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CYTOTOXIC EFFECT OF FREE BLEOMYCIN A_5-IRON (II) COMPLEX AND ITS CONJUGATES WITH CONCANAVALIN A, INSULIN AND CALCITONIN ON MOUSE THYMOCYTES

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SUMMARY: The possibility of using the antibiotic bleomycin as a part of a hybrid molecule consisting of a targeting fragment and a generator of reactive oxygen species has been investigated. The bleomycin-iron (II) complex was shown to destroy the plasma membrane of thymocytes by producing reactive oxygen species. Antioxidants protected the cells from destruction thus pointing to its free-radical mechanism. The protective effects of catalase and superoxide dismutase indicate that superoxide radical and hydrogen peroxide being formed during autooxidation of the complex are involved in cell damage. The covalent binding of bleomycin to targeting molecules (concanavalin A, insulin, and calcitonin) enhanced the ability of the bleomycin-iron (II) complex to destroy the plasma membrane of thymocytes.

The selective damage and destruction of leukemic, tumor and virus-affected cells is an important task for the present-day medicine (1, 2, 3). To cope with it, considerable research effort has been recently devoted to the synthesis of hybrid molecules consisting of a targeting fragment (e.g., antibody, hormone) and a fragment possessing cytotoxic activity. The present study was aimed to finding out whether bleomycin can be used as such a cytotoxic fragment.

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Abbreviations used: PBS, phosphate buffer solution; TBA, thiobarbituric acid; MDA, malone dialdehyde; DMSO, dimethylsulphoxide.

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Bleomycins comprise a group of structurally similar glycopeptide antibiotics, which are using for the treatment of tumor diseases. They act by forming a complex with iron (III) in equimolar ratio, which can be reduced by ascorbic acid, thiole compounds, NAD(P)H and other reducing agents, present in the organism and oxidized to yield reactive oxygen species. Due to its high affinity to DNA, bleomycin intercalates into the DNA molecule and causes single- and double-strand breaks (4).

Bleomycin membranolytic action examined in this study also represents a great interest. The cells plasma membranes damage was shown also to be a free radical process. Bleomycin ability to damage target cells membranes by active oxygen species generation enables its using as toxic fragment of hybrid molecules. The covalently bonded bleomycin with such targeting molecules as calcitonin, insulin and concanavalin A, which receptors are represented on thymocytes, has higher ability to destroy the cell membranes then non-modified antibiotic.

MATERIALS AND METHODS

Bleomycin A_5 was purchased from Riga Institute of Organic Synthesis, Latvia; bleomycin was [^{3}H]-labeled in the Moscow Institute of Molecular Genetics.

The thymocytes of CBA mice were isolated by the method of (5). The number of viable cells was estimated by staining with the vital dye trypan blue. The isolated cell suspension contained 80-90% of viable cells. The incubation mixture contained $2x10^6$ cells per ml.

The covalent binding of bleomycin to the targeting proteins was brought about using dimethyl adipimidate by the method of (6). Conjugates were purified by gel-permeation chromatography. Bleomycin-concanavalin A conjugate was purified on 1x20 cm column with Sephadex G-200 in 100 mM ammonium acetate buffer, pH 6.5. Conjugates of bleomycin with insulin and calcitonin were desalted on 0.9x60 column with Sephadex G-25 "superfine" and purified by high performance gel permeation chromatography on 7.5x600 mm TSK-2000SW column (LKB) in 100 mM ammonium acetate buffer, pH 6.5. Conjugates containing fractions were lyophilized. Stoichiometry of bleomycin and targeting molecules in conjugates was defined using [^{3}H]-bleomycin.

Bleomycin-iron complexes were prepared by incubation of the antibiotic or conjugates with iron (II) sulfate or iron (III) chloride as described in (7).

Bleomycin and conjugates cytotoxity was measured using DNA intercalating reagent Hoechst 33342 (bis-benzimide), which fluorescenced only in complex with DNA, just as cell damage was accompanied by Hoechst 33342 reagent release and fluorescence decrease. The reagent Hoechst 33342 was added to the thymocyte

suspension to a concentration of 1 mg/ml and incubated at 37°C for 1 hour and washed 3 times by PBS buffer as described in (8), directly after that the cytotoxity tests were performed. The destruction of thymocytes was assessed by fluorescence quenching of the Hoechst 33342 reagent at excitation and emission wavelengths of 355 and 450 nm, respectively.

The total yield of lipid peroxidation and DNA degradation products was evaluated spectrophotometrically by color reaction with TBA as described in (7).

Cytochrome c reductase activity was determined from cytochrome c reduction rate measured using the extinction coefficient of 21 $mM^{-1}cm^{-1}$ for 550 nm (9). Incubation mixture of 1 ml contained $1x10^6$ cells in the Hanks solution, 60 μM cytochrome c. Measurements were carried out at 37°C.

RESULTS AND DISCUSSION

The complex of the glycopeptide antibiotic bleomycin with iron generates reactive oxygen species in a redox cycle similar to the monooxygenase cycle of cytochrome P-450 (4, 10, 11). Bleomycin-iron (III) complex can be reduced by various electron donors present in the organism, such as ascorbic acid, thiole compounds, hydrogen peroxide, etc. (12). It was interesting to find out whether the ability of the complex to generate reactive oxygen species can be used for destruction of target cells.

The addition of bleomycin-iron (II) or bleomycin-iron (III) complex to thymocytes suspension led to the fluorescence quenching of the Hoechst 33342 reagent (Fig. 1). The addition of either bleomycin or iron salts alone did not cause cell lysis. The cytolytic activity of bleomycin-iron (III) was the same as that of bleomycin-iron (II) indicating that the former complex can be readily reduced by lymphocytes (Fig. 1). Taking into account the earlier observation that bleomycin-iron (III) complex can be reduced by isolated NADPH-cytochrome P-450 reductase (13) we measured the cytochrome c reductase activity of thymocytes in the absence and presence of NAD(P)H (Table 1). As seen from the table, thymocytes possess both theirs own and NAD(P)H-dependent cytochrome c reductase activity. This activity is one of the ways of bleomycin-iron (III) complex reduction and also takes part in its cytolytic action. In addition, the complex can be reduced non-enzymatically by hydrogen peroxide, ascorbate, thioles, etc. (12).

As bleomycin action has a free radical nature, we investigated catalase, superoxide dismutase and antioxidants ability to protected the plasma membrane of thymocytes from bleomycin-mediated

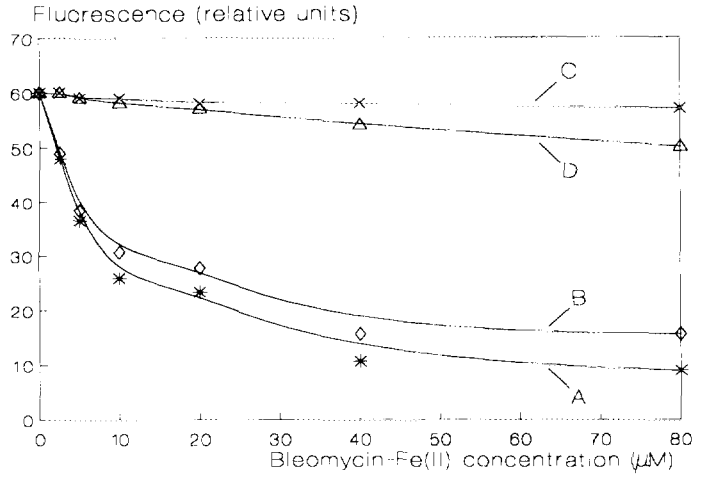

Fig. 1. Relationship between the decrease in the fluorescence of the Hoechst 33342 reagent during thymocytes destruction and the concentration of bleomycin-iron (II) complex (A), bleomycin-iron (III) complex (B), bleomycin (C), and iron (II) sulfate (D). Isolated cells previously incubated with Hoechst 33342 reagent and washed 3 times were suspended in the PBS buffer. Incubation mixture of 1 ml contained $2x10^6$ cells. The Hoechst 33342 reagent fluorescence changing was measured on excitation wavelength 355 nm and emission wavelength 420 nm at 37°C.

destruction. As it is shown on Fig. 2, bleomycin action was decreased in presence of antioxidants. That points to the possible involvement of superoxide anion, hydrogen peroxide, hydroxyl radical and other radicals in this process. This conclusion is confirmed by the formation of TBA-reactive products during cell destruction caused by the bleomycin-iron complex (Fig. 3).

The results obtained indicate that bleomycin-iron (II) and bleomycin-iron (III) complexes possess identical cytolytic activities.

TABLE 1
The own and NAD(P)H-dependent cytochrome c reductase activity of thymocytes

Cosubstrate	Cytochrome c reductase activity ($nMxml^{-1}xmin^{-1}$)
NADPH	20.2±0.74
NADH	25.9±0.72
None	4.9±0.21

Incubation mixture of 1 ml contained $1x10^6$ cells in the Hanks solution, 60 μM cytochrome c, and 50 μM NADH or NADPH (if indicated). Measurements were carried out at 37°C.

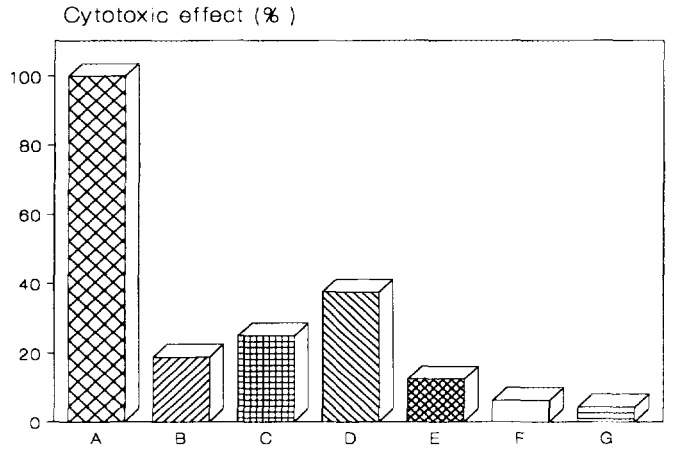

Fig. 2. Effect of catalase, superoxide dismutase, and antioxidants on the cytolytic effect of bleomycin-iron (II) complex. Incubation mixture of 1 ml contained $2x10^6$ cells. Fluorescence was measured as described in Fig. 1. Incubation time: 5 min. A - control: the cells were incubated with 25 μM bleomycin-iron (II) alone; B - as in case A, but with $12x10^3$ U/ml catalase; C - with $26x10^3$ U/ml superoxide dismutase; D - with 100 μM mannitol; E - with 20 μM ethanol, F - with 100 μM ionol (2,6-di-tert-butyl-4-methylphenol), G - with 25 μM DMSO.

This fact and the existence of reductase activity in lymphocytes mean that the complex can function in the catalytic regime due to enzymatic and non-enzymatic reduction in lymphocytes. The

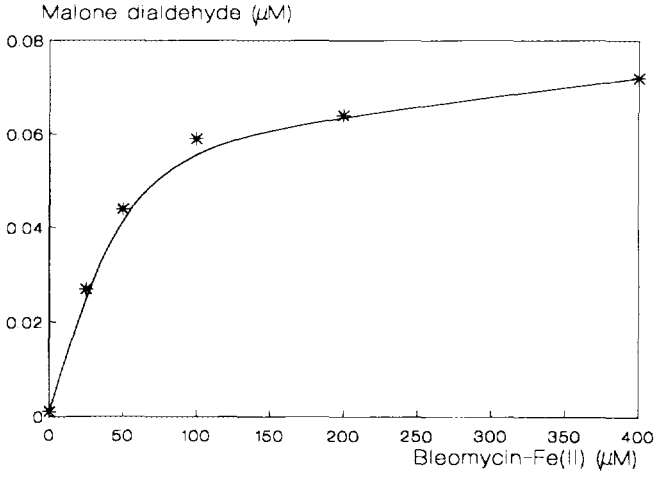

Fig. 3. Formation of TBA-reactive products of lipid peroxidation and DNA degradation during cell destruction by bleomycin-iron (II) complex. Incubation mixture of 1 ml contained $5x10^6$ cells. Incubation time: 15 min. at 37°C. MDA concentration was measured spectrophotometrically, λ=532 nm, $\varepsilon=1.6x10^5xM^{-1}xcm^{-1}$.

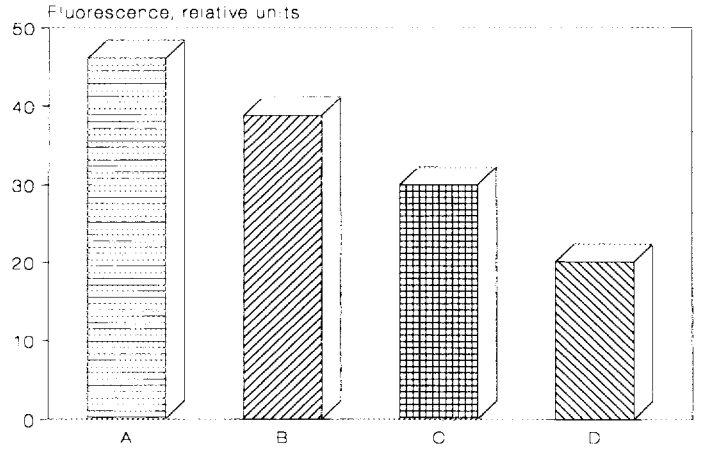

Fig. 4. Changes in fluorescence of Hoechst 33342 reagent during the destruction of thymocytes with bleomycin-iron (II) (A) and conjugates bleomycin-concanavalin A (B), bleomycin-insulin (C), and bleomycin-calcitonin (D); bleomycin concentrations were 25 μM in all cases. Incubation mixture of 1 ml contained $5x10^6$ cells. Incubation time: 5 min. Fluorescence was measured as described in Fig. 1.

protective effects of catalase, superoxide dismutase and antioxidants indicate to the radical mechanism of cell damage.

Selectivity in bleomycin action can be attained by binding the antibiotic to a fragment that imparts the complex affinity to certain cells. For this purpose antibodies or their fab-fragments, proteins specific for receptors on the cell surface, as well as synthetic targeting peptides can be used. The conjugation of bleomycin with the targeting fragment must not affect its ability to generate reactive oxygen species.

Bleomycin was conjugated with the T-cell mitogen concanavalin A, as well as with insulin and calcitonin because receptors for these molecules were present on the surface of thymus cells. Bifunctional cross-linking reagent dimethyl adipimidate was used for cross-linking. As shown in our laboratory earlier, such conjugation did not deprive bleomycin of the ability to generate reactive oxygen species. Using [^{3}H]-bleomycin correlation between bleomycin and concanavalin A, insulin, and calcitonin in conjugates was found to be 14:1, 1:1 and 1:1, respectively. Concanavalin A, insulin, and calcitonin alone did not affect the viability of the target cells, whereas their conjugates with bleomycin-iron (II) possessed higher cytolytic activities than did the free bleomycin-iron complex (Fig. 4), where bleomycin-calcitonin conjugate was the most active.

Conjugation of the bleomycin-iron complex with concanavalin A, insulin and calcitonin enhances the cytolytic effect. Bleomycin therefore can be used as a part of a hybrid molecule destroying the plasma membranes of target cells through generation of reactive oxygen species.

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