

# Deacetylase Activity of Human Tumor Cells Producing Immunosuppressive Aminosugars: Its Possible Role in Resistance to Cell-mediated Cytotoxicity<sup>1</sup>

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## Abstract

In the present study, we examined the presence of deacetylases capable of producing free hexosamines, which we have shown earlier to be immunosuppressive against human natural killer (NK) cell-mediated cytotoxicity, from *N*-acetylhexosamines in human tumor cells. When human NK-resistant colon cancer cells (Colo-320DM) were incubated with acetyl-D-[1,6-<sup>3</sup>H(N)]glucosamine, a significant conversion to [<sup>3</sup>H]glucosamine occurred. Deacetylation was demonstrated as a change of the substrate radioactivity into free glucosamine trapped by a cation exchange resin, and this was subsequently confirmed by paper chromatography. This deacetylase activity was detected in other NK-resistant tumor cell lines, especially in freshly isolated human renal and breast cancer cells and testicular seminoma cells. However, no deacetylase activity was detected in NK-sensitive target cells such as K562, MOLT-4, or HL-60 cells. The ability to produce free hexosamines from *N*-acetylated aminosugars may provide a new mechanism for the escape of tumor cells from the attack of immune effector cells such as NK cells.

## Introduction

We have previously shown that human NK<sup>3</sup> cytotoxicity can be inhibited by simple hexosamines such as D-mannosamine, D-galactosamine, and D-glucosamine (1). The inhibitory effect of aminosugars was demonstrated to be due to ATP deprivation of the effector cells, apparently resulting from the high oxygen demand of the *N*-acetylation and phosphorylation of the free aminosugars (1). *N*-Acetylated hexosamines do not similarly deprive cells of oxygen, and it is known that hexosamines generally exist as acetylated forms in nature (2). Furthermore, acetylated hexosamines do not inhibit the NK cell-mediated cytotoxicity, probably because they are not taken up by mammalian cells (3, 4). However, it is known that under certain conditions *N*-acetylhexosamines can be deacetylated by enzymatic processes (5). If free hexosamines would be secreted or shed from tumor cell-surfaces, they might be inhibitory on immune reactions and provide a mechanism of escape from the host effector cell-mediated surveillance. Tumor cells themselves proved to require considerably higher concentrations of hexosamines than lymphoid effector cells before their energy metabolism was impaired (1).

Bacterial *N*-acetylglucosamine deacetylase activity has been originally demonstrated in *Escherichia coli* (5); this enzyme acts on monomeric *N*-acetylglucosamine. Several *N*-deacetylases are known to act on bound *N*-acetylglucosamine. Recently, deacetylation of uridine diphosphate-bound acetylglucosamine has been shown to be an es-

sential step in the synthesis of lipid A of the endotoxin in gram-negative bacteria (6, 7). Also, deacetylated glucosamine has been demonstrated in cell wall peptidoglycan of *Bacillus cereus* and shown to be responsible for the notable resistance of these bacteria to lysozyme (8). Recently, the delineation of the primary structure of a fungal chitin deacetylase has suggested the existence of a group of evolutionarily distant proteins with structural and functional homology (19). In animal cells, the polymer modification process in the biosynthesis of heparin/heparan sulfate is initiated by *N*-deacetylation followed by *N*-sulfation of the polymer-bound *N*-acetylglucosamine, and the enzyme involved in this switch has been partially characterized (9, 18).

Based upon these considerations, we setup experiments to explore for the presence of GlcNAc deacetylase activity potentially capable of producing free hexosamines in human tumor cells and cell lines using monomeric substrates as targets. Our results indicated the presence of deacetylase activity in human tumor cells which correlated with the degree of resistance of the cells to NK cell-mediated cytotoxicity.

## Materials and Methods

**Cells.** Cultured cell lines used in the present study were Colo-320DM (human colon cancer cells), K562 (human myelogenous leukemia cells), U937 (human monocytic leukemia cells), and Raji (human Burkitt's lymphoma cells) obtained from the American Tissue Type Culture Collection (Rockville, MD). NUGC-4 (a human gastric cancer cell line) cells were provided by the Second Department of Surgery, Nagoya University (Nagoya, Japan). Fresh renal cell carcinoma and testicular seminoma cells were isolated from surgical specimens which were minced with scissors and incubated with a mixture of trypsin (0.25 mg/ml), DNase (0.25 mg/ml), and collagenase (0.25 mg/ml) with gentle mechanical stirring for 40 min at room temperature. Uninvolved kidney and testicular tissue cells adjacent to primary tumors were also obtained by the same method. The viable cells were harvested over Ficoll-Hypaque gradients. Cell viability was over 80%.

**Cell Culture and Medium.** Complete medium was prepared from RPMI 1640 (Gibco, Paisley, United Kingdom), 10% heat-inactivated fetal calf serum (Gibco), 0.29 mg/ml glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C in 5% CO<sub>2</sub> humidified air. In *E. coli*, the incubation of bacteria with *N*-acetylglucosamine reportedly induces the deacetylase activity (5). Therefore, for some of the assays as indicated in the text, the cells were incubated for 2 days in glucose-free RPMI 1640 (Gibco) supplemented with 1 mg/ml GlcNAc, 2% fetal calf serum, and the regular supplements.

**Cell Preparation.** Peripheral blood mononuclear cells were isolated from heparinized whole blood of normal volunteers by Ficoll-Hypaque gradient centrifugation. Adherent cells were removed by plastic adherence and filtration through nylon wool columns. Peripheral blood lymphocytes were further centrifuged on discontinuous Percoll (Pharmacia Fine Chemical AB, Uppsala, Sweden) gradients, as reported previously (1).

**Bacteria.** *E. coli* strain K12 was grown in yeast nitrogen base (pH 7.2) containing 1 mg/ml of added GlcNAc for 18 h, washed in PBS, and lyophilized. The lyophilized bacteria were resuspended in 1 ml of PBS, sonicated by a cell disruptor, and centrifuged (16,000 × g) for 20 min. The supernatants were used in the present study as positive controls.

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<sup>3</sup> The abbreviations used are: NK, natural killer; GlcNAc, *N*-acetyl-D-glucosamine; PBS, phosphate-buffered saline; MRA, *Mycoplasma* removal agent; PMA, phorbol myristate acetate.

**Detection of *Mycoplasma* in Culture and Treatment with *Mycoplasma* Removal Agent.** To exclude the possibility of contribution of *Mycoplasma* to deacetylase activity, the cells were stained with H $\ddot{o}$ chst fluorochrome bis-benzimid H33258 (10). When *Mycoplasma* infection was identified, cells were treated with 0.5  $\mu$ g/ml MRA (ICN Biomedicals, Tokyo, Japan) for 10 days. Removal of *Mycoplasma* was ascertained by the above-mentioned staining, and cells were subject to the deacetylase activity assay.

**Cytotoxicity Assay.** A standard 4-h  $^{51}\text{Cr}$  release assay was used to measure cytotoxicity as reported previously (1). Target cells were labeled with 100  $\mu$ Ci of  $\text{Na}_2^{51}\text{CrO}_4$  (Amersham International, Amersham, United Kingdom). Effector cells and target cells were mixed in a total volume of 0.2 ml/well and incubated for 4 h at 37°C in 5%  $\text{CO}_2$ ; then one-half of the supernatants were collected for counting. Maximum release was produced by lysing target cells with 1% Triton X-100 (BDH Chemicals, Poole, United Kingdom). The percentage of specific lysis was obtained by

$$\% \text{ of cytolysis} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100$$

All determinations were made in triplicate, and data were expressed as % cytolysis.

**Detection of Deacetylase Activity by Ion-Exchange Resins.** Cells (usually  $5 \times 10^6$ ) were washed in PBS twice, resuspended in 1 ml of PBS containing 0.5% bovine serum albumin, and then incubated with 5 or 10  $\mu$ Ci of acetyl-[1,6- $^3\text{H}(\text{N})$ ]-glucosamine (specific activity, 64.1 Ci/mmol; NEN, Boston, MA), acetyl-D-[1,6- $^3\text{H}(\text{N})$ ]-galactosamine (specific activity, 15 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO), or acetyl-D-[1,6- $^3\text{H}(\text{N})$ ]-mannosamine (specific activity, 40 Ci/mmol; NEN, Boston, MA) for 2–18 h. After centrifugation (400  $\times$  g), the supernatants were transferred to AG 50W-X8 cation exchange resin (200 to 400 mesh;  $[\text{H}^+]$  form; Bio-Rad; 10 ml) which had been prepacked in Econopack Bio-Rad columns. After washing in 10 volumes of water, the radioactive hexosamines adsorbed to resin were eluted in 3-ml fractions of 0.2 M pyridine acetate (pH 5.0) or 0.5 M HCl. One hundred  $\mu$ l of the eluted samples were mixed with 2.9 ml of  $\beta$ -scintillation liquid (LKB Wallac/Pharmacia, Uppsala, Sweden) and counted for radioactivity.

**Identification of Free Glucosamine in Eluted Fractions.** To identify the presence of free glucosamine, the pyridine acetate in the sample solutions was removed by lyophilization, and the samples were subjected to paper chromatography as previously reported (11). Shortly, chromatography on Whatman No. 3 CHR paper was carried out with *n*-butanol:ethanol:water, 10:1:2 by volume, and the running time was 16 h. The radioactivity in paper chromatograms was analyzed by cutting the 3-cm-wide lanes into strips of 3  $\times$  1 cm that were counted in a cocktail containing 5.5 g Permablend (United Technologies Packard, Downers Grove, IL) in 1 liter of toluene. Spots of the unlabeled marker saccharides on paper chromatograms were visualized by using ammoniacal silver nitrate.

## Results and Discussion

**Successful Demonstration of Deacetylase Activity in NK-resistant Human Colon Cancer Cells.** NK-resistant Colo-320DM cells were incubated with 10  $\mu$ Ci of acetyl-D-[1,6- $^3\text{H}(\text{N})$ ]-glucosamine for 18 h, and the supernatants were loaded on cation exchange resin columns (AG50W-X8;  $\text{H}^+$ -form). As shown in Fig. 1, significant radioactivity from tumor cell line supernatants was retained by the column and eluted with 0.2 M pyridine acetate, suggesting the presence of enzyme activity able to produce free [ $^3\text{H}$ ]glucosamine from the labeled *N*-acetylated glucosamine substrate. To exclude trivial nonspecific binding or other technical problems, it was necessary to show that the retained radioactivity really represented free [ $^3\text{H}$ ]glucosamine. Therefore, pyridine acetate was removed by lyophilization, and the samples were subjected to paper chromatography. As shown in Fig. 2, most of the radioactivity was found comigrating with glucosamine.

It has been reported that *E. coli* strain K12 grown in the presence of GlcNAc contains high *N*-acetylglucosamine deacetylase activity (5). Therefore, we used this strain grown in the presence of 1 mg/ml

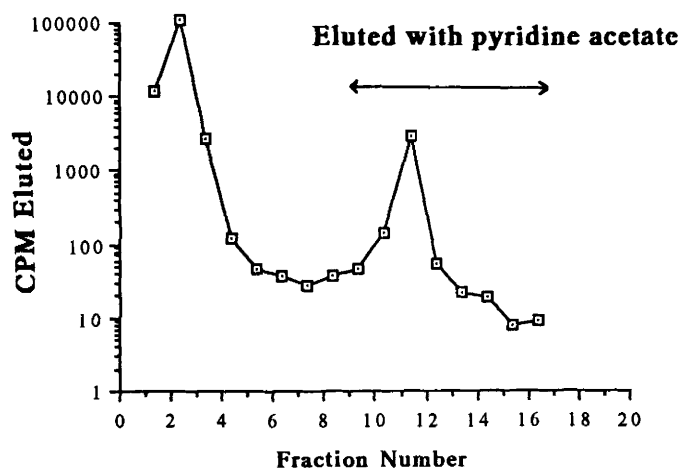


Fig. 1. Deacetylase activity in human colon cancer cells (Colo-320DM). Cells ( $5 \times 10^6$  cells) were washed twice in PBS, resuspended in 1 ml of PBS containing 0.5% bovine serum albumin, and incubated with 10  $\mu$ Ci of  $^3\text{H}$ -acetyl-D-glucosamine for 18 h. After centrifugation (400  $\times$  g), the supernatant was transferred to a 10-ml column of AG50W-X8 ( $\text{H}^+$ ) cation exchange resin. After washing in 10 volumes of water (fractions 1–8), the attached radioactivity was eluted with 0.2 M pyridine acetate (pH 5.0) (fractions 9–16, 3 ml/fraction).

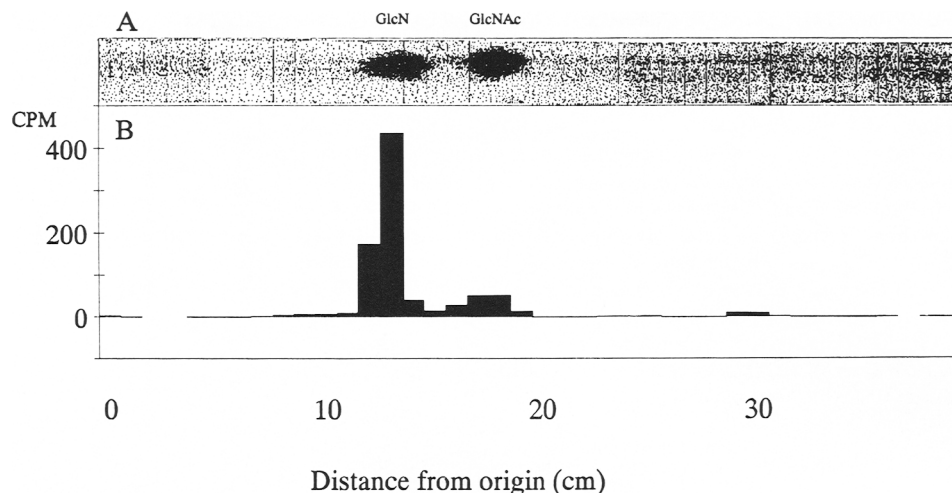
GlcNAc for the positive control of the assay. When the bacterial homogenates were incubated with acetyl-D-[1,6- $^3\text{H}(\text{N})$ ]-glucosamine and subsequently run on cation exchange resins, significant radioactivity was retained by the cation exchanger column and eluted with pyridine acetate (Table 1, experiment 1). This confirmed the presence of deacetylase in *E. coli* grown in GlcNAc. Taken together, the data thus show that deacetylase activity is present in cultured human carcinoma cells.

**Effect of Incubation Time on Enzyme Activity and Its Substrate Specificity.** When the effect of incubation time on deacetylase activity was examined, it was higher after 18 h of incubation than in 2 h of incubation (Table 1, experiment 2). Substrate specificity was also examined. When Colo-320DM cells were incubated with various *N*-acetyl- $^3\text{H}$ -hexosamines, the highest deacetylase activity was detected with GlcNAc (Table 1, experiment 3), but it was also clear that *N*-acetylgalactosamine and *N*-acetylmannosamine were substrates for deacetylation, although less efficiently. It is not known whether bound *N*-acetylhexosamine residues found in the saccharides of glycoproteins or glycolipids are deacetylated by this enzyme.

**Contribution of *Mycoplasma* Contamination to Deacetylase Activity.** In the course of the study, it turned out that some of our Colo-320DM cells were infected with *Mycoplasma*. Therefore, the contribution of *Mycoplasma* to deacetylase activity was examined. *Mycoplasma*-infected Colo-320DM cells were treated with MRA for 10 days. This duration of treatment is recommended by the manufacturer for the complete cure of *Mycoplasma*. After treating with MRA, *Mycoplasma* contamination was rechecked with H $\ddot{o}$ chst fluorochrome staining at the same time with the deacetylase assay. The deacetylase activity of Colo-320DM cells was not affected by MRA treatment in spite of morphologically ascertained cure of the *Mycoplasma* infection (Table 1, experiment 4). We also purposely infected K562 and HL-60 cells with *Mycoplasma* aliquots from infected cultures but could not detect deacetylase activity (data not shown). Thus, we consider it unlikely that *Mycoplasma* contamination contributed to the deacetylase activity of cells.

**Relationship of Deacetylase Activity to NK Resistance.** Glucosamine, galactosamine, and mannosamine are highly inhibitory to human NK cytotoxicity *in vitro* (1). To examine the role of the deacetylase activity in providing free aminosugars in the cellular vicinity and thus possibly providing protection against NK effector cells, we examined the deacetylase activity of various NK-sensitive

Fig. 2. Direct demonstration of free glucosamine in the samples eluted with pyridine acetate from the cation exchange resin. A, pyridine acetate fractions from Colo-320DM cells were lyophilized for removal of the pyridine acetate; the samples were redissolved in water, mixed with unlabeled glucosamine and GlcNAc, and subjected to paper chromatography. Unlabeled glucosamine and GlcNAc were also subjected to paper chromatography at the same time to identify the position of each sugar responsible for the radioactivity. B, the sample lane on the Whatman paper was cut in 1-cm stripes which were soaked in 200  $\mu$ l of  $\beta$ -scintillation liquid and counted for radioactivity.



and -resistant tumor cells. As can be seen in Table 1, the NK-resistant tumor cell lines COLO-320 (colon carcinoma line), NUGC-4 (gastric carcinoma line), and Raji (human Burkitt's lymphoma) showed significant deacetylase enzyme activity (Table 1, experiment 5). Furthermore, freshly isolated human tumor cells such as human renal cell carcinoma, testicular seminoma cells, and breast cancer cells also showed a significant deacetylase activity (data not shown). Specially, cells from a renal cell carcinoma showed remarkably high deacetylase activity which was on a per cell basis almost 30 times higher than in COLO-320DM cells (data not shown). Freshly isolated human tumor cells are generally NK resistant (12). In contrast, very little deacetylase activity was detected in NK-sensitive cell line cells such as K562, MOLT-4 cells, or HL-60 cells, with some exception of moderately positive deacetylase activity in U937 cells (Table 1, experiment 5; Fig. 3A). The incubation of these cells in the presence of 1 mg/ml GlcNAc, which is reportedly able to induce deacetylase activity in *E. coli* (5), did not induce the enzyme activity in these cells either (data not shown). However, the treatment of HL-60 cells with PMA ( $10^{-8}$  M),

known to push HL-60 cells towards monocyte-macrophage phenotypic differentiation, in the presence of 1 mg/ml GlcNAc induced significant deacetylase activity (Fig. 3A). A concomitant increase in the resistance of HL-60 cells to NK-mediated cytotoxicity was also seen (Fig. 3B) as has been reported earlier (13). These results thus suggested the possibility that the deacetylase activity of tumor cells might be associated with NK resistance. We have shown previously that hexosamines are highly suppressive on NK cytotoxicity at 2–25 mM, whereas these concentrations did not affect the growth or viability of the target tumor cell lines (1). Below these concentrations, hexosamines (specifically glucosamine) are not toxic and are metabolized to glycogen (14) or utilized for the synthesis of glycoproteins (15). Simple calculations of the range of concentrations of free aminosugars measured in the cell supernatants of the present experiments show that they are at least two orders of magnitude below the inhibitory concentrations above. However, no attempts have been made to study and implement possibly more optimal conditions for the reaction to improve the yield, and some of the liberated [ $^3$ H]glucosamine may have

Table 1 Deacetylase activity in various conditions and tumor cells

Experiment	Cells or bacteria	Incubation with [ $^3$ H]acetylhexosamine <sup>a</sup>	H <sub>2</sub> O eluates cpm	Deacetylase activity <sup>b</sup> (cpm) <sup>c</sup>	Conversion to [ $^3$ H]hexosamine
1	<i>E. coli</i> (K12) <sup>d</sup>	GlcNAc (10 $\mu$ Ci, 18 h)	245,899	6,540	2.66
2	Colo-320DM	GlcNAc (5 $\mu$ Ci, 2 h)	74,594	373	0.50
	Colo-320DM	GlcNAc (5 $\mu$ Ci, 18 h)	86,300	837	0.96
3	Colo-320DM	GlcNAc (5 $\mu$ Ci, 18 h)	73,374	1,085	1.46
	Colo-320DM	GalNAc (5 $\mu$ Ci, 18 h)	60,647	360	0.59
	Colo-320DM	ManNAc (5 $\mu$ Ci, 18 h)	80,975	135	0.17
4	Colo-320DM <sup>e</sup>	GlcNAc (10 $\mu$ Ci, 18 h)	254,873	1,200	0.47
	Colo-320DM (MRA-treated) <sup>f</sup>	GlcNAc (10 $\mu$ Ci, 18 h)	251,430	1,247	0.49
5	K562	GlcNAc (5 $\mu$ Ci, 18 h)	72,041	111	0.14
	MOLT-4	GlcNAc (5 $\mu$ Ci, 18 h)	67,201	124	0.18
	U937	GlcNAc (5 $\mu$ Ci, 18 h)	56,837	667	1.17
	Raji	GlcNAc (5 $\mu$ Ci, 18 h)	42,850	1,049	2.44
	NUGC-4	GlcNAc (5 $\mu$ Ci, 18 h)	82,489	990	1.20

<sup>a</sup> Cells ( $2 \times 10^6$  cells) were incubated with 5 or 10  $\mu$ Ci of [ $^3$ H]GlcNAc, [ $^3$ H]acetyl-galactosamine (GalNAc), or [ $^3$ H]acetyl-mannosamine (ManNAc) in PBS containing 0.5 bovine serum albumin for 2 to 18 h. After centrifugation ( $400 \times g$ ), supernatants were loaded on a column of AG 50W-X8(H<sup>+</sup>) cation exchange resins.

<sup>b</sup> After washing in water, the radioactive *N*-acetyl-hexosamines adsorbed to resins were eluted with 0.2 M pyridine acetate and the radioactivity of each fraction (100  $\mu$ l) was counted. Deacetylase activity was calculated as a sum of positive counts (cpm) over the background elution. The background was set as the eluted radioactivity of control experiment in which only radioactive hexosamines in 0.5% bovine serum albumin/PBS (without tumor cells or bacteria) was challenged on AG 50W-X8(H<sup>+</sup>) cation exchange resins.

<sup>c</sup> The counting efficiency (dpm/cpm) was 50 in all experiments.

<sup>d</sup> Lyophilized bacteria (2.5 mg) were resuspended in 1 ml of PBS, sonicated by cell-disruptor, and centrifuged ( $16,000 \times g$ ) for 20 min. The supernatants were used in the present study as positive controls.

<sup>e</sup> *Mycoplasma*-infected Colo320-DM cells.

<sup>f</sup> *Mycoplasma*-infected Colo-320DM cells were treated with MRA for 10 days. The cure of the infection was checked by staining with H $\ddot{o}$ chst fluorochrome bisbenzimid H33258.

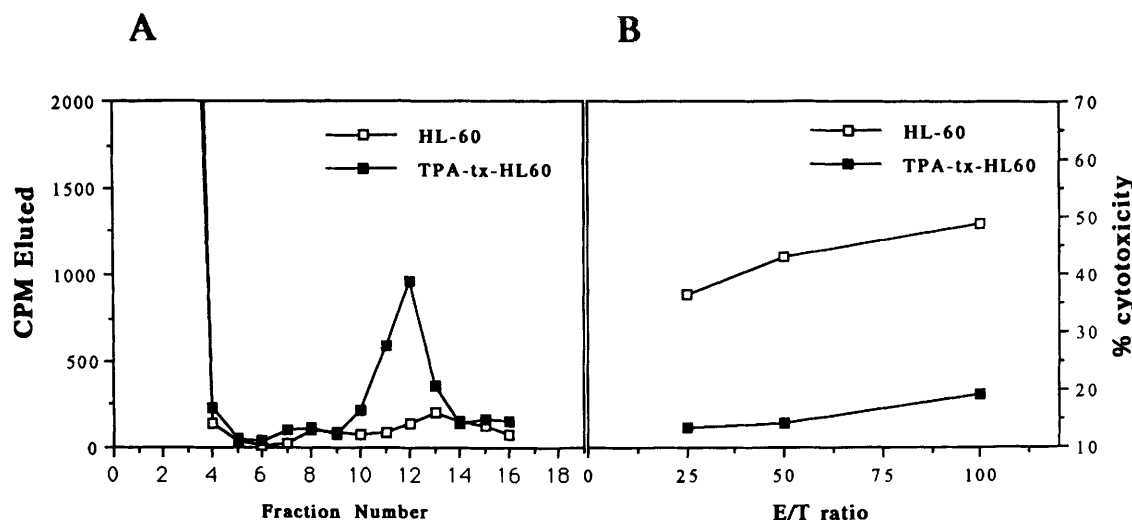


Fig. 3. A, TPA-induced deacetylase activity in HL-60 cells. HL-60 cells were incubated with  $10^{-8}$  M PMA for 2 days and washed in PBS thoroughly. Both HL-60 and TPA-treated HL-60 cells were incubated with  $^3\text{H}$ -acetyl-D-glucosamine for 18 h, and the supernatants of those cells were subjected to cation exchange resins. After washing in water (fractions 1–8), the positive radioactivity was eluted with 0.5 M HCl (fractions 9–16). B, NK susceptibility of HL-60 and PMA-treated HL-60 cells. The same TPA-untreated and treated HL-60 cells as above were tested for sensitivity to NK killing using Percoll density gradient-enriched human large granular lymphocytes as effectors. NK activity was determined in a 4-h  $^{51}\text{Cr}$  release assay. Mean  $\pm$  SD of triplicate samples.

entered the cells, thus escaping detection. Local conditions in tissues probably vary greatly, and also time may be a critical factor for potentially effective concentrations to be reached *in vivo*. Furthermore, it is possible that the deacetylase activity might be decreased during the culture of tumor cells.

We do not know the relationship of the deacetylase(s) revealed in the present experiments using monosaccharides as substrates to those described with bound hexosamine substrates in certain bacterial systems (see below). The characterization of any of these enzymes at the protein or gene level has not been performed to our knowledge, and thus direct resistance transfer experiments or cellular localization studies must await further advances in this area. Hexosamines are contained as components of bacteria such as cell walls or of their products (16). In most gram-negative bacteria, UDP-bound GlcNAc is enzymatically deacetylated, and one fatty acid is added to the deacetylated glucosamine for the synthesis of the lipid A of bacterial lipopolysaccharides (6, 7). A significant amount of glucosamine with free amino residues has been reported to be present in the cell wall polypeptidoglycan of *Bacillus cereus* (8), and the presence of deacetylase to convert polysaccharide-bound GlcNAc residues to glucosamine has also been reported (17). Interestingly, this bacterial cell wall with deacetylated glucosamine residues is responsible for the resistance of the microbe to lysozyme digestion (8). It is thus a significant factor in the bacterial evasion of the host defense; lipid A of bacterial lipopolysaccharide also plays a major role as a survival factor. It may well be that one key enzyme in the synthesis of these substances might have evolved to counteract other host surveillance mechanisms, such as cell-mediated immunity, and then also was adopted by tumor cells for the same purpose.

The presence of a hexosamine deacetylation step is an essential part of heparin/heparan sulfate synthesis in animal cells. In this system, analysed in a mouse mastocytoma model, the activity was intimately associated with sulfotransferase activity replacing the acetyl with a sulfate group, and very few *N*-unsubstituted glucosamine residues remained. The combined deacetylation-sulfation activities always migrated with a single  $M_r$  110,000 protein (9), and it has been shown that the same protein catalyzes both deacetylation and sulfation during the biosynthesis of heparin sulfate in the rat liver (18). The relationship of the deacetylation step in these systems and the present study describing significant production of free unsubstituted aminosugars remains

to be explored as well as the possible evolutionary homologies of the enzymes involved (19). In particular, the possible association with NK resistance might shed further light on the critical question of how malignant cells escape from the attack of immune effector cells.

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