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High Levels of RAE-1 Isoforms on Mouse Tumor Cell Lines Assessed by Anti-"pan" RAE-1 Antibody Confer Tumor Susceptibility to NK Cells

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Two sublines of the benzpyrene-induced mouse hepatoma cell line, G-1 and G-5, showed low and high metastatic ability, respectively, to the lung. We produced a polyclonal antibody (pAb) against RAE-1α. Five isoforms of RAE-1 have been identified to date, and this pAb recognized all isoforms and was named anti-"pan" RAE-1 pAb. The level of RAE-1 was ~5-fold higher in G-5 than in G-1, which was almost RAE-1-negative, as determined using anti-pan RAE-1 pAb. Expression levels of other markers including MHC class I (MHC-I) and Qa-1b were very low and indistinguishable in these sublines. NK-mediated cytotoxicity was determined with these sublines; G-5 was highly susceptible to NK-mediated cytolysis, while G-1 was relatively resistant. The NK-mediated G-5 > G-1 killing profile was diminished if the G-5 cells were pretreated with $F(ab)'_2$ of anti-pan RAE-1 pAb. G-1, when transfected with *Rae-1α* cDNA, acquired NK-responsiveness similar to that of G-5. These and additional data using mouse cell lines with low MHC-I levels and various RAE-1 levels also demonstrated that RAE-1 level is critically associated with NK-susceptibility in tumor cells.

NK cells and cytotoxic T cells (CTL) are major effector cells in the immune system, providing protection against certain tumors and microbial infections (1, 2). Clonal proliferation of CTL is induced by mature dendritic cells that present antigenic peptides on their MHC-I for CTL to discriminate between self and malignantly transformed cells. The maturation process of dendritic cells depends on preceding activation of innate immunity where adjuvant-active microbial components and inflammatory cytokines are involved. On the other hand, the molecular mechanisms allowing NK cells to discriminate between normal versus affected cells remain largely undefined. It appears, however, that NK cell function is regulated by a delicate balance between the engagement of activation and inhibitory receptors (3, 4). Many of the activating NK cell receptors (e.g., CD16, KIR2DS, Ly49D/H, CD94/NKG2C) are noncovalently associated with membrane-anchored adaptors that possess immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic domains (5). These adaptor proteins, which include Fcγ R1, CD3 and DAP12, stimulate NK cells by recruiting and activating the Syk and ZAP70 protein tyrosine kinases (5). The receptor-based regulation governs NK activity. Thus, molecular bases for activation of CTL and NK cells appear to be rooted in the innate immune system.

Wu *et al.* reported that DAP 10, a membrane adaptor molecule similar to DAP12, forms an activating NK cell receptor complex with a C-type lectin-like molecule, NKG2D (6). Unlike most other NK cell receptors that are expressed on minor subsets of NK or T cells, the NKG2D/DAP10 receptor complex is on all NK cells, CD8+ αβ TCR+ T cells, and γδ TCR+ T cells (7), implying a broader and more primary role in immune responses in mice. NKG2D ligands were discovered as RAE-1 family proteins, RAE-1α, β, γ, and δ, and H60 (~25% homologous to RAE-1) in a recent study (8).

We first identified RAE-1α, β, and γ as retinoic acid-inducible genes on mouse F9 cells with low but significant homology to mouse MHC-I (9, 10). RAE-1 family proteins were GPI-anchored membrane proteins with no association with β_2-microglobulin (10). The function of this family, however, remained elusive until the report by Cerwenka *et al.* (8) highlighting the under-

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lying link between the structure and function of RAE-1.

Here, we successfully produced a pAb against RAE-1α and found that this pAb recognized all the RAE-1 family proteins reported to date. We designated this pAb as anti-pan RAE-1 pAb, and used it to test the reported function of RAE-1.

MATERIALS AND METHODS

Cell lines, antibodies and reagents. The mouse cell lines used in this study except for G-1 and G-5 were gifts from Sumitomo Seiyaku Co., Osaka. G-1 and G-5 cells were established in our laboratory as reported previously (11). Cells were maintained in RPMI 1640 containing 10% FCS. Cultures were maintained in a 5% CO_2/95% air atmosphere at 37°C. A rabbit kidney cell line, RK13, was from the ATCC collection.

G-1 cells were transfected with cDNA/plasmid of RAE-1α according to the method described previously (11). Briefly, RAE-1α cDNA was placed in the mammalian expression vector pEFBOS using standard gene manipulation techniques (12). To obtain G-1 cells stably expressing RAE-1α protein, G-1 cells (80% confluency on 100-mm dishes) were transfected with RAE-1α/pEFBOS (20 μg) with Lipofectamine (30 μg). The empty vector (20 μg) was used for transfection instead of RAE-1α/pEFBOS for preparation of control cells. Selection was started by addition of G418 (final concentration, 200 μg/ml) to the culture medium. The G-1 cells expressing the expected protein were screened by flow cytometry using the pAb against RAE-1α and subcloned by limiting dilution. Three independent clones of G-1/RAE-1α with different levels of RAE-1α protein were finally established.

Monoclonal antibodies (mAbs) against mouse MHC class I (MHC-I) and Qa-1b (HLA-E homologue) were purchased from Pharmingen-Fujisawa (Tokyo, Japan). Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Wako Pure Chemicals (Osaka, Japan). Treatment of cells with PI-PLC was performed according to a method previously described (13).

Production of antibody against RAE-1. A polyclonal antibody (pAb) against mouse RAE-1α was raised in a rabbit. The protocol for rapid antibody production was reported previously (14). Briefly, RK13 cells (1×10^7) were transiently transfected with RAE-1α/pEFBOS using Lipofectamine 2000 (LIFE Technologies). After 2 days, transfected RK13 cells were collected with 10 mM EDTA–PBS, washed with PBS three times, and suspended in 0.5 ml of PBS. Then, RK13 cell suspensions were mixed with 0.6 ml of Freund's complete adjuvant (FCA) (DIFCO, Detroit, MI) and extensively agitated. The mixture was used to immunize rabbits four times at 7-day intervals with a boost-injection before drawing blood. The antisera were harvested by centrifugation. IgG was purified from the sera according to the standard method (13). $F(ab)'_2$ was prepared with pepsin by the reported method (15). The recognition profile of this antibody toward the RAE-1 family proteins were determined using Ba/F3 cell clones expressing RAE-1α, β, γ, δ, or ϵ by flow cytometry, and all of them were found to be reacted with this antibody (T. Pertel and L. Lanier, unpublished observations). RAE-1ϵ is the newest member of the RAE-1 family registered by Lanier LL (AY054973), and Girardi *et al.* and Oppenheim *et al.* (AY056835) in GenBank.

Two-step solubilization of proteins. Cells (4×10^7 cells) were washed in Dulbecco's phosphate-buffered saline (D-PBS) and solubilized with 0.4 ml of lysis buffer 1 (D-PBS containing 10 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), and 1% Triton X-100). After standing at 4°C for 30 min, supernatants (Triton-soluble fraction) were separated by centrifugation at 10,000*g* for 3 min. The 10,000*g* pellets of the Triton X-100 lysates were further solubilized with 0.4 ml of lysis buffer 2 (D-PBS containing 10 mM EDTA, 1 mM PMSF, and 60 mM octylglucoside) at 4°C for 30 min with constant agitation, and sup 2 (octylglucoside-soluble fraction) was obtained by centrifugation at 15,000*g* for 1 h at 4°C (16). Supernatants were mixed with SDS–PAGE sample buffer (nonreducing) and stored at −30°C until subjected to electrophoresis.

Western blotting. Protein samples (50 μl) were resolved on SDS–PAGE (10% or 12.5% gels) and electroblotted onto nitrocellulose membranes. Membranes were blocked with 10% nonfat dry milk in Tris-buffered saline (TBS) and probed with the first Abs (1 μg/ml) at room temperature for 1 h. After 5 washes in washing buffer (0.1% Tween 20 in TBS), membranes were incubated with 1:10,000 diluted horseradish peroxidase-conjugated goat anti-mouse (or -rabbit) IgG (Bio-Rad, Richmond, CA) at room temperature for 1 h and then washed 7 times before detection with an ECL system (Amersham Life Science, Arlington Heights, IL).

Flow cytometry. Cells (5×10^5) were incubated with 30 μl of 20 μg/ml anti-pan RAE-1 pAb or 30 μl of 5 μg/ml monoclonal antibody (mAb) against MHC-I or Qa-1b for 1 h at 4°C. After three washes, cells were treated with FITC-conjugated secondary antibody. The stained cells were analyzed using a FACSCalibur. Mean fluorescence shift (MFS) was evaluated on the attached computer.

LDH release cytotoxicity assay. NK cells were prepared according to the method described previously with slight modifications (8). Briefly, spleen cells were collected from female B6 mice. The cells were dispersed, filtered with a nylon wool column to remove B cells and allowed to adhere to an FCS-coated dish to remove macrophages/monocytes. The cells depleted of B cells and monocytes were treated with 1000 U/ml of recombinant mouse IL-2 (PeproTec Ltd., NJ) in RPMI 1640/10% FCS/5×10^5 M of 2-mercaptoethanol for 7 days at 37°C. The cells bound to the dish were used as effector cells for NK cytotoxicity.

Various concentrations of effector cells were mixed with constant amounts of target tumor cells ($0.4–3 \times 10^4$/well) in 96-well plates. In some experiments, cells were treated with 20 or 80 μg/ml of $F(ab)'_2$ or intact anti-pan RAE-1 pAb. The plates were centrifuged at 500 rpm for 5 min at 4°C, and incubated for 4 h at 37°C in the presence of pAb or $F(ab)'_2$. The plate was again centrifuged to harvest the supernatants, which contained LDH. The LDH assay kit was purchased from Takara, Co., Ltd., Tokyo, and the assay was performed according to the manufacturer's booklet. The experiments were performed three times in triplicate. NK-mediated killing was determined according to the formula:

$$\%\text{Cytotoxicity} = \text{LDH (target)} - \text{LDH (spontaneous)}/\text{LDH (100\% killed)} - \text{LDH (spontaneous)}.$$

RESULTS

Levels of Total RAE-1 and MHC1 on Mouse Tumor Cell Lines

MHC-I, Qa-1b and RAE-1 levels were determined with various mouse tumor cell lines using anti-class I Ab and anti-pan RAE-1 pAb by flow cytometry (Fig. 1, Table 1). The levels of these antigens are shown by mean fluorescence shift (MFS). Except EL-4, L5178Y, and Colon26, these cell lines were virtually negative for MHC-I (MFS < 0.25) as well as Qa-1b (MFS < 0.5). Activated NK and T cells are known to induce interferon γ (IFNγ) and MHC-I were up-regulated by IFNγ in most of these cell lines (Table 1). Qa-1b was minimally up-regulated in G-1, G-5 and Colon26 but not

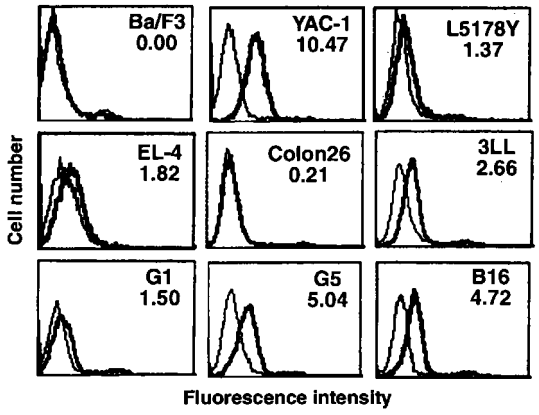

FIG. 1. Levels of RAE-1 determined by flow cytometry. Mouse tumor cell lines (5×10^5 cells) were incubated with anti-pan RAE-1 pAb (thick lines) or control rabbit IgG (thin lines) followed by FITC-labeled anti-rabbit IgG antibody. Cells were then analyzed by flow cytometry. Ba/F3, which was negative in RAE-1, was used as a control to evaluate appropriate concentrations of antibodies to determine specific and nonspecific shifts (data not shown). The values of MFS of each cell line are shown in the insets.

other cell lines (Table 1). These cell lines could be targets for NK cells as NK inhibitory receptors miss their ligands on tumor cells under the conditions where IFNγ is not yet induced. Various levels of RAE-1 were found to be expressed on these cell lines (Fig. 1). The levels of RAE-1 protein were high (>10) in YAC-1, a typical target of NK, moderate (2–10) in B16, G-5 and 3LL, and low (<2) in Ba/F3, LS178Y, EL-4, Colon26 and G-1 (Fig. 1). The levels of RAE-1 protein in these cell lines were assessed by Western blotting (Fig. 2a). The amounts of RAE-1 protein were not always in parallel with the surface levels in these cell lines, suggesting the presence of an internal pool. The relevant band of RAE-1 was a broad single band with $M_r \sim 40$ kDa. This band disappeared after PI-PLC treatment on Western (not shown) and flow cytometric analyses (Fig. 2b), confirming that all RAE-1 proteins were GPI-anchored forms recognized by anti-pan RAE-1 pAb.

Relationship between NK Sensitivity and RAE-1 Level in Mouse Tumor Cell Lines

G-1 and G-5 are sublines of mouse hepatoma cell lines derived from the same parent mass (11). The metastatic properties of G-1 and G-5 were completely altered during the passage of nonmetastatic and metastatic clones in the lung (11). The physicochemical features of G-1 and G-5 are quite similar (17) but the levels of RAE-1 were high in G-5 and low in G-1 (Fig. 1). We tested the NK-susceptibility using these sublines. Polyclonal mouse NK cells prepared from B6 mice, which express NKG2D, were cultured in IL-2 for 7 days (8). These cells were used as effectors in the cytotoxicity assay. G-5 cells were more effectively killed by the activated NK cells than G-1 cells (Fig. 3a). Similar results were obtained with different batches of NK cells. The high susceptibility of G-5 to NK was in part cancelled by treatment with $F(ab)'_2$ of anti-pan RAE-1 pAb (Fig. 3b). Intact pAb on the other hand failed to cancel NK cytotoxicity toward G-5 cells (Fig. 3b); Fc portion of IgG may sustain NK cytotoxicity in a RAE-1-independent manner. Thus, RAE-1 family proteins appear to participate in the high susceptibility of G-5 to NK cells. Expression study was performed to confirm this result. G-1 cells were transfected with *Rae-1*α cDNA and G-1 clones with stable expression of RAE-1α were established (Fig. 3c). One of the G-1 clones with a similar expression level of RAE-1α to that of G-5 were tested for NK sensitivity and the results indicated that G-1 cells were converted to NK-highly sensitive in parallel with RAE-1 expression (Fig. 3c). Again, RAE-1 conferred NK susceptibility on this NK-relatively resistant subline.

Mouse tumor cell lines with low (MFS < 0.25) and moderate MHC-I levels (2.5 < MFS < 4.5) were next used to test NK sensitivity. E/T = 10 was chosen for analysis, since this point reflected an appropriate dose for semiquantitative analysis of NK activity (not shown). The RAE-1 levels of these cell lines were roughly correlated with NK susceptibility (Fig. 4).

TABLE 1

Levels of H-2 and Qa-1b and Their IFN-γ-Responsiveness in Mouse Tumor Cell Lines

	Levels of MHC (MFS)							
Molecules	YAC-1	G-1	G-5	EL-4	3LL	B16	L5178Y	Colon26
Qa-1b	0.01	0.45	0.34	0.01	0	0	0	0
H-2Db	0.01	ND	ND	2.96	0.02	0	ND	ND
H-2Kb	0.05	ND	ND	2.66	0	0	ND	ND
H-2Dd	ND	0.21	0.14	ND	ND	ND	2.98	4.27
IFN-γ inducibility								
Qa-1b	−	+	+	−	−	−	−	+
H-2	−	+	+	+	−	+	+	++

Note. MFS up-regulation: −, <2.5; +, 10–25; ++, >25.

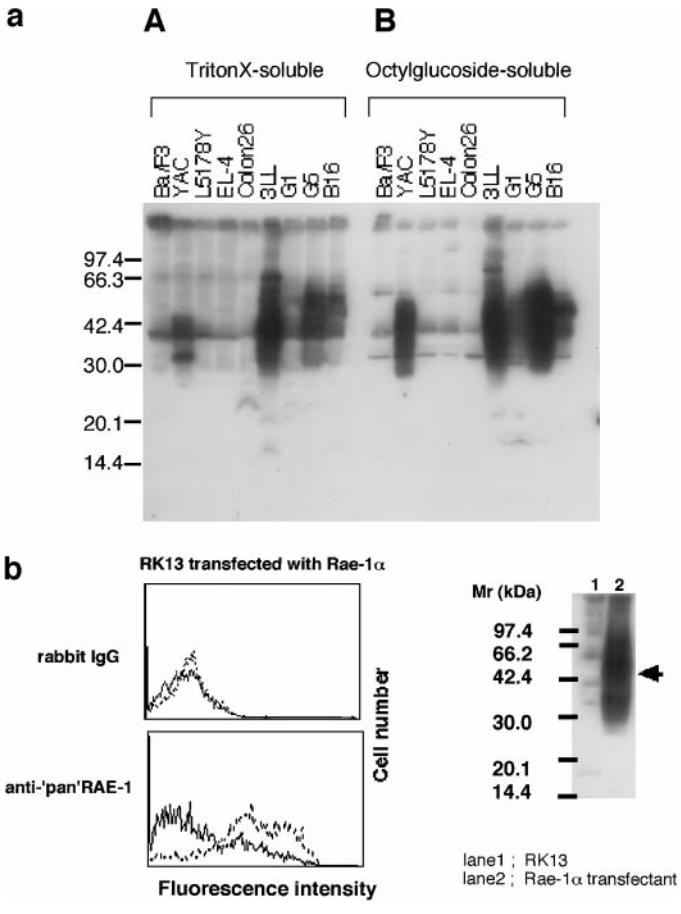

FIG. 2. Immunoblotting profiles of RAE-1 in various cell lines. (a) Mouse cell lines (5×10^5 cells) were treated with Triton X-100 and octylglycoside (see Materials and Methods) and the lysates were extracted. The lysates was subjected to SDS–PAGE (12.5% gel) under non-reducing conditions and transferred onto nitrocellulose sheets. Immunoblotting was performed using the pAb against RAE-1 and HRP-labeled second antibodies. Mouse IgG and normal rabbit serum (NRS) were used as controls. Triton X-100-soluble fractions are shown in A and octylglycoside-soluble fractions are in B. The multiple bands profiles were observed in each cell line, suggesting the different composition or sugar modification of RAE-1 family proteins in each cell line. Molecular markers are shown to the left. (b) RK cells (2×10^6 cells) transfected with Rae-1α/PEFBOS were treated with PI-PLC (solid lines) or buffer only (broken lines) as described previously (15). The cells were analyzed by flow cytometry using anti-pan RAE-1 pAb or control rabbit IgG and FITC-labeled second antibodies as in Fig. 1 (right panel). Intact RK13 (lane 1) and Rae-1 transfectant (lane 2) were solubilized by octylglycoside and analyzed by Western blotting as in (a) (right panel). The arrow indicates ~40 kDa proteins of RAE-1α.

Thus, at least total RAE-1 protein levels are likely to be associated with NK sensitivity irrespective of a certain degree of MHC-I expression in mouse tumor cells.

DISCUSSION

Establishing anti-pan RAE-1 pAb enabled us to conduct semi-quantitative analysis of total RAE-1 and RAE-1 functional blocking. This study using our antibody experimentally furthered our knowledge of tumor cell killing by NK cells: 1. RAE-1 expression (MFS $>$ 2.0) renders tumor cells NK-sensitive irrespective of MHC-I levels (MFS $<$ 4.5); 2. RAE-1 levels roughly paralleled NK sensitivity in tumor cells; 3. Blocking RAE-1 makes tumor cells NK-resistant.

Cerwenka *et al.* first demonstrated that artificial expression of RAE-1γ on RAE-1-negative cells rendered them susceptible to attack by NK cells (8). After completing this study, several reports indicated that NK cell-mediated tumor cell rejection is accomplished by expressing RAE-1γ on tumor cells (18, 19). In those studies, the levels of RAE-1 were evaluated with NKG2D with Ig tag + second Ab, because of the lack of an Ab that directly recognized RAE-1. The present results confirmed these findings using a specific antibody against pan RAE-1 and experimentally supported the importance of RAE-1 in induction of NK-mediated cytotoxicity. The results can be evaluated semiquantitatively as a requirement of MFS $>$ 2.0 of total RAE-1 for NKG2D-mediated NK cytotoxicity.

Rae-1 was initially found as a retinoic acid-inducible gene in our group (9). Retinoic acid has been used as an anti-acute promyelocytic leukemia (APL) chemotherapic agent (20). Retinoic acid is an inducer of diffentiation to mature APL to granulocytes (21). The antitumor function of retinoic acid may not be so simple, since it may simultaneously induce RAE-1 on leukemia cells to make the tumor cells NK-sensitive. Tumor cells may be cleared by two different mechanisms, differentiation and NK-mediated cytolysis. As human homologues of *Rae-1* may exist in the genome (22) and human MICA and MICB are functional homologues of mouse RAE-1 (23), this point should be examined using leukemia cells from patients treated with retinoic acid.

Further studies are needed to examine whether distinct environmental factors (cytokines, viral infection, etc.) can differentially induce the RAE-1 family genes resulting in modulation of tumor immunity. Further information concerning the regulation of these ligands might lead to potential therapeutic strategies against tumors. The RAE-1 mRNAs are produced during fetal development (9). After all, we should consider the possibility that RAE-1 serves as ligands for receptors other than NKG2D for developmental patterning as in other innate immune receptors such as Toll-like receptors (TLRs). In addition, it would be interesting whether the difference of metastatic ability between G-1 and G-5 (11, 17) is associated with RAE-1 functions.

Actually, we found conditions where tumor cells were killed by NK therapy by our anti-pan RAE-1 pAb: MFS of total RAE-1 expression $>$2.0 and MFS of MHC-I levels $<$4.5. Additional or alternative treatment including CTL induction will be needed to clear tumor cells if they show high levels of MHC-I or low

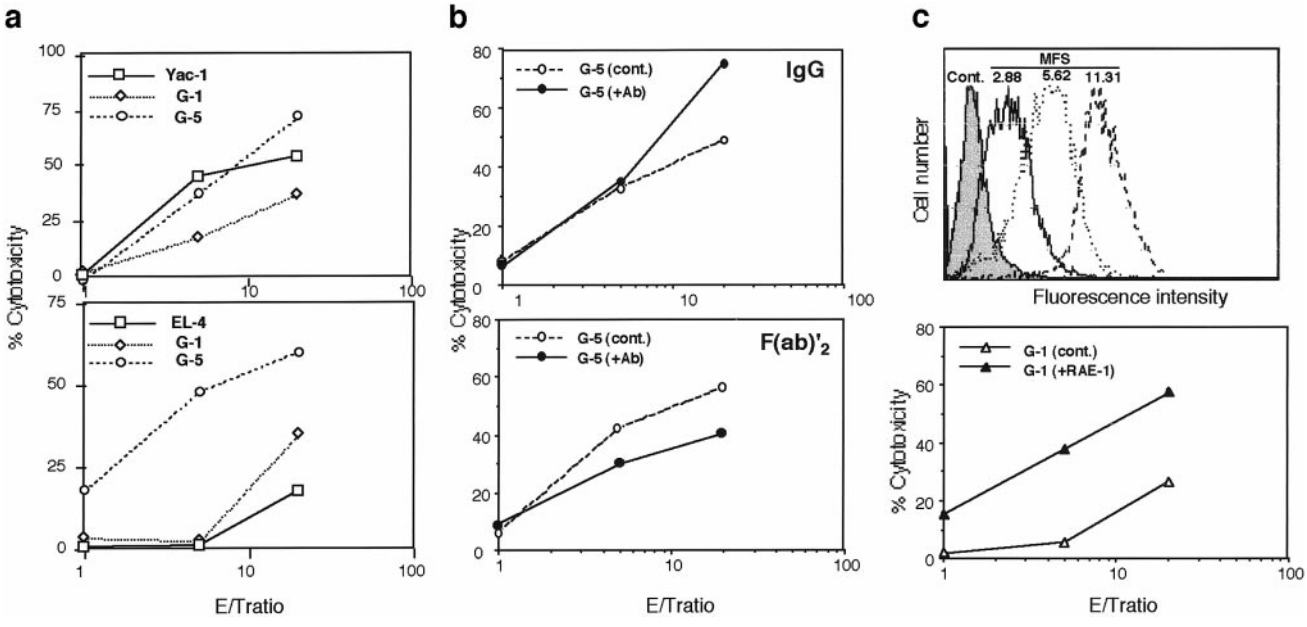

FIG. 3. NK-mediated cytolysis of G-1 and G-5 cells. (a) 4-h LDH release assays were performed using IL-2-activated NK cells from B6 mice at the indicated effector to target ratios. Open diamonds, G-1 cell killing; open circles, G-5 cell killing; open squares, Yac-1 (positive control) or EL-4 (negative control). Three experiments were resulted in similar tendencies. One of the three experiments is shown. (b) The NK-cytotoxicity assay was performed as in (a) with G-5 cells treated with anti-pan RAE-1 pAb (upper panel) or its $F(ab)'_2$ form (lower panel). Two doses (20 and 80 μg/ml) of pAb and its $F(ab)'_2$ form were employed as described under Materials and Methods, and the data using the high dose of pAb and $F(ab)'_2$ are shown as representatives. Open circles with broken lines, G-5 cell killing; closed circles with solid lines, killing of G-5 cells treated with anti-pan RAE-1 pAb or $F(ab)'_2$. A representative one of the three experiments is shown. (c) NK sensitivity of G-1 cells transfected with *Rae-1α* cDNA was tested as in (a). The three stable clones of G-1 with the constant levels of RAE-1α were established by cDNA transfection of *Rae-1α* (upper panel). The levels of RAE-1α were tested with these clones by flow cytometer using anti-pan RAE-1 pAb and second antibody, and one with a similar level (MFS = 5.62) to that of G-5 was chosen for the NK-cytotoxic assay. Cont., intact G-1 cells. The assay was performed as in (a) (lower panel). Open triangles, intact G-1 (control); closed triangles, G-1 transfected with Rae-1α. Two additional experiments confirmed the data shown here.

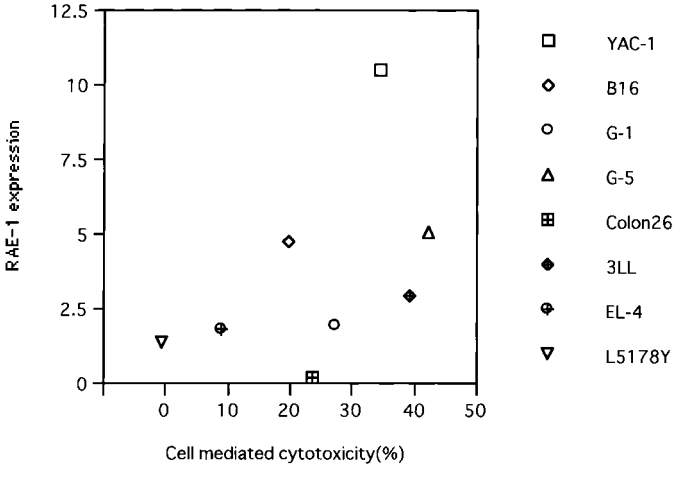

FIG. 4. Relationship between RAE-1 level and NK cytotoxicity in mouse tumor cell lines. The cell lines listed on the right were used for this study. The levels of RAE-1 proteins were assessed by flow cytometry as shown in Fig. 1. NK cytolysis assay was performed as described under Materials and Methods. E/T ratio in this study was fixed as 10. Although statistical significance was difficult to obtain from this limited scale of analysis, a correlation between RAE-1 levels and NK sensitivity appears to be present.

levels of RAE-1 expression. These combinational conditions will be attained by gene therapy or chemotherapy. In the future, NK ligand therapy may be adaptable to patients with cancer in combination with CTL-induction therapy. TLRs on dendritic cells can be activated by microbial components namely PAMP (pathogen-associated molecular pattern), which may be crucial for effective antigen-presentation by dendritic cells followed by CTL induction. Many bacterial PAMPs are ligands for different sets of TLRs and induce differential immune responses in dendritic cells (24). Finally, innate immune therapy for cancer will be able to be achieved by simultaneous activation of CTL and NK cells, which cooperatively cover high and low MHC-I-bearing tumors as immune targets. Notably, both are effectors inducible by PAMP-mediated dendritic cell maturation (25).

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