these cMAbs present in the tumor microenvironment substantially increased the ability of A-NK cells to localize to the tumor. This observation, confirmed in our in vivo experiments in nude mice and combined with our previous data indicating that NK cells are the only effectors mediating antibody-dependent cellular cytotoxicity in the presence of cMab (20), strongly supports the role for NK cells not only in spontaneous or natural but also in tumor-specific immunity at a time when antitumor Ab is present in vivo.

In vivo experiments, using a perilesional route of effector cell delivery, indicated that both types of effector cells significantly contributed to the observed decreases in metastases and prevention of the weight loss in treated mice (Table 4). These in vivo data were subsequently extended and confirmed with larger groups of mice. Perilesional rather than i.v. delivery of effector cells was used in view of a limited number of CTLs available for these experiments. The results of the comparison suggested that both types of effector cells were equally able to reduce metastatic disease and protect from a weight loss in animals bearing tumors at the time of perilesional adoptive therapy. However, from the viewpoint of therapeutic feasibility, A-NK cells have considerable advantages over CD8+ CTLs. A-NK cells are non-MHC restricted and do not require prior sensitization with autologous tumor or peptide-pulsed antigen presenting cells in

### Table 4

<table>
<thead>
<tr>
<th>Therapy</th>
<th>% lymph nodes with metastases</th>
<th>% mean area of metastases</th>
<th>Body weight, g (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>55 (11/20)</td>
<td>30</td>
<td>20 ± 1.3</td>
</tr>
<tr>
<td>Not treated (controls)</td>
<td></td>
<td></td>
<td>15.8 ± 1.2</td>
</tr>
<tr>
<td>A-NK therapy</td>
<td>23 (6/26)</td>
<td>5.8</td>
<td>18.9 ± 1.8</td>
</tr>
<tr>
<td>CD8+ T therapy</td>
<td>30 (8/26)</td>
<td>5.6</td>
<td>22.7 ± 1.5</td>
</tr>
</tbody>
</table>

*Established 7-day OSC-19 tumors were treated by perilesional injections of A-NK cells or the CD8+ CTL line (six nude mice in each group) according to the schedule described in "Materials and Methods." On day 21, mice were weighed and sacrificed, and their tissues were embedded for histology.

*bDifferences between control and treated mice were significant at *P* < 0.05. The data in parentheses are the number of lymph nodes with metastases among total examined."

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Figure 6: Effects of DiO or Dil on cytotoxicity of A-NK cells against PCI-13 targets or on proliferation of A-NK cells. In A, the cells were labeled with various concentrations of dye for 5 min and then tested in a 4-h 51Cr-release assay. In B, A-NK cells (1 × 10⁶/well) were labeled with DiO or Dil and incubated in the presence of IL-2 (600 IU/ml) for 24 h. [3H]Tdr incorporation was measured. Columns, mean cpm obtained from triplicate wells; bars, SD.

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tumor antigen(s) via the TCR-CD3 complex. In fact, the CTLs were more effective than A-NK cells in killing tumor targets in 24-h assays. In contrast, A-NK cells were consistently more effective than CTLs in killing PCI-13 targets by perforin-mediated lysis. Because both types of effector cells were IL-2 activated and both presumably contained cellular components necessary for perforin-mediated lysis (37), the reason for this
long-term cultures and yet appear to be able to bind and selectively destroy tumor cells, sparing normal tissue cells. The feasibility for reproducible in vitro generation of A-NK cells through a relatively easy and cost-effective process of adhesion to plastic followed by culture in the presence of IL-2 is also important. We have demonstrated that autologous or allogeneic A-NK cells can be generated in numbers sufficient for therapy from patients with cancer (9, 10) and have successfully cultured these effector cells for human therapy (9, 10). To the best of our knowledge, it has not been feasible to culture human tumor-specific CTL lines or clones in long-term cultures, including bioreactors, to obtain cell numbers needed for therapeutic delivery. Because A-NK cells express FcγRIII, they can be armed with tumor-specific Abs and directed to the tumor based on the specificity of the Abs for tumor-associated antigens. Like CTLs, A-NK cells have the ability to mediate lysis not only via the perforin pathway but also via other mechanisms, including cytokine/cytokine receptor (e.g., the tumor necrosis factor receptor family) pathways (26, 38). The potential of A-NK cells for in situ release of a broad spectrum of cytokines and enzymes, facilitating degradation of extracellular matrix components (11) further strengthens the impression that these cells might be as potent antitumor effector cells as CTLs and thus might play a key role not only in the elimination of circulating tumor cells but also in the destruction of tumor metastases in solid tissues. There is evidence from numerous studies that NK cells are residents of many human tissues, including liver (4, 39) and thus are present and poised to perform effector functions whenever necessary. We believe that A-NK cells are a biologically significant subset of effector cells endowed with characteristics that facilitate their entry into tissue and recruitment (via cytokines) of other inflammatory cells. Although recent advances in gene or vaccination therapies have shifted interest away from adoptive therapy of cancer patients, this study serves to emphasize that both A-NK cells and CTLs have a considerable therapeutic potential, which should not be disregarded or overlooked in a design of future clinical trials.

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