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Vitamin E Increases the Growth Inhibitory and Differentiating Effects of Tumor Therapeutic Agents on Neuroblastoma and Glioma Cells in Culture[1] (40840)

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Vitamin E possesses antioxidative properties and has been presumed to participate only in antioxidative activities of cells *in vivo* (1). Sodium ascorbate (vitamin C), another antioxidant, possesses antitumor activity both *in vivo* (2) and *in vitro* (3–4). In addition to its antitumor activity as a single agent, sodium ascorbate potentiates the effect of ionizing radiation and certain chemotherapeutic agents on neuroblastoma (NB) cells in culture (3–4). These properties of sodium ascorbate are not shared by another antioxidant, glutathione (4). A recent study has show (5) that vitamin C potentiates the effect of BCNU in murine CNS leukemia. We have reported (6) that vitamin E exhibits anticancer properties by increasing the expression of morphological differentiation in NB (NBP_2 clone) cell culture and by enhancing the growth inhibition and morphological differentiation produced by ionizing radiation on NB cells. Because of its potential contribution in the treatment of neoplastic disease, the effects of vitamin E in combination with several pharmacological agents were studied to determine whether vitamin E would potentiate their growth inhibitory effect (due to cell death and reduction of cell division) on NB cells and glioma cells in culture. We now report that vitamin E in combination with some chemotherapeutic agents produces synergistic or additive growth inhibition on both glioma and NB cells (depending upon the specific agent and cell line). Finally, the combined treatment of vitamin E and pharmacological agents induces morphological differentiation or enhances the effect of differentiating agents.

Materials and methods. Mouse neuroblastoma clone NBP_2, which contains both tyrosine hydroxylase (TH) and choline acetyltransferase (CAT) activities, was used in this study (7). Rat glioma clone (C-6) (8) of passages 30–42 (9) was used in this investigation. Neuroblastoma cells were grown in F12-medium containing 10% agammaglobulin newborn calf serum, whereas glioma cells were grown in F12 medium containing 10% fetal calf serum. Both media contained antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Aquasol vitamin E (DL-alphatocopheryl acetate, water solubilized with polysorbate 80; 50 IU/ml was diluted with water. All solutions were protected from light and stored at 4°C. To study the effect of the combination of vitamin E and chemotherapeutic agents on morphological changes, cells (50,000) were plated in Lux tissue culture dishes (60 mm), and vitamin E and pharmacological agents were added 24 hr later. The medium and drug were changed 2 days after treatment and the number of morphologically differentiated cells was determined 3 days after treatment. Cells with cytoplasmic processes greater than 50 μm in length were considered morphologically differentiated (10).

To study the effect of vitamin E and pharmacological agents on growth inhibition (due to cell death and reduction of cell division), cells (5×10^4 NB cells; 10^5 glioma) were plated in Lux tissue culture dishes (60 mm) and vitamin E and drugs were added 24 hr later. The cell number per dish was determined 3 days after treatment. Cell viability was determined by counting the number of cells stained with trypan blue (0.2% in saline) among attached cell population before removing them for counting. For counting purposes NB cells were re-

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moved from the dish by incubating them in the presence of pancreatin solution (0.25% in Ca-free MEM) for 10 min; whereas glioma cells were washed twice with phosphate-buffered saline and then incubated in the presence of trypsin solution (0.25% in Ca-free MEM) for 40 min. Cells were counted by a Coulter Counter. The stained cells were considered dead and this number was subtracted from the total in order to obtain the number of viable cells/dish.

The effect of vincristine in combination with vitamin E was investigated in greater detail than the other agents. The effects of other pharmacological agents such as bleomycin, adriamycin, 5-fluouracil (5-FU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC), mutamycin, *cis*-diamminedichloroplatinum II, chlorozotocin, sodium butyrate, prostaglandin E_1 (PGE_1), 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine (R020-1724), and papaverine were studied at a concentration which by itself inhibited growth by 50% or less. All compounds except PGE_1, R020-1724, and papaverine were water soluble. PGE_1, R020-1724, and papaverine were dissolved in 50% ethyl alcohol. These solutions were stored at 4°C.

Results. Effect on growth inhibition. Vitamin E markedly potentiated the growth inhibitory effect (due to cell death and reduction in cell division) of vincristine on neuroblastoma (Fig. 1) and glioma cells (Fig. 2) in culture. Vitamin E concentration of as little as 0.01 IU/ml was sufficient to enhance the vincristine cytotoxicity on NB cells (Fig. 3) and glioma cells (Fig. 4). The number of trypan blue-stained cells in control and treated glioma cell cultures was <1%. However, the number of trypan blue-stained cells in NB culture depended upon the treatment. Control NB cultures

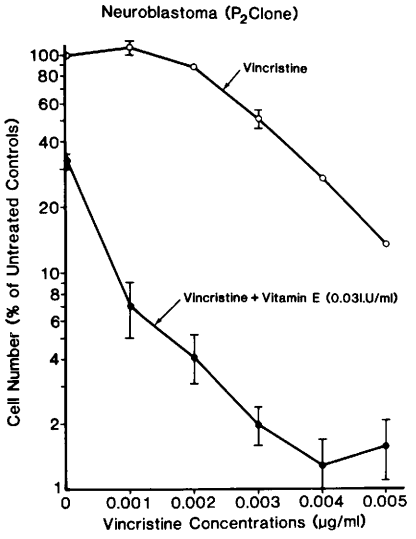

FIG. 1. Neuroblastoma cells (50,000) were plated in Lux culture dishes (60 mm), and vincristine and vitamin E were added 24 hr later. Drugs and medium were changed 2 days after treatment. The cell number and the number of trypan blue-stained cells were determined 3 days after treatment. The number of stained cells was subtracted from the total number of cells to obtain viable cells per dish. The average value of control cultures was considered 100%. Each value represents an average of at least six samples. The bar of each point is standard deviation. The bars not shown in figure were equal to sizes of symbols.

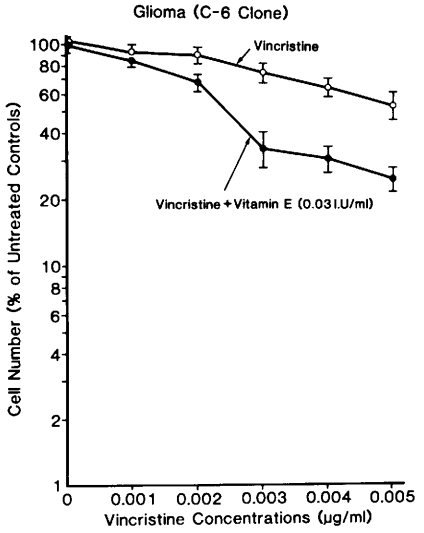

FIG. 2. Glioma cells (100,000) were plated in Lux tissue culture dishes (60 mm), and vincristine and vitamin E were added 24 hr later. Drugs and medium were changed 2 days after treatment. The cell number and the number of trypan blue-stained cells were determined 3 days after treatment. The number of stained cells was subtracted from the total number of cells to obtain viable cells per dish. The average value of control cultures was considered 100%. Each value represents an average of at least six samples. The bar of each point is standard deviation.

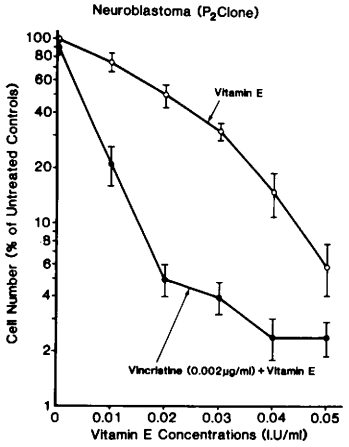

FIG. 3. Neuroblastoma cells (50,000) were plated in Lux culture dishes (60 mm), and vincristine and vitamin E were added 24 hr later. Drugs and medium were changed 2 days after treatment. The cell number and the number of trypan blue-stained cells were determined 3 days after treatment. The number of stained cells was subtracted from the total number of cells to obtain viable cells per dish. The average value of control cultures was considered 100%. Each value represents an average of at least six samples. The bar of each point is standard deviation.

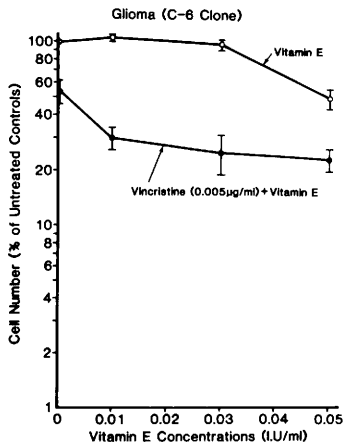

FIG. 4. Glioma cells (100,000) were plated in Lux tissue culture dishes (60 mm), and vincristine and vitamin E were added 24 hr later. Drugs and medium were changed 2 days after treatment. The cell number and the number of trypan blue-stained cells were determined 3 days after treatment. The number of stained cells was subtracted from the total number of cells to obtain viable cells per dish. The average value of control cultures was considered 100%. Each value represents an average of at least six samples. The bar of each point is standard deviation.

and NB cultures treated with vitamin E (0.02 IU/ml) or vincristine (0.002 μg/ml) contained <1% trypan blue-stained cells. The number of stained cells increased at higher concentrations of vitamin E or vitamin E plus vincristine.

Vitamin E (0.02 IU/ml) in combination with 5-FU, adriamycin, R020-1724, sodium butyrate, chlorozotocin, or PGE_1 produced a synergistic effect on growth inhibition of neurobastoma cells, whereas, vitamin E in combination with bleomycin, CCNU, DTIC, mutamycin, or *cis*-diammine-dichloroplatinum II produced an additive effect on growth inhibition of NB cells (Table I). In glioma cell culture, vitamin E in combination with CCNU, or R020-1724, produced a synergistic effect on growth inhibition, whereas, vitamin E in combination with bleomycin, 5-FU, adriamycin, DTIC, papaverine, mutamycin, and *cis*-platinum produced an additive effect (Table I). Vitamin E failed to enhance the effect of sodium butyrate, PGE_1, and chlorozotocin on glioma cells (Table I). Neuroblastoma cells were more sensitive to vitamin E, bleomycin, 5-FU, adriamycin, CCNU, DTIC, sodium butyrate, R020-1724, or papaverine than glioma cells by the criterion of growth inhibition (Table I). Both cell types were equally sensitive to chlorozotocin, *cis*-platinum, and mutamycin by the same criterion (Table I).

Effect on morphological differentiation. PGE_1, R020-1724, papaverine (10), and vitamin E (6) are known to cause morphological differentiation of neuroblastoma cells in culture. The present study shows that adriamycin (0.001–0.002 μg/ml), mutamycin (0.1–0.3 μg/ml, or bleomycin (0.004 U/ml) by themselves induced a marked increase in morphological differentiation associated with an increase in the size and soma of cells (Table II). The *cis*-platinum increased the expression of morphological changes only slightly. Most of the drugs used in the study except vincristine and 5-FU increased the size of cells with or without morphological differentiation. The effect of combined treatment of vitamin E with

TABLE I. EFFECT OF VITAMINE E IN COMBINATION WITH PHARMACOLOGICAL AGENTS ON NEUROBLASTOMA (P_2) AND GLIOMA (C-6) CELLS IN CULTURE[a]

Treatments	Cell number (% of untreated control)	
	Glioma	Neuroblastoma
Vitamin E	50 ± 6[b] (0.05 IU/ml)	53 ± 5 (0.02 IU/ml)
Bleomycin	77 ± 5 (0.004 U/ml)	54 ± 4 (0.002 U/ml)
Bleomycin + vitamin E	42 ± 3[c]	29 ± 3[c]
5-FU	71 ± 7 (0.1 μg/ml)	63 ± 4 (0.03 μg/ml)
5-FU + vitamin E	32 ± 3[c]	15 ± 3[d]
Adriamycin	36 ± 4 (0.004 μg/ml)	42 ± 3 (0.001 μg/ml)
Adriamycin + vitamin E	22 ± 2[c]	12 ± 1.5[d]
CCNU	43 ± 3 (20 μg/ml)	55 ± 6 (10 μg/ml)
CCNU + vitamin E	11 ± 2[d]	38 ± 3[c]
DTIC	57 ± 4 (20 μg/ml)	60 ± 5 (4 μg/ml)
DTIC + vitamin E	35 ± 5[c]	31 ± 4[c]
Sodium butyrate (NaB)	78 ± 4 (0.5 mM)	87 ± 6 (0.25 mM)
NaB + vitamin E	45 ± 6[e]	36 ± 6[d]
R020-1724	73 ± 6 (100 μg/ml)	45 ± 4 (100 μg/ml)
R020-1724 + vitamin E	16 ± 3[d]	10 ± 1.8[d]
Prostaglandin E_1 (PGE_1)	78 ± 7 (5 μg/ml)	40 ± 5 (10 μg/ml)
PGE_1 + vitamin E	46 ± 5[e]	9 ± 1.3[d]
Papaverine (μg/ml)	63 ± 5 (10 μg/ml)	41 ± 4 (5 μg/ml)
Papaverine + vitamine E	25 ± 4[c]	18 ± 3[c]
Mutamycin	46 ± 4 (0.01 μg/ml)	48 ± 4 (0.01 μg/ml)
Mutamycin + vitamin E	23 ± 3[c]	24 ± 4[c]
Chlorozotocin	84 ± 7 (2 μg/ml)	82 ± 4 (2 μg/ml)
Chlorozotocin + vitamin E	52 ± 5[e]	36 ± 3[d]
cis-platinum	54 ± 4 (0.2 μg/ml)	43 ± 5 (0.2 μg/ml)
cis-platinum + vitamin E	29 ± 5[c]	18 ± 5[c]

[a] Cells (5×10^4, NB; 10^5, glioma) were plated in Lux tissue culture dishes (60 mm), and drugs were added 24 hr after plating. Fresh media and drugs were added 2 days after treatment. The number of viable cells were determined 3 days after treatment. The values in treated groups were expressed as percentage of untreated controls. The number of cells per dish in untreated control, NB and glioma cells were 81×10^4 and 68×10^4, respectively. Each value represents an average of at least six samples.

[b] Standard deviation.

To test whether the drug had any effect at all in combination with vitamin E in enhancing the growth inhibition of cells, the mean percentage of untreated control cultures for the drug + vitamin E-treated cultures with that of vitamin E-treated cultures was compared by use of two independent sample t tests at $P = 0.01$. If no significant effect of the drug was found, no further analysis was done. If there was a significant effect of the drug in conjunction with vitamin E, then an additional test was performed to establish whether the combination of vitamin E and a drug produced an additive or a synergistic effect.

[c] Additive effect.

[d] Synergistic effect.

[e] No effect.

pharmacological agents on morphological differentiation was also studied. The size of cells increased in NB culture treated with vitamin E and a pharmacological agent more than observed in NB culture treated with the individual agent (data not shown). Vitamin E at a concentration of 0.02 IU/ml did not significantly increase the expression of morphological differentiation in NB cells. Bleomycin, CCNU, and chlorozotocin at concentrations used in this study did not increase the morphological differentiation of NB cells, but when they were used in combination with vitamin E, the number of morphologically differentiated NB cells was dramatically increased (Table II). Vitamin E also markedly enhanced the differentiating effects of adriamycin, PGE_1, R020-1724, papaverine, mutamycin, and *cis*-platinum (Table II).

Treatment of glioma cells with vitamin E and pharmacological agents did not induce or increase the expression of morphological changes (data not shown).

TABLE II. EFFECT OF VITAMIN E IN COMBINATION WITH PHARMACOLOGICAL AGENTS ON NEUROBLASTOMA CELLS IN CULTURE[a]

Treatments	Morphological differentiation (% of total cells)	Trypan blue-stained cells (% of total cells)
Control	3 ± 0.6[b]	<1
Vitamin E (0.02 μg/ml)	5 ± 2	2 ± 0.4
Adriamycin (0.001 μg/ml)	35 ± 4	3 ± 1
Adriamycin + vitamin E	66 ± 6	7 ± 2
CCNU (10 μg/ml)	4 ± 1	<1
CCNU + vitamin E	42 ± 5	2 ± 0.2
Prostaglandin E_1 (10 μg/ml)	55 ± 3	<1
Prostaglandin + vitamin E	96 ± 3	2 ± 0.3
Bleomycin (0.002 U/ml)	4 ± 1	<1
Bleomycin + vitamin E	68 ± 6	2 ± 1
Papaverine (5 μg/ml)	20 ± 4	<1
Papaverine + vitamin E	67 ± 3	<1
R020-1724 (100 μg/ml)	27 ± 4	<1
R020-1724 + vitamin E	79 ± 3	<1
cis-platinum (0.2 μg/ml)	12 ± 2	<1
cis-platinum + vitamin E	69 ± 6	3 ± 0.2
Mutamycin (0.01 μg/ml)	11 ± 2	<1
mutamycin + vitamin E	48 ± 4	<1
Chlorozotocin (2 μg/ml)	2 ± 0.3	<1
Chlorozotocin + vitamin E	30 ± 3	<1

[a] Cells (5×10^4) were plated in Lux tissue culture dishes (60 mm), and drugs were added 24 hr after plating. Fresh growth medium and drug were added 2 days after treatment, and the number of morphologically differentiated and trypan blue-stained cells were determined 3 days after treatment. Each value represents an average of at least six samples.

[b] Standard deviation.

Discussion. The present study shows that vitamin E in combination with several pharmacological agents produces an additive or synergistic effect on growth inhibition of NB and glioma cells in culture, depending upon the particular pharmacological agent and cell line. In addition, vitamin E enhances the differentiating effect of adriamycin, prostaglandin E_1, R020-1724, papaverine, mutamycin, and *cis*-platinum. The combined treatment of vitamin E with bleomycin, CCNU, or chlorozotocin induces a marked increase in morphological differentiation on NB cells; the individual agent by itself does not cause any significant increase in morphological differentiation. No such effect is observed on glioma cells in culture. It has been reported (6) that vitamin E alone induces morphological differentiation in neuroblastoma cells which is primarily irreversible. Such differentiated neuroblastoma cells induced by other agents are not tumorigenic (10). Earlier studies (10) have shown that the inhibition of cell division is not a prerequisite for the expression of morphological differentiation, however, most of the differentiated functions in neuroblastoma cells are fully expressed after the inhibition of cell division. This concept is further supported by the present finding in which all agents inhibit cell division, but only some of them cause morphological differentiation.

Although both vitamin E and vitamin C are reducing agents, their effects on NB and glioma cells in part differ. For example, vitamin E increases the expression of morphological differentiation on NB cells, whereas, vitamin C does not (3). Vitamin E increases the differentiating effect of certain pharmacological agents, whereas, vitamin C does not (3). Both vitamin E and vitamin C enhance the growth inhibitory effect of several pharmacological agents, but their effects quantitatively differ. For example, vitamin E in combination with vincristine produces a synergistic effect on NB cells, whereas, vitamin C under a simi-

lar condition does not (3), except at higher vincristine doses (4). Both vitamins E and C (3) in combination with 5-FU produce a synergistic effect on NB cells for the criterion of growth inhibition. Vitamin E in combination with DTIC produces an additive effect on NB cells for the criterion of growth inhibition, whereas, vitamin C completely prevents the cytotoxic effect of DTIC (3). Thus, the mechanisms of vitamin E effects are not entirely due to its reducing property. The exact mechanism of the effects of vitamin E remains to be elucidated; however, we have observed (unpublished observation) that vitamin E is bound with protein receptors. A significant amount of vitamin E was bound with the chromatin fraction of glioma and NB cells, suggesting the possibility that vitamin E may regulate the gene expression in mammalian cells.

If the present results can be applicable to *in vivo*, the combination of vitamin E with some of the currently used therapeutic agents may help to improve the present management of metastatic NB and other neoplasms. Such an adjuvant could reduce the dose requirements of cytotoxic drugs without reducing the cell killing or differentiating effects. The present study also points out the possibility that vitamin E may modulate the effect of various pharmacological agents, but the extent of modification may depend upon the cell type and upon the particular pharmacological agent.

Summary. The effects of vitamin E in combination with several pharmacological agents on neuroblastoma (NBP_2) and glioma (C-6) cells in culture for the criteria of growth inhibition (due to cell death and reduction of cell division) and morphological changes were studied. Vitamin E in combination with 5-FU, adriamycin, R020-1724, vincristine, sodium butyrate, chlorozotocin, or PGE_1 produced a synergistic effect on growth inhibition of NB cells, whereas, vitamin E in combination with bleomycin, CCNU, DTIC, mutamycin, or *cis*-platinum produced an additive effect for the same criterion. In glioma cell culture, vitamin E in combination with vincristine, R020-1724, and CCNU produced a synergistic effect on growth inhibition; whereas, vitamin E in combination with bleomycin, 5-FU, adriamycin, DTIC, papaverine, mutamycin, and *cis*-platinum produced an additive effect for the same criterion. Vitamin E failed to enhance the effect of sodium butyrate, PGE_1, chlorozotocin on glioma cells. Bleomycin, CCNU, chlorozotocin, or vitamin E at concentrations used in this study did not increase the morphological differentiation of NB cells, however, vitamin E in combination with one of these agents markedly enhanced the number of morphologically differentiated NB cells. Adriamycin, PGE_1, R020-1724, papaverine, mutamycin, and *cis*-platinum increased the expression of morphological differentiation of NB cells, but vitamin E enhanced the effect of these agents for this criterion. The treatment of glioma cells with vitamin E and pharmacological agents did not cause any significant change in morphological differentiation. These data also suggest that vitamin E may modulate the effects of pharmacological agents.

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