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Minoru Ono and Noriaki Tanaka

Abstract

Spleen cells from tumor-bearing mice showed decreased natural killer (NK) activity and decreased binding to target cells with progression of the tumor. Treatment of spleen cells from tumor-bearing mice with vibrio cholerae neuraminidase (VCN) increased the cytotoxicity to a level twice or more as high as that of untreated cells, but the same treatment of spleen cells from normal mice had no or little effect. On the other hand, neither in spleen cells from tumor-bearing mice nor in those from normal mice, the VCN treatment had no effect on their binding to M-HeLa cells. The suppression of NK activity by preincubation with serum from tumor-bearing mice or prostaglandin E2 was completely abolished by VCN treatment. The above results indicate that VCN treatment of lymphocytes might augment NK activity by an antagonistic effect against an immune suppressive factor.

KEYWORDS: NK cell, neuraminidase, tumor-bearing serum, target-binding cell, MH-134 hepatoma.

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Augmentation of Natural Killer Activity by Neuraminidase Treatment of Lymphocytes from Tumor-Bearing Mice

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Spleen cells from tumor-bearing mice showed decreased natural killer (NK) activity and decreased binding to target cells with progression of the tumor. Treatment of spleen cells from tumor-bearing mice with vibrio cholerae neuraminidase (VCN) increased the cytotoxicity to a level twice or more as high as that of untreated cells, but the same treatment of spleen cells from normal mice had no or little effect. On the other hand, neither in spleen cells from tumor-bearing mice nor in those from normal mice, the VCN treatment had no effect on their binding to M-HeLa cells. The suppression of NK activity by preincubation with serum from tumor-bearing mice or prostaglandin E_2 was completely abolished by VCN treatment. The above results indicate that VCN treatment of lymphocytes might augment NK activity by an antagonistic effect against an immune suppressive factor.

Key words : NK cell, neuraminidase, tumor-bearing serum, target-binding cell, MH-134 hepatoma.

Natural cytotoxicity may play an important role in immune surveillance against tumors and viral infection in experimental animals and in man (1-3). Diminished natural killer (NK) activity has been found in tumor-bearing animals (4) and in humans with malignant disease, especially those with widely disseminated tumors (5-7). The mechanism of suppression in these situations is not clearly understood. It has been reported that the treatment of peripheral blood lymphocytes from healthy donors with vibrio cholerae neuraminidase (VCN) increases their cytotoxic activity against tumor cells *in vitro* (8). In order to analyze this phenomenon, changes in NK activity and in target binding of spleen cells after treatment with VCN were studied in tumor-bearing mice.

Materials and Methods

Mice and Tumors. Inbred C3H/He and CBA mice, 8-10 weeks of age, were supplied by the Mouse Colony of the Okayama University Medical School.

MH-134 ascites hepatoma cells (1×10^6 cells/0.1 ml) were inoculated subcutaneously in the back of C3H/He mice. Spleen and peripheral blood samples were taken at various intervals after tumor inoculation to obtain spleen cells and serum.

Effector Cells. Spleens were removed aseptically and cut into small pieces in culture medium (CM) consisting of Rosewell Park Memorial Institute Tissue Culture Medium 1640 (RPMI 1640) with 100 μg/ml streptomycin, 100 U/ml penicillin (Meiji Pharmaceutical Co., Ltd.), 25 mM N'-2-hydroxy ethylpiperazene N'-2-ethane sulfonic acid (HEPES) and 10% heat-inactivated fetal calf serum (FCS, GIBCO). The cell suspension and spleen fragments were passed through #150 plat-

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inum mesh, and the erythrocytes were lysed with 0.75% NH_4Cl-tris buffer at room temperature for 1 minute. The cells were then centrifuged and washed twice with RPMI 1640 and once with CM solution.

Reagents. Serum from tumor-bearing mice was obtained 17 days after the MH-134 tumor inoculation and inactivated by heating at 56°C for 30 min. Vibrio cholerae neuraminidase (VCN) was dissolved in culture medium. Aspirin (500 μg/kg) or indomethacin (5 mg/kg) dissolved in saline was administrated for 7 days after the MH-134 tumor inoculation and again 24 h before measuring the NK activity. Prostaglandin E_2 (PGE_2) was dissolved in 95% ethanol and then diluted in culture medium to the desired concentration. Ethanol up to 0.01% did not affect cell viability or NK activity. IFN (mouse crude interferon) produced by L-cells derived from mouse fibroblasts was used.

Treatment of spleen cells with each reagent. Spleen cells (2×10^7 cells) of normal mice or MH-134 tumor-bearing mice were treated with serum from tumor-bearing mice, VCN (0-250 U/ml), PGE_2 (10^{-6} M) or IFN (4×10^3 U/ml) for 1 h at 37°C and washed three times with CM.

Target cells and NK assay. The details of the NK assay have been described elsewhere (4). M-HeLa cells, measles virus infected HeLa cells, and YAC-1 cells, Molony virus induced lymphoma cells of A/Sn mice, were used as target cells in the NK assay. The target cells were labeled with 200 μCi of $Na_2[^{51}Cr]O_4$ (New England Nuclear, Boston, MA., specific activity 200-500 Ci/g) for 60 minutes in 0.5 ml of CM. The target cells were then washed 3 times with CM and added to effector cells at an effector cell to target cell (E/T) ratio of 50 or 100 in flat-bottomed micro-test III tissue culture plates (Falcon 3072). The total volume was adjusted to 200 μl/well. The plates were incubated at 37°C under a humidified 5% CO_2 atmosphere for 6 hours (for YAC-1) or 12 h (for M-HeLa). The culture supernatants were then harvested, and radioactivity was counted in a gamma-counter. Spontaneous release was measured by incubating the targets in CM alone. The percentage of lysis (NK activity) was calculated by the following formula :

$$\% \text{ cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100.$$

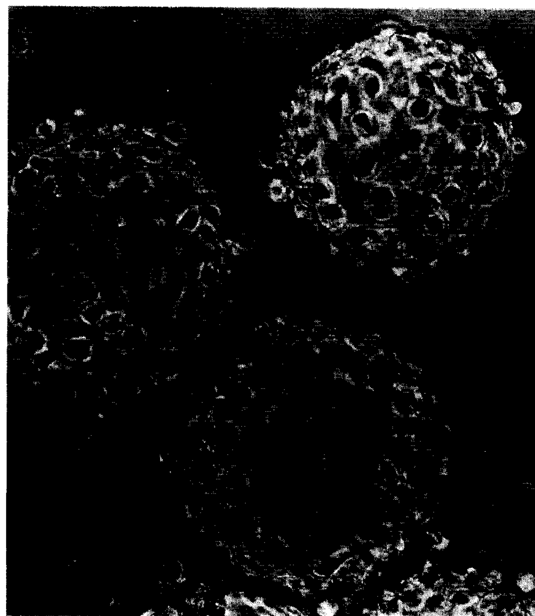

Fig. 1 The microscopic appearance of Superbeads nearly confluent with M-HeLa target cells. $\times100$.

Target binding of effector cells. As an assay for lymphocyte binding with target cells, we applied method of Saksela (9). This method, using Superbeads (Flow Laboratories, U.S.A.), detects the effector-target conjugate, making quantification easy with a gamma-counter. After Superbeads (0.2 mg in 1 ml) were incubated with 2.5×10^6 target cells for 5 days in a tissue culture flask (Falcon 3013, U.S.A.), the Superbead surfaces were nearly saturated with target cells (Fig.1). Two hundred μCi of ^{51}Cr was added to 2×10^6 lymphocytes, and then the mixture was incubated at 37°C for 1 h. The lymphocytes were washed three times. The lymphocytes (1×10^6) were mixed with 0.2 ml (0.04 mg) of Superbeads with the attached target cells, and incubated for 6 h. The suspension was gently removed to test tubes filled with FCS, and the tubes were left to stand for 30 minutes. Non-adherent lymphocytes were collected from the upper layer, and lymphocytes adherent to the beads from the bottom of the tube. Radioactivity of each fraction was compared with Superbeads pretreated only with the medium. The percent of binding cells was calculated in accordance with the following formula :

$$\% \text{ binding cells} = \left(\frac{\text{cpm of lymphocytes adhering to experimental beads}}{\text{cpm of total lymphocytes}} - \frac{\text{cpm of lymphocytes adhering to control beads}}{\text{cpm of total lymphocytes}} \right) \times 100.$$

Results

Kinetics of target binding and natural killing of normal lymphocytes. The binding of normal lymphocytes to target cells increased rapidly during the first hour, reached a peak at 3 h, and decreased slowly thereafter. On the other hand, the natural killing progressed slowly for the first 6 h, then the activity rapidly elevated, and reached a plateau at 12 h (Fig. 2). Thus, there was a long interval of 9 hours between the maximal binding and the maximal killing.

Changes in NK activity and target binding of spleen cells by tumor progression. Both NK activity and binding of spleen cells to target cells decreased with the progression of the tumor. The decrease in NK activity was more marked than that in target binding. The spleen cells obtained from tumor-bearing mice 17 days after the MH-134 tumor inoculation showed little NK activity.

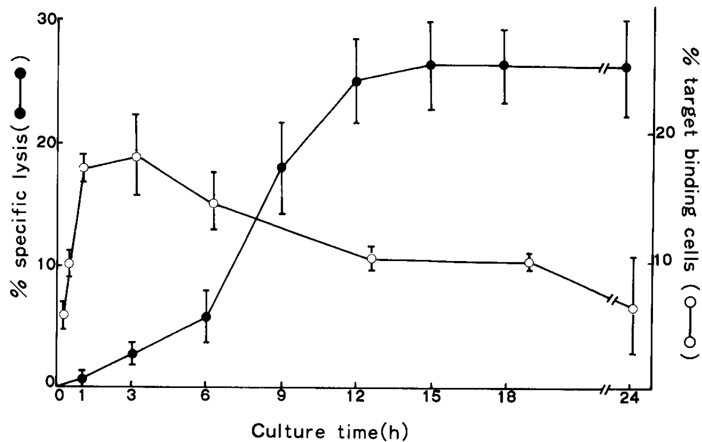

Fig. 2 Kinetics of natural killing (●——●) and target binding (○——○) of normal spleen cells against M-HeLa target cells. The values are expressed as the mean ± S. D. of 3 separate experiments.

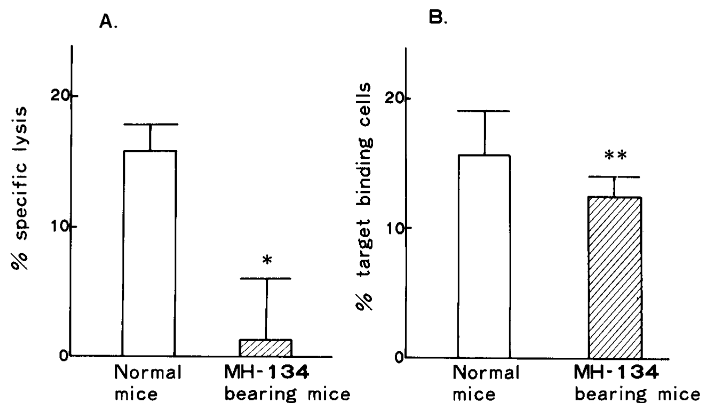

Fig. 3 Changes in natural killing (A) and target binding (B) of spleen cells from MH-134 tumor-bearing mice. The values are expressed as the mean ± S. D. Significance of the difference from normal mice : $^{*}p < 0.001$, $^{**}p < 0.1$ (Student's t-test).

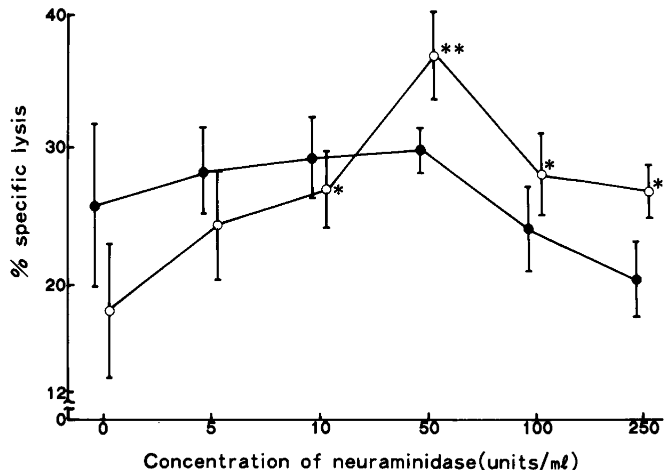

Fig. 4 The effect of various concentrations of neuraminidase (VCN) on NK activity of spleen cells from normal (●——●) and MH-134 tumor-bearing mice (○——○) against M-HeLa target cells. Significance of the difference from spleen cells treated with medium only : $^*p < 0.01$, $^{**}p < 0.001$ (Student's t-test). The values are expressed as the mean ± S. D.

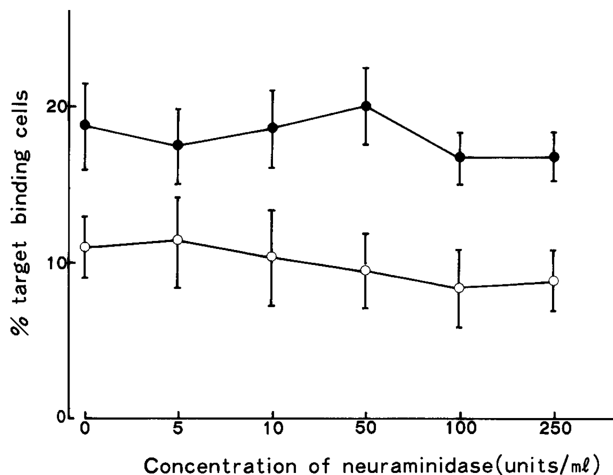

Fig. 5 The effect of neuraminidase (VCN) treatment on binding to M-HeLa target cells of spleen cells from normal mice (●——●) and tumor-bearing mice (○——○). Spleen cells were obtained 16 days after the MH-134 inoculation.

Binding to target cells was retained at a low level, but showed no further reduction until death (Fig. 3).

Influence of vibrio cholerae neuraminidase (VCN) treatment of effector cells on the NK activity and target binding. The changes in NK activity and binding activity against M-HeLa cells after treatment with VCN (5 U/ml to 250 U/ml) were examined. The NK activity of normal spleen cells exhibited no significant change after the treatment, but in the spleen cells 16 days after tumor inoculation, the NK activity was augmented after VCN treatment (50 U/ml), showing a peak activity exceeding that of normal spleen cells (Fig. 4). On the contrary, the binding activity to target cells was not influenced significantly by VCN treatment in normal spleen cells or in spleen cells from tumor-bearing mice (Fig. 5).

When using YAC-1 cells as target cells, the VCN treatment brought a dose dependent increase in NK activity of not only the normal spleen cells but also the spleen cells from tumor-bearing mice (Fig. 6). The activity of the latter exceeded that of the former at VCN concentrations of 50 U/ml to 100 U/ml.

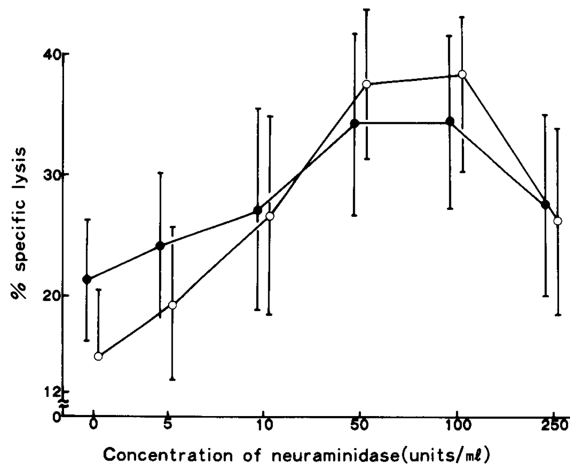

Fig. 6 The effect of various concentrations of neuraminidase (VCN) on NK activity of spleen cells from normal mice (●——●) and MH-134 tumor-bearing mice (○——○) against YAC-1 target cells. The values are expressed as the mean ± S. D.

The suppressive effect of the serum from tumor-bearing mice and the recovery from the suppression by VCN treatment. NK activity of tumor-bearing mice disappeared completely 17 days after tumor inoculation. When normal spleen cells were incubated at 37℃ for 60 min with serum from the tumor-bearing mice, the NK activity and target binding were both inhibited. This inhibitory effect was noted up to a 50-fold dilution of the original serum (data not shown). The target binding of lymphocytes was less affected by the serum than NK activity. When the spleen cells were preincubated with VCN, the binding activity recovered to the normal level, and the NK activity exceeded the normal level by 150% augmentation (Table 1).

Effect of in vivo administration of aspirin or indomethacin on NK suppression of serum from tumor-bearing mice. Tumor-bearing C3H/He mice 17 days after the MH-134 tumor inoculation, expected to have serum suppressive to NK cells, were treated previously with an oral administration of aspirin (500 μg/kg) or indomethacin (5 mg/kg). Twenty-four hours after the last administration, sera were obtained to test for the presence of a suppressive factor against NK activity. The serum from tumor-bearing mice without pretreatment blocked considerably the NK activity of normal spleen cells.

Table 1 Influence of serum from tumor-bearing mice and neuraminidase (VCN) on NK activity and binding to target cells

Source of Cells	Treatment	% Cytotoxicity[a]		% Binding
		YAC-1	M-HeLa	
Normal spleen	Medium	31.0±1.5[b]	26.3±2.2	19.8±0.8
	Normal serum	30.6±1.2	21.0±2.1	14.8±1.1
	MH-134 serum	15.6±1.0	9.6±1.4	11.8±1.1
	MH-134 serum+VCN[c]	45.5±2.3*	36.6±2.7*	17.1±1.6

a : YAC-1 cells (1×10^4) or M-HeLa cells (5×10^3) labeled with ^{51}Cr, and effector cells (E/T = 100 : 1) were mixed, cultured at 37℃ with 5% CO_2 for 12 hours (M-HeLa) or 6 hours (YAC-1).

b : Mean± S. D. Significance of the difference from MH-134 serum, $^*p < 0.01$.

c : Spleen cells (2×10^6) were treated for 1 h with VCN (50 U/ml) and serum 16 days after the MH-134 transplantation.

Table 2 Effect of *in vivo* administration of aspirin or indomethacin on NK suppression of serum from tumor-bearing mice

Source of Cells	Treatment with serum from		% Cytotoxicity[a]	
			Target cells	
			M-HeLa	YAC-1
Normal spleen	Normal mice	Saline	15.8±2.0[b]	22.4±3.5
		Aspirin	13.2±2.8	23.0±4.2
		Indomethacin	15.2±2.5	21.4±2.9
	MH-134 mice	Saline[c]	3.3±4.2	7.6±2.8
		Aspirin[c]	13.0±3.4	19.6±3.3
		Indomethacin[c]	13.6±3.0	18.7±3.4

a : YAC-1 cells (1×10^4) or M-HeLa cells (5×10^3) labeled with ^{51}Cr, and effector cells (E/T = 50) were mixed, cultured at 37°C with 5% CO_2 for 12 hours (M-HeLa) or 6 h (YAC-1).
b : Percent cytotoxicity, mean±S. D.
c : 17 days after MH-134 transplantation.

By the previous administration of these agents, the serum lost its suppressive function completely (Table 2).

Effect of VCN on PGE_2 depressed NK activity. It has already been shown that NK activity can be inhibited by PGE_2. This experiment was undertaken to examine the effect of VCN or interferon treatment on NK activity and target binding of spleen cells which were pretreated with PGE_2. Treatment with VCN returned the depressed NK activity of PGE_2-pretreated normal spleen cells to the normal level, as did IFN. In PGE_2-pretreated lymphocytes from tumor-bearing mice, in which NK activity was almost negligible, NK activity returned to the normal level after VCN treatment. On the other hand, IFN was not so effective. Target binding of spleen cells was slightly decreased by PGE_2, but it was not affected by successive treatment with VCN or IFN (Table 3).

Discussion

Diminished NK activity has been found in tumor-bearing animals (4) and in humans with malignant disease (5-7). It has been reported that cancer patients who had diminished NK activity did not have decreased numbers of large granular lympho-

Table 3 Effect of neuraminidase VCN on PGE_2 pretreated NK cells

Source of Cells	PGE_2 Pretreatment[a]	% Cytotoxicity[b]			% Binding		
		Treatment[c]			Treatment[c]		
		None	IFN	VCN	None	IFN	VCN
Normal	−	25.8±6.2[d]	40.2±4.6	29.8±1.6	17.7±3.4	18.2±4.1	16.3±3.5
	+	13.3±2.8	27.7±3.2	25.2±3.7	15.2±3.5	17.0±3.2	16.4±2.9
MH-134[e]	−	14.0±5.1	32.3±4.3	36.7±5.6	13.5±2.6	12.0±3.7	10.2±3.4
	+	5.7±2.0	19.6±1.8	26.1±2.8	8.9±4.3	11.3±2.8	8.8±3.3

a : Spleen cells were pretreated for 30 min with medium only (−) or 10^{-6}M PGE_2 (+).
b : See Table 2. M-HeLa were used as target cells.
c : Treatment with IFN (4×10^3 U/ml) or VCN (50 U/ml) for 1 h at 37°C.
d : Percent cytotoxicity, mean±S. D.
e : Tumor-bearing mice 10 days after the MH-134 transplantation.

cytes (LGL) in comparison with normal donors (10). In this study, we observed that both the NK activity and target binding of spleen cells were decreased in tumor-bearing mice. The former was more affected than the latter.

The existence of cells and factors regulating the level of NK activity both *in vivo* and *in vitro* is suggested by experimental evidence. Interferon enhances the activity of NK cells (11, 12). Prostaglandin has both an inhibitory effect and a stimulatory effect on NK cells (13, 14). In humans, carcinomatous pleural effusion was found to contain macrophages which suppress NK activity (15). In our study, the preincubation of normal spleen cells with serum of tumor-bearing mice was found to decrease both NK activity and target binding of effectors. The suppressive effect of the serum was eliminated when the tumor-bearing mice were administered either aspirin or indomethacin. Therefore, prostaglandin appears to be related to the phenomenon of NK inhibition by the tumor-bearing mouse serum. Serum factors, such as $\alpha 1$ antitrypsin (16) and immunosuppressive substance (ISS) (17) are known to be NK inhibitors. These substances, which are serum components elevated during acute inflammation, have been implicated in the suppression of various immunological responses. Binding of substances to cell membrane may result in a marked increase in cell surface associated sialic acid and hence in negative surface charge. Such an increase in negative surface charge could alter antigenic recognition, cellular incorporation and specific membrane-associated events leading to lymphoid cell activation.

Neuraminidase treatment has been shown to enhance the attachment of effector and target cells (18) and to augment the immune response *in vivo* (19) and *in vitro* (20). It has been reported that neuraminidase increases both cell-mediated cytotoxicity (CMC) and antibody-dependent cellular cytotoxicity (ADCC) (21). In the present study, the treatment of spleen cells from tumor-bearing mice was found to augment the depressed NK activity to an extent exceeding that of the normal level.

There are several possible explanations for the activation effect. A change in the topologic distribution and exposure of hidden receptor-sites could increase the contact between the attacker and the targets cells. Sialogylcoproteins and sialic acid could contribute to the negative charge of the cell surface, so that removal of sialic acid by neuraminidase would markedly decrease the electro-negative charge. It is possible that mild proteolysis with neuraminidase reduces the repulsive forces and increases contact between reacting cells. Non-specific blocking factors or specific blocking immune complexes could be removed by proteolysis. Stimulation of cellular metabolic activity could result from enzyme treatment.

It was reported that the enhanced cell mediated cytotoxicity following VCN-treatment may result from an activation of killer cells, including VCN-treated T-lymphocytes which possess an increased capacity to attach to target cells (8). Our studies have indicated that NK activity was enhanced following exposure of lymphocytes to VCN, whereas target binding activity was not significantly changed. This seeming discrepancy is not contradictory since, in Galili's study, the increased attachment of VCN-treated T-lymphocytes was not associated with the increased killer activity of T-lymphocytes themselves. Therefore, the augmentation of NK activity was not thought to be related to increased contact of target cells and effector cells, nor to exposure of the receptor.

It was found by several investigators that immunoglobulins or other serum factors are involved in some way with NK cells. Cell

bound monomeric IgG can inhibit NK activity (22). This inhibition is caused by attachment of IgG via Fc γ receptors ($Fc\gamma R$) to NK cells. Since VCN treatment is known to augment $Fc\gamma R$ expression (23), but the VCN-induced $Fc\gamma R$ expression was not associated with enhanced cytotoxic activity in an assay for ADCC and spontaneous CMC (23), it can be deduced that VCN treatment can restore the NK activity suppressed by cytophilic IgG.

Consequently, from studies in the literature and our own *in vitro* expriments we adopt the last of the above mentioned possible explanations. As VCN treatment is known to influence ATPase on the cell membrane (24) and to activate lymphocytes (25), it is reasonable to speculate that the VCN treatment augments NK activity via cellular metabolic activity.

In the present study, VCN treatment of spleen cells from tumor-bearing mice produced a prominent increase in NK activity, whereas the same treatment of normal spleen cells had no effect on NK activity against M-HeLa cells and some effect against YAC-1 cells. In a study on human peripheral blood lymphocytes, NK activity of normal lymphocytes against M-HeLa cells was not augmented by VCN. On the other hand, lymphocytes of cancer patients were activated (26).

It seems that the augmentation of NK activity by VCN treatment is more effective in the immuno-suppressive state, such as in the case of malignant disease, than in the normal state. In cancer patients and tumor-bearing animals, PGE_2 might play an important role in the low NK cell activity. VCN treatment which had no significant effect on normal spleen cells augmented the PGE_2-depressed NK activity of cells from tumor-bearing mice to the normal level.

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