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# Vitamin $B_{12b}$ increases the cytotoxicity of short-time exposure to ascorbic acid, inducing oxidative burst and iron-dependent DNA damage

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**Abstract**

It has been found previously that hydroxycobalamine (vitamin $B_{12b}$) amplifies significantly the cytotoxic effect of ascorbic acid (vitamin C) added to cells for a long period of time (48 h). However, according to pharmacokinetics, the concentration of vitamin C in vivo decreases to a physiological value within a short period of time (2–3 h) after the injection. Therefore, in this study we examined the cytotoxic effect of a short-time (up to 2 h) exposure of human larynx carcinoma HEp-2 cells to a combination of vitamins $B_{12b}$ and C ($B_{12b}$+C). The kinetics of the $B_{12b}$+C-caused extracellular oxidative burst in this time interval was also explored. Vitamin $B_{12b}$ combined with ascorbic acid provoked a rapid accumulation of extracellular hydrogen peroxide followed by intracellular oxidative stress, DNA single-strand breaks, and the initiation of apoptosis. The chelators of iron phenanthroline and desferrioxamine prevented $B_{12b}$+C-induced DNA single-strand breaks and cell death but not the accumulation of $H_2O_2$ in culture medium. The nonthiol antioxidants pyruvate and catalase were effective in preventing the prooxidant and cytotoxic effects of $B_{12b}$+C. Thiols, when added simultaneously with the combined vitamins, inhibited these effects only partially (*N*-acetylcysteine, GSH) or even amplified them (dithiothreitol). The results obtained point to the determining role of oxidative burst and iron-dependent DNA damage in the cytotoxic effect of short-time exposure to $B_{12b}$+C combination.


*Keywords:* Ascorbic acid; Vitamin $B_{12b}$; Cytotoxicity; DNA single-strand break; Hydrogen peroxide; Oxidative stress; Tumor cell

## 1. Introduction

Ascorbic acid, a well-known antioxidant, is used as a vitamin in common medicine practice. There were numerous attempts to apply ascorbic acid (vitamin C) or sodium ascorbate in antitumor therapy. Thus, it was reported that ascorbate given intravenously at a dose of 10 g daily is effective in treating some cancers and in improving patient's well-being (Padayatty and Levine, 2001; Chen et al., 2005). Ascorbic acid influences the expression of some genes by improving the cell response to DNA damage, suppressing the regulation of E6 oncoprotein and transcription factor AP1 and modulating the apoptosis (Reddy et al., 2001; Wozniak and Blasiak, 2004). Ascorbate increases the susceptibility of tumor cells to the cytotoxic action of cisplatin, etoposide (Reddy et al., 2001), and vanadyl sulfate (Wozniak and Blasiak, 2004). The accumulation of ascorbic acid in tumor cells and a low resistance of tumor cells to megadoses of ascorbate as compared to normal cells were shown (Kimoto et al., 1983; Taper and Roberfroid, 1992; Reddy et al., 2001; Chen et al., 2005). Thus, there are reasons to suggest that ascorbate produces a selective cytotoxic effect on tumor cells. However, recent clinical evidence indicates that the role of ascorbate in cancer treatment is unclear and should be examined anew (Padayatty et al., 2004).

The prooxidant effect of ascorbic acid combined with metals of transient valence (Fe, Cu) or quinones, which makes itself evident in the generation of reactive oxygen species, is well-known (Buettner and Jurkiewicz, 1996; Kohen and Nyska, 2002). New antitumor agents based on the combinations of ascorbate with copper organocomplexes (Kimoto et al., 1983) or vitamin $K_3$ and other quinones (Jarabak and Jarabak, 1995; Jamison et al., 2004) were developed. The antitumor effect of

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ascorbic acid combined with vitamin $B_{12b}$ ($B_{12b}$+C), which includes a metal of transient valence, cobalt, was also revealed in some works (Poydock et al., 1979; Poydock, 1991). However, in other investigations the antitumor action of $B_{12b}$+C combination was not confirmed (Newell et al., 1981). Recently a new attempt was made to evaluate the antitumor potential of the combination. It was shown that $B_{12b}$+C possesses the therapeutic activity against solid and ascitic tumors in mice and improves significantly the antitumor effect of cisplatin and vincristin (Vol'pin et al., 1998). The combination of vitamin C and $B_{12b}$ had a cytotoxic effect in vitro at pharmacological concentrations of the components at which they were nontoxic when administered separately (Akatov at al., 2000). These results were obtained if tumor cells were exposed to the vitamins for a long period of time (2 days). However, according to pharmacokinetics, the concentration of vitamin C in vivo decreases to a physiological value during a short period of time (2–3 h) after injection (Padayatty et al., 2004). Therefore, the significance of the damaging effect of combined vitamins was not clear enough since the prooxidant and cytotoxic effects of a short-time exposure of tumor cells to the combination were not investigated. In this study we explored the extracellular and intracellular oxidative stresses, DNA damage, and the initiation of death of carcinoma HEp-2 cells during a short-time exposure to $B_{12b}$+C combination. In addition, the role of intracellular iron in $B_{12b}$+C cytotoxicity and the ability of some antioxidants to prevent the toxicity were studied.

## 2. Materials and methods

### 2.1. Materials

*N*-acetylcysteine, glutathione, dithiothreitol, pyruvate, and 1,10-phenanthroline were purchased from MPbiomedicals (USA); 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) was from Molecular Probes Inc. (USA); and fetal bovine serum was from HyClone (USA). Other chemicals were from Sigma (USA).

### 2.2. Cell culture

Human epidermoid larynx carcinoma (HEp-2) cells were obtained from the Russian Cell Culture Collection (Institute of Cytology, Russian Academy of Sciences, St. Petersburg). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 80 mg/l gentamycin, and 20 mM sodium bicarbonate at 37 °C in an atmosphere of 5% $CO_2$.

### 2.3. Drug treatment and cytotoxicity assay

The concentrations of $B_{12b}$ and vitamin C used in the present study were 25 μM and 500 μM, respectively. Freshly prepared solutions of the vitamins or hydrogen peroxide in DMEM were added to culture medium 24 h after seeding the cells. The antioxidants and the chelator phenanthroline, desferrioxamine, and neocuproine were added to the cultures 1–2 h before, or simultaneously with, the administration of the vitamins, and pH of the medium was adjusted to 7.4 by NaOH.

For cytotoxicity assay, cells were seeded in 96-well microplates or culture dishes (Corning, USA) at a concentration of $5\times10^4$ cells/ml ($5\times10^3$ cells in 100 μl/well), and a day after the seeding the vitamins were added. To provide a short-time exposure of cells to $B_{12b}$+C, the toxic action of the combination was interrupted by replacing the incubation medium with a fresh growth medium. Cytotoxicity was evaluated using the crystal violet assay by the ratio of optical densities at 560 nm in treated and untreated (control) cultures at 48 h after adding the toxic agents (Nomizu et al., 1995). The optical density value was in direct proportion to the number of viable cells.

The percent of dead cells was estimated by the trypan blue exclusion assay after the trypsinization of cell cultures.

### 2.4. DNA fragmentation assay

For the detection of internucleosomal DNA cleavage, DNA samples of $3–5\times10^5$ cells were subjected to electrophoresis in 1.2% agarose gel as described earlier (Ren et al., 2001). Gels were stained for 20 min with ethidium bromide (0.5 μg/ml).

### 2.5. Single cell gel electrophoresis (comet assay)

The assay used was a modification of the method described by Doulias et al. (2001). Microscope slides were coated with 1.5% agarose and allowed to dry. Cells were washed twice with PBS and trypsinized for 2 min. Then the trypsinization was stopped by adding the growth medium supplemented with 10% fetal bovine serum. Approximately 10,000 cells in 10 μl of the medium were suspended at 37 °C in 70 μl of 1% low-melting-point agarose, and the suspension was spread on a precoated slide, covered with a 25-mm coverslip, and kept at 4 °C for 5 min. A third agarose layer was added (80 μl of low-melting-point agarose), and the slides were allowed to stand at 4 °C until the layer hardened. After the hardening, the slides were immersed in a freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris at pH 10, 1% Triton X-100 v/v, 10% dimethyl sulfoxide) at 4 °C for at least 1 h and then placed in a single row to a horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA, pH > 13, at 4 °C for 20 min to allow the separation of two DNA strands (alkaline unwinding). Electrophoresis was performed in the unwinding solution at 25 V (1 V/cm) and 300 mA for 20 min. The slides were then washed three times with 0.4 M Tris, pH 7.4, at 4 °C for 5 min each and stained with Hoechst 33342 (1 μg/ml, 5 min). The pictures of 50 randomly selected comets per slide were captured at a magnification of ×400 using an epifluorescence microscope. The image analysis of the data was made using the program CometScore™ (TriTek Corp., USA). The degree of DNA damage (amount of DNA single-strand breaks) was estimated as the mean percentage of fluorescence in comet tails.

### 2.6. Assessment of apoptosis in situ

All cells, including floating and trypsinized cells, were harvested after the treatment with $B_{12b}$+C and stained simultaneously with the fluorescent DNA-binding dyes Hoechst

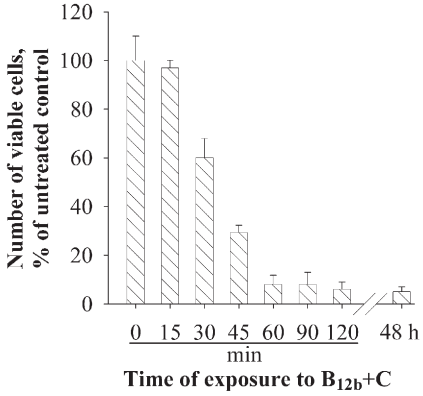


Fig. 1. Cytotoxic effect of a short-time exposure to the combined vitamins $B_{12b}$+C. The vitamin combination was added 24 h after seeding HEp-2 cells. The effect of $B_{12b}$+C was interrupted by replacing the culture medium with a fresh growth medium. The cytotoxicity was estimated at 48 h after the addition of the vitamins by the crystal violet assay (see Materials and methods). The data are the means ± S.E.M. of five separate experiments.

33342 (1 μg/ml) and ethidium bromide (1 μg/ml) to evaluate cell viability and to determine the type of cell death (necrosis or apoptosis). Cells with condensed or fragmented chromatin or with marginal allocation of chromatin were counted as apoptotic (Cohen, 1993). At least 300 cells were analyzed by fluorescent microscopy. The percentage of apoptotic cells to the total cell number was determined.

### 2.7. Flow cytometry analysis of DNA

Floating and trypsinized cells were harvested after the treatment with $B_{12b}$+C, washed with PBS, and fixed in 70% ethanol. Then cells were stained with Hoechst 33258 (5 μg/ml) and analyzed on the FL4 channel of a PARTEC III flow cytometer.

### 2.8. Detection of hydrogen peroxide accumulation caused by $B_{12b}$ + C in culture medium

The concentration of hydrogen peroxide in culture medium was estimated using an $O_2$ electrode. Cell-free growth medium or a cell culture a day after seeding the cells ($3.5 \times 10^5$) in T-25 flask was supplemented with $B_{12b}$ and ascorbic acid and incubated at 37 °C in an atmosphere of 5% $CO_2$. Before the experiment, a 1-ml chamber supplied with an $O_2$-electrode was filled with air-saturated PBS (pH 7.4) for electrode stabilization during 30 min. Then PBS was replaced by a sample of culture medium being examined. After a 1-min stabilization of the electrode, catalase (10 μl of PBS solution containing 1000 U) was added through a hole in the cap of the chamber, and the hydrogen peroxide content was determined from an increase in $O_2$ concentration. For each sample, the calibration of the electrode was carried out by adding 10 μl of 10 mM $H_2O_2$ solution.

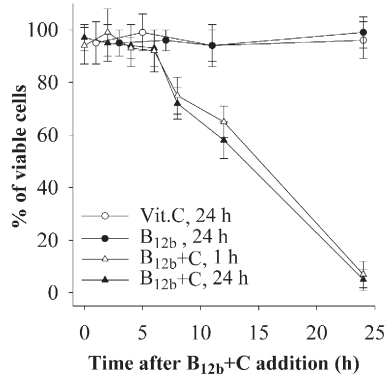


Fig. 2. Cell death during a 24-h or after a 1-h exposure of cells to the combination $B_{12b}$+C. The vitamins were added 24 h after cell seeding. After the 1-h exposure, cells were incubated in a fresh growth medium. The percent of dead cells in a population was estimated by the trypan blue assay. The data are the means ± S.E.M. of four separate experiments.

### 2.9. Assay of intracellular oxidative activity

The intracellular oxidative activity was assessed using the oxidant-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA). To determine whether extracellular oxidative stress induces any increase in intracellular oxidative

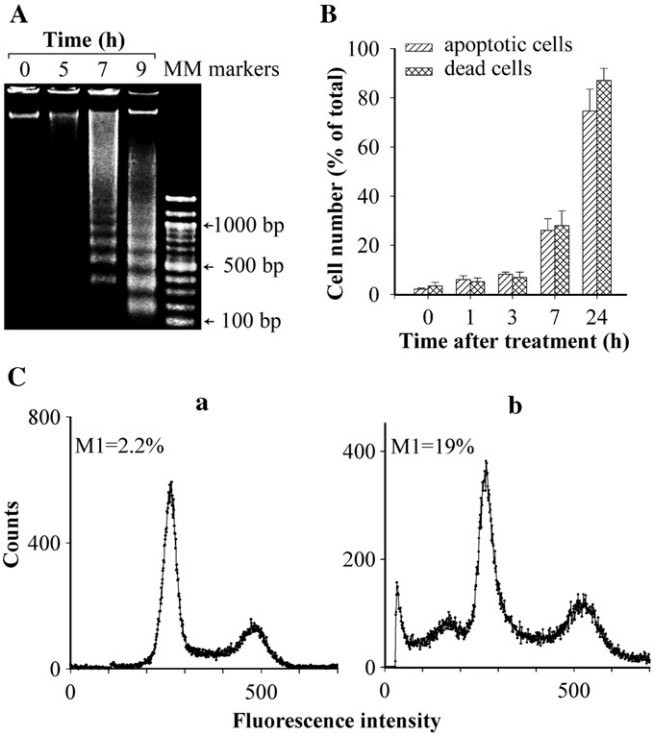


Fig. 3. Apoptosis in HEp-2 cells after a 1-h treatment with $B_{12b}$+C. After the treatment, cells were incubated in a fresh growth medium. For analysis of DNA fragmentation (A), 1 μg of DNA was loaded on a 1.2% agarose gel and electrophoresed. Gels were stained with ethidium bromide (0.5 μg/ml). Lane 0, control; 5, 7, and 9 h indicate a period of time after the 1-h treatment with $B_{12b}$+C; MM, molecular mass markers. (B) The amount of apoptotic and dead cells (percent of the total cell number) was determined by double staining with Hoechst 33342 and ethidium bromide. The condensation, fragmentation or marginal allocation of chromatin was regarded as evidence of apoptosis. At least 300 cells were analyzed by fluorescent microscopy. The values are the means ± S.E.M. of five separate experiments. (C) The appearance of sub-G1 cells was detected by flow cytometry after staining with Hoechst 33258 at 6 h after the treatment; (a) untreated control. M1 is a cell population in the sub-G1 phase of the cycle.

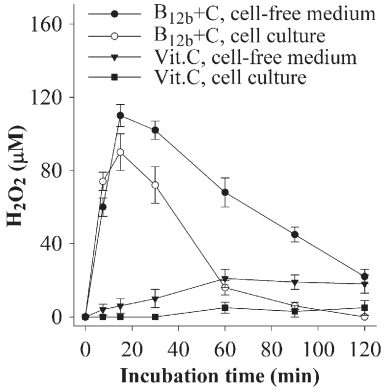


Fig. 4. Kinetics of $H_2O_2$ accumulation in growth medium after the addition of $B_{12b}$+C. The vitamins (alone and in combination) were administered to DMEM supplemented with 10% serum in T-25 culture flasks (7 ml per flask) without cells or 24 h after seeding the cells at a density of $3.5\times10^5$ cells/flask. The concentration of $H_2O_2$ was estimated by an $O_2$-electrode. The data are the means±S.E.M. of three separate experiments.

activity, cells were detached by trypsinization, suspended in growth medium ($10^5$ cells/ml), and stained for 30 min with 40 μM $H_2$DCFDA taken from a 10 mM stock solution in dimethyl sulfoxide. After staining, cells were washed, resuspended in growth medium, and treated with $B_{12b}$+C or $H_2O_2$ added as a bolus. The staining and treatment procedures were performed in the dark at 37 °C with stirring. Then cells were washed with, and resuspended in, cold PBS at a concentration of $5\times10^5$ cells/ml and analyzed by a PARTEC III flow cytometer (FL1 channel). To detect changes in the level of intracellularly generated reactive oxygen species (Pantopoulos et al., 1997), a cell monolayer was trypsinized after $B_{12b}$+C or $H_2O_2$ treatment, and cells were suspended in growth medium, stained with 40 μM $H_2$DCFDA for 30 min, washed, and analyzed as described above. The experimental parameters (e.g., time period of $B_{12b}$+C treatment, the interval between the addition of the dye and the treatment, the duration of staining, and procedure of trypsinization) were routine and were thoroughly controlled in the experiments.

### 2.10. Statistical analysis

Each experiment was performed at least three times. All the values represent the means±S.E.M. The statistical significance of the results was analyzed using the Student's *t*-test.

## 3. Results

### 3.1. Initiation of apoptotic cell death by a short-time exposure to $B_{12b}$+C

Ascorbic acid at concentrations above 10 mM had a toxic effect on HEp-2 cells. The cytotoxicity of vitamin $B_{12b}$ was not revealed at concentrations up to 3 mM. As was indicated in the Methods section, the concentrations of $B_{12b}$ and vitamin C used in the present study were 25 μM and 500 μM, respectively. When administered alone, 25 μM $B_{12b}$ and 500 μM vitamin C produced no cytotoxic effect, whereas in combination they caused the death of 90–95% of HEp-2 cells by 24 h after their addition. A 15-min exposure to $B_{12b}$+C induced no cytotoxic effect. The cytotoxicity makes itself evident when the vitamins were removed 30 min after the addition and later (Fig. 1). The kinetics of cell death after a 1-h exposure to $B_{12b}$+C was identical to that observed when the combination was not removed during 24 h (Fig. 2). The amount of viable cells began to decrease at 7 h after the addition of $B_{12b}$+C to reach minimum values at 24 h.

A short-time (1-h) exposure of HEp-2 cells to the combination $B_{12b}$+C initiated apoptotic cell death. Apoptosis was confirmed by the ladder-like DNA fragmentation, which was revealed at 6 h after the exposure (Fig. 3A). Moreover, a significant increase in the number of apoptotic cells with aberrant chromatin was observed beginning from 4 h after the addition of $B_{12b}$+C (Fig. 3B). The results of flow cytometry showed an increase in the percent of sub-$G_1$-cells at 6 h after the exposure (Fig. 3C), which also indicated the onset of apoptosis.

### 3.2. Kinetics of $B_{12b}$+C-caused accumulation of hydrogen peroxide in extracellular medium

We found a rapid accumulation of $H_2O_2$ to a concentration of 110±16 μM in cell-free culture medium within 7–15 min after the addition of $B_{12b}$+C (first stage), which was followed by a decrease in the hydrogen peroxide level during the next hour (second stage) (Fig. 4). This $B_{12b}$+C-caused oxidative burst in culture medium did not depend on pH in the range 6.6–8.1. In cell cultures, the amplitude of the $H_2O_2$ burst was less and the following decrease in $H_2O_2$ concentration was more rapid than in the cell-free growth medium. The addition of ascorbate alone (0.5 mM) initiated a low accumulation of hydrogen peroxide in the cell-free growth medium (up to 20 μM) and no accumulation in cell culture (Fig. 4). Vitamin $B_{12b}$ alone (25 μM) induced no accumulation of $H_2O_2$ in the cell-free medium and in a culture during a 2-h exposure.

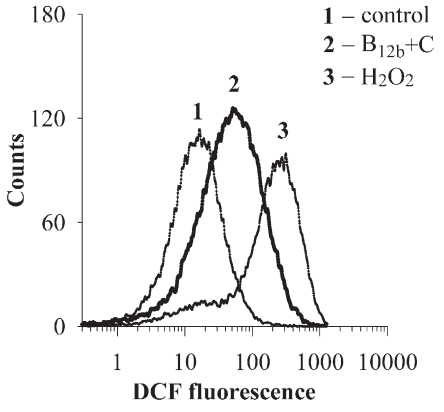


Fig. 5. Intracellular oxidative stress during the treatment of cells with $B_{12b}$+C. Suspended cells were stained with 40 μM $H_2$DCFDA for 30 min in growth medium before a 30-min incubation with $B_{12b}$+C or 2 mM hydrogen peroxide as a positive control (see Materials and methods). After washing the cells from the toxic agents and resuspending them in PBS, the fluorescence of dichlorofluorescein (DCF) in cells was measured by flow cytometry; $n$=20,000 cells/analysis. Three separate experiments gave similar results.

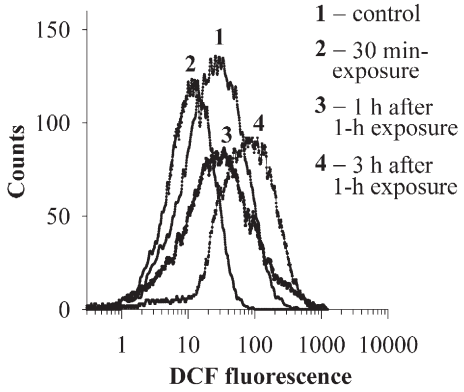


Fig. 6. Imbalance in the cell redox system after a short-time exposure to $B_{12b}$+C. Cells were trypsinized after a 30-min incubation with $B_{12b}$+C (2), or 1 h (3) and 3 h (4) after a 1-h exposure to $B_{12b}$+C, stained with 40 μM $H_2$DCFDA for 30 min in a fresh growth medium at 37 °C in the dark, washed, resuspended in PBS, and the fluorescence of dichlorofluorescein (DCF) in treated and control untreated cells (1) was analyzed using a flow cytometer; $n$=20,000 cells/analysis. The data were obtained in one of three separate experiments that gave similar results.

### 3.3. Intracellular oxidative stress induced by $B_{12b}$+C

We assumed that the $B_{12b}$+C-mediated extracellular oxidative stress gives rise to intracellular oxidative stress. Indeed, the fluorescence of $H_2$DCFDA-loaded cells increased two to three times after 15- and 30-min treatment with $B_{12b}$+C (Fig. 5). The addition of 2 mM $H_2O_2$ as a positive control caused a more pronounced (about twentyfold) increase in cell fluorescence intensity (Fig. 5). After the first hour of incubation with $B_{12b}$+C, the fluorescence of cells decreased to the control level, and this decrease coincided with the termination of oxidative stress in the culture medium. These data indicate that, during the extracellular oxidative burst induced by the combination, intracellular oxidative stress occurred. Catalase (200 U/ml) and GSH (5 mM, 1-h preincubation) completely inhibited the $B_{12b}$+C-caused intracellular oxidative stress. Ascorbate alone provoked no change in the fluorescence of $H_2$DCFDA-loaded cells.

We determined whether the intracellular oxidative stress revealed during the 1-h exposure to the $B_{12b}$+C combination was provoked by the inflow of extracellular $H_2O_2$ or by an increase in the activity of intracellularly generated reactive oxygen species. To do this, we measured the fluorescence intensity in cells stained with $H_2$DCFDA after incubation with $B_{12b}$+C and washing the vitamins (see Materials and methods). It was found that a 30- and 60-min treatment leads to a 30–50% decrease in cell fluorescence intensity, indicating a drop in the activity of intracellularly generated reactive oxygen species (Fig. 6). An hour after the 1-h exposure with the vitamins, the fluorescence intensity was the same as in control untreated cells, and 2–5 h after the exposure, a two- to fourfold increase in the intensity occurred (Fig. 6).These results suggest that, during the initiation of cell death, the activity of the cellular antioxidant system was higher than in control untreated cells but insufficient to prevent the intracellular oxidative stress caused by the penetration of extracellular $H_2O_2$ into cells. The development of intracellular oxidative stress after the initiation of cell death by a short-time exposure to $B_{12b}$+C points to the impairment of the redox balance during the execution of the apoptotic program.

### 3.4. DNA damage in cells during a short-time exposure to $B_{12b}$+C

The comet assay revealed that a 1-h exposure to vitamin C alone induced only insignificant DNA breaks (10.1±1.6% in comet tails), which were completely repaired during 4 h. Vitamin $B_{12b}$ alone induced no DNA damage. In cells treated with the combination of the vitamins, DNA strand breaks were detected as early as at 5 and 15 min of $B_{12b}$+C action, but these lesions were completely repaired after a 4-h incubation of treated cells in a $B_{12b}$+C-free growth medium (Fig. 7). After a 30-min incubation with $B_{12b}$+C, the repair of DNA breaks occurred only in a half of the cell population, and practically no DNA repair took place after a 45–60-min treatment (Fig. 7). Catalase completely prevented the $B_{12b}$+C-caused DNA damage. Thus, the initiation of cell death by the combined vitamins occurred during the accumulation of unrepaired DNA lesions.

### 3.5. Prevention of $B_{12b}$+C cytotoxicity by chelators of iron

It is known that the cytotoxic effect of hydrogen peroxide is inhibited by the $Fe^{2+}$ chelator phenanthroline and by desferrioxamine, a $Fe^{3+}$ and, to a lesser extent, $Fe^{2+}$ chelator (Halliwell and Gutteridge, 1986; Byrnes, 1996), which are able to penetrate into cells. In our study, phenanthroline (30 μM) and desferrioxamine (0.3 mM) added 2 h prior to the treatment strongly suppressed the cytotoxic effects of combined vitamins and $H_2O_2$ (Fig. 8). When added simultaneously with $B_{12b}$+C, the chelators prevented cell death to a lesser extent (Fig. 8). Phenanthroline and desferrioxamine themselves at doses below or equal to 30 μM and 0.3 mM, respectively, revealed no cytotoxicity in our study. As indicated by the comet assay, a 2-h preincubation with the chelators completely inhibited DNA damage within the first

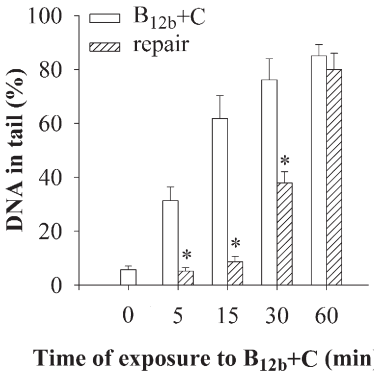


Fig. 7. Repair of DNA damage after a short-time exposure of HEp-2 cells to $B_{12b}$+C. The percentage of tail DNA after the treatment with $B_{12b}$+C or after the treatment and subsequent 4-h incubation in $B_{12b}$+C-free growth medium was estimated by the comet assay. The data are the means±S.E.M. of three separate experiments (*$P$<0.05 vs. treatment without 4-h repair).

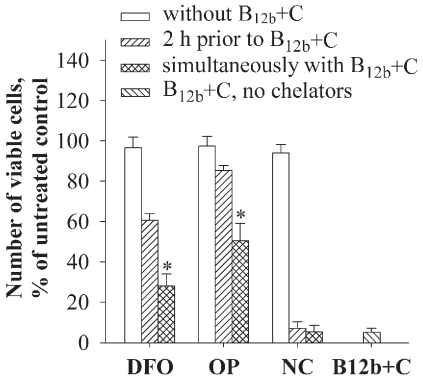


Fig. 8. Protection of HEp-2 cells by chelators against the toxicity of $B_{12b}$+C. A culture was supplemented with desferrioxamine (DFO, 0.3 mM), phenanthroline (OP, 30 μM) or neocuproine (NC, 10 μM) 2 h prior to, or simultaneously with, the addition of $B_{12b}$+C and incubated with the vitamins and a chelator for 1 h. The effects of DFO, OP, and NC alone added for 3 h (no $B_{12b}$+C), or $B_{12b}$+C combination added for 1 h are presented for comparison. After incubation, the medium was replaced by a fresh growth medium, then cultures were cultivated for 48 h, and the cytotoxicity was estimated by the crystal violet assay. The data are the means±S.E.M. of four separate experiments. The difference between cultures supplemented with DFO and OP 2 h prior to, or simultaneously with, the addition of $B_{12b}$+C was significant (*$P$<0.05).

hour of treatment with $B_{12b}$+C. The amount of DNA in comet tails after the treatment was 5.8±1.1% with phenanthroline and 5.1±1.6% with desferrioxamine vs. 5.2±1.3% in untreated control cells. The chelators at the doses used did not influence the $B_{12b}$+C-induced accumulation of $H_2O_2$. The concentration of hydrogen peroxide reached 101±11 μM with phenanthroline and 94±19 μM with desferrioxamine vs. 110±16 μM without the chelators at 15 min after the addition of the vitamins to the cell-free medium, i.e., at the moment of culmination of the oxidative burst.

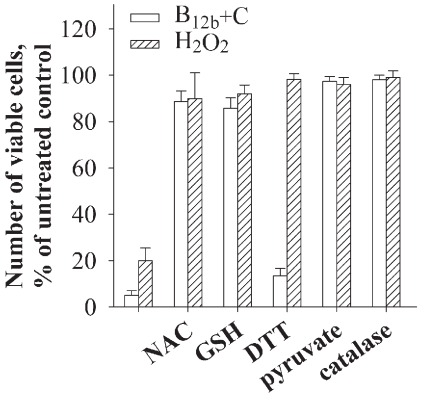


Fig. 9. Modulation of $B_{12b}$+C cytotoxicity by some antioxidants. HEp-2 cells were preincubated for 1 h in growth medium containing a thiol (10 mM *N*-acetylcysteine (NAC), 5 mM GSH, or 2 mM dithiothreitol (DTT)), pyruvate (10 mM), or catalase (200 U/ml). Then $B_{12b}$+C or 100 μM $H_2O_2$ was added for 1 h and the cells were washed and incubated for 48 h in growth medium. The cytotoxicity was estimated by the crystal violet assay. The data are the means± S.E.M. of five separate experiments. The difference between number of viable cells of cultures treated with $B_{12b}$+C and $H_2O_2$ in the presence or absence of an antioxidant (except for DTT added to $B_{12b}$+C) was significant ($P$<0.05).

We also tested the effect of neocuproine, a copper chelator, on the cytotoxicity of $B_{12b}$+C. Neocuproine alone at concentrations above 10 μM produced the cytotoxic effect. At doses below 10 μM, neocuproine added simultaneously with, or 2 h prior to, the combined vitamins was ineffective in preventing the cytotoxicity of 1-h treatment by $B_{12b}$+C (Fig. 8).

### *3.6. Modulation of the prooxidant and cytotoxic effects of $B_{12b}$+C by some antioxidants*

Since the accumulation of $H_2O_2$ up to a concentration of 100 μM occurs during the reaction of vitamin C with $B_{12b}$, the HEp-2 cell viability after $B_{12b}$+C and $H_2O_2$ treatment was compared. The cytotoxic effect of a 1-h incubation of cells with the $B_{12b}$+C combination was found to be more pronounced than that of 100 μM $H_2O_2$ added as a bolus (Fig. 9). The cell number at 48 h after the 1-h exposure to $H_2O_2$ and $B_{12b}$+C was 20.5±5.5% and 5±2% vs. control, respectively ($P$<0.05).

The incubation of cells with *N*-acetylcysteine (10 mM) or GSH (5 mM) for 1 h prior to the addition of $B_{12b}$+C significantly increased cell survival compared with experiments in which the

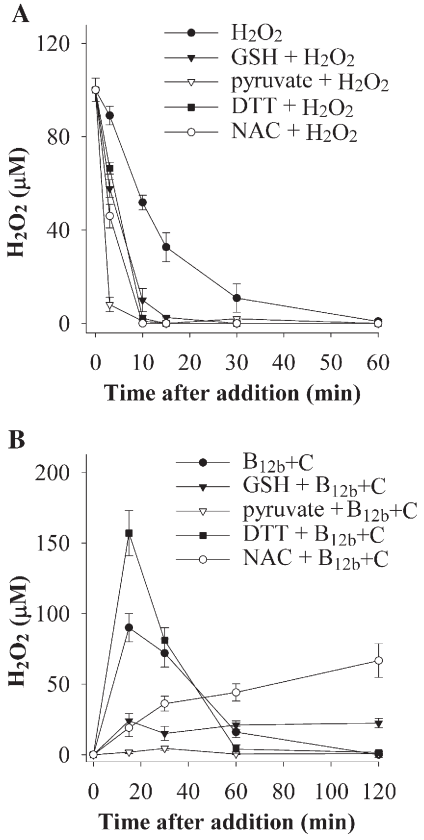


Fig. 10. Effect of some antioxidants on the decomposition of exogenously added $H_2O_2$ (A) and $B_{12b}$+C-induced accumulation of $H_2O_2$ (B) in culture medium. The vitamins $B_{12b}$ and C or 100 μM $H_2O_2$ were administered to a culture 24 h after the seeding of cells at a density of $3.5\times10^5$ cells/T-25 flask. Antioxidants (5 mM GSH, 10 mM NAC, 10 mM pyruvate, and 2 mM DTT) were added immediately prior to the addition of $B_{12b}$+C or $H_2O_2$. The level of $H_2O_2$ in the medium was determined by an $O_2$-electrode from an increase in the $O_2$ concentration after the addition of 1000 U/ml catalase. The data are the means± S.E.M. of three separate experiments.

antioxidants and $B_{12b}$+C were simultaneously added to the incubation medium (85.8±4.5 vs. 65.0±5.5% for GSH, and 88.6±8.3% vs. 56.4±10.1% for *N*-acetylcysteine; $P<0.05$). The protective effect of *N*-acetylcysteine (10 mM) or GSH (5 mM) against 100 μM hydrogen peroxide (Fig. 9) did not depend on whether the antioxidants were added 1 h prior to, or simultaneously with, $H_2O_2$. The addition of *N*-acetylcysteine and GSH to cell cultures enhanced the degradation of exogenous hydrogen peroxide (Fig. 10A). These antioxidants considerably (nearly fourfold) reduced the peak of the $B_{12b}$+C-caused accumulation of hydrogen peroxide in culture medium; however, they increased the exogenous $H_2O_2$ level between 1 and 2 h of $B_{12b}$+C action (Fig. 10B). A 2-h treatment with *N*-acetylcysteine or GSH alone was nontoxic and induced the accumulation of $H_2O_2$ in culture medium to concentrations of no more than 8±4 μM and 6±4 μM, respectively. As opposed to *N*-acetylcysteine and GSH, pyruvate (10 mM), similar to catalase (200 U/ml), completely prevented cell death, when added simultaneously with, or 1 h prior to, a 1-h incubation with $B_{12b}$+C or 100 μM hydrogen peroxide (Fig. 9). Both catalase and pyruvate, a well-known scavenger of $H_2O_2$, completely suppressed the $B_{12b}$+C-induced $H_2O_2$ accumulation in the medium (Fig. 10B). Pyruvate alone (10 mM) induced no accumulation of $H_2O_2$ in the culture during a 2-h exposure (3± 2 μM).

Dithiothreitol (2 and 10 mM), which is widely used for the protection of cells against the cytotoxic action of peroxides, completely prevented HEp-2 cell death when added for 1 h with 100 μM $H_2O_2$. However, when administered either 1 h prior to, or simultaneously with, $B_{12b}$+C it did not protect the cells from the toxic effect of the combined vitamins (Fig. 9). Dithiothreitol rapidly decomposed hydrogen peroxide added as a bolus but significantly increased the $B_{12b}$+C-caused $H_2O_2$ accumulation in culture medium (Fig. 10B). Dithiothreitol alone did not induce the generation of $H_2O_2$ in the culture during a 2-h incubation (2± 2 μM). These results suggest that thiol antioxidants take part in redox reactions with $B_{12b}$.

## 4. Discussion

It has been reported previously that vitamin $B_{12b}$ dramatically increased the cytotoxicity of ascorbate when the combination of the vitamins was given for a long period of time, i.e., 48 h (Akatov et al., 2000). In this work, we investigated the prooxidant and toxic effects of a short-time exposure of HEp-2 cells to the combination $B_{12b}$+C. The duration of the exposure (up to 2 h) was comparable with the pharmacokinetics of ascorbate added in high doses (Padayatty et al., 2004). We evaluated the time of initiation of cell death by $B_{12b}$+C to be 30–60 min and the lag time of the initiation to be 15 min. These data suggest that the pharmacokinetics of ascorbic acid given in pharmacological doses, i.e., the 1-h half-period of excretion of excessive ascorbate (Padayatty et al., 2004), makes the cytotoxic action of the combined vitamins in vivo quite probable. The conclusion points to the validity of attempts to apply the combined vitamins in antitumor therapy.

The prooxidant effect of the combination makes itself evident in the generation of an oxidant, namely $H_2O_2$. The results of the study indicated that the kinetics of extracellular oxidative stress produced in a culture of HEp-2 cells by $B_{12b}$+C was biphasic. At the initial stage (15 min), a burst of hydrogen peroxide accumulation in culture medium occurred. The second stage, the decrease in $H_2O_2$, was more prolonged than the stage of $H_2O_2$ accumulation. Evidently, both the accumulation and elimination of $H_2O_2$ were produced by $B_{12b}$+C (Fig. 4) since cobalt of $B_{12b}$ catalyzes the reduction of oxygen to superoxide anion and hydrogen peroxide, as well as the reduction of $H_2O_2$ to hydroxyl radical (Vol'pin et al., 1998). In addition to the $B_{12b}$-mediated catalysis, the elimination of extracellular hydrogen peroxide at the second stage of the oxidative stress could be accelerated by cells (Fig. 4). The total time of $B_{12b}$+C-caused extracellular oxidative stress did not exceed 1 h, and the initiation of apoptotic cell death took place during the second stage of the oxidative burst induced by the combination. It should be noted that the concentration of $H_2O_2$ accumulated in culture medium after the addition of ascorbate alone was significantly lower than that reported by other authors (Clement et al., 2001; Long and Halliwell, 2001). They revealed the accumulation of 40 and 110 μM $H_2O_2$ in growth medium by 1 h after the addition of 0.2 and 1 mM ascorbate, respectively. This discrepancy can be explained by the fact that the accumulation of $H_2O_2$ in growth medium containing ascorbic acid depends on the lot of serum used (our unpublished data).

We showed that the extracellular oxidative stress caused by a short-time exposure to $B_{12b}$+C provoked an intracellular oxidative stress in spite of an increase in the antioxidant activity of cells. The increase in the antioxidant activity is probably an adaptive response to the inflow of exogenous hydrogen peroxide into cells. The impairment of the cell redox system took place only after the $B_{12b}$+C-induced oxidative stress, during the execution of apoptosis. The prevention of $B_{12b}$+C-mediated intracellular oxidative stress by antioxidants, which also abolished the cytotoxic effect of the combination, indicates a decisive role of the stress in the initiation of cell death. However, the accumulation of hydrogen peroxide by the combined vitamins was a necessary but not a sufficient condition for the realization of their cytotoxicity. Our results show that the iron chelators phenanthroline and desferrioxamine did protect cells against the cytotoxicity and genotoxicity of $B_{12b}$+C though they did not prevent the generation of hydrogen peroxide by the combination. A chelator of copper neocuproine did not prevent the cytotoxicity of the combined vitamins. These results suggest that hydroxyl radicals produced by the Fenton reaction of $H_2O_2$ with intracellular iron rather than copper play a crucial role in DNA damage and the initiation of cell death by the combined vitamins. The conclusion about the determining role of intracellular iron takes into account the facts that the stability constants (log β) for the chelator phenanthroline and desferrioxamine complexed with iron and copper greatly differ (e.g., 30.6 and 14.0 for $Fe^{3+}$-desferrioxamine and $Cu^{2+}$-desferrioxamine, respectively), and the concentration of intracellular iron is well above that of copper (Galaris et al., 2002). Thus, it can be suggested that the early unrepaired iron-dependent DNA single-strand breaks are a trigger in the initiation of cell death by the combined vitamins.

Our data showed that the protective effects of some antioxidants against $H_2O_2$ and $B_{12b}$+C differ considerably in magnitude though the cytotoxic action of the combined vitamins is mediated by the generation of hydrogen peroxide. The greatest difference was revealed with dithiothreitol that efficiently protected cells from exogenous hydrogen peroxide added as a bolus but was unable to prevent $B_{12b}$+C cytotoxicity. Our preliminary data suggest that dithiothreitol combined with $B_{12b}$ exerts a prooxidant effect via redox chemistry similar to the combinations of the vitamins $B_{12b}$ and C (Akatov et al., 2000) or thiols with iron (Held et al., 1996; Taatjes et al., 1997). It is quite possible that dithiothreitol reduces $Co^{3+}$ of $B_{12b}$ to $Co^{2+}$, and $Co^{2+}$ reduces oxygen to superoxide anion, $H_2O_2$ and then to hydroxyl radical. The thiol antioxidants *N*-acetylcysteine and GSH, when added with $B_{12b}$+C, also participated in redox reactions, decreasing significantly the hydrogen peroxide accumulation. Surprisingly, the addition of *N*-acetylcysteine and GSH simultaneously with the vitamin combination, as opposed to preincubation with the thiols, incompletely prevented the cytotoxicity, though the $H_2O_2$ concentration in cultures during the 1-h incubation did not exceed 20–30 μM. This partial retention of toxicity can be explained by the fact that incubation of cells at a low but persistent concentration of $H_2O_2$ is comparable in the magnitude of the effect with a single bolus addition of hydrogen peroxide at a large cytotoxic dose (Pantopoulos et al., 1997). The protective effect of a 1-h preincubation with *N*-acetylcysteine or GSH against $B_{12b}$+C may be due to the well-known elevation of the intracellular GSH level caused by this treatment (Banki et al., 1996).

In summary, a short-time (1 h) exposure to the combined vitamins $B_{12b}$ and C produces the prooxidant and cytotoxic effects on HEp-2 cells. The $B_{12b}$+C-mediated extracellular oxidative burst gives rise to intracellular oxidative stress, which induces, with the participation of intracellular iron, early DNA single-strand breaks, leading to the initiation of apoptotic cell death. Some nonthiol antioxidants and thiols prevent the cytotoxic effect of $B_{12b}$+C, whereas dithiothreitol amplifies it.

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