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Communication

Sensitivity of Vi phages III to γ-radiation in the presence of cisplatin

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In this study we determined Vi bacteriophage III sensitivity to native cisplatin, γ radiation (^{60}Co) or to irradiated cisplatin, and checked the possibility of enhanced Vi bacteriophage III inactivation under combined exposure to cisplatin and γ radiation. We used highly purified phage suspensions in 0.9% NaCl solution or phosphate-buffered saline. Phage suspensions were titrated using a double agar layer method. Our study implies that survival of Vi bacteriophage III shows an exponential inverse correlation with cisplatin concentration in the incubation medium and the time of phage incubation in the presence of cisplatin. The use of irradiated cisplatin reduces phage survival in comparison with suspensions containing non-irradiated cisplatin. Irradiation of phage suspension with cisplatin causes a significant increase of phage inactivation in comparison with either treatment alone. Our results suggest that presence of cisplatin in irradiated medium enhances the radiobiological effect on Vi bacteriophages III.

Keywords: cisplatin, Vi bacteriophage III, cytotoxicity, irradiation

The use of *cis*-diamminedichloroplatinum (cisplatin, CDDP) is of interest from the point of view of two overlapping areas: clinical and basic sciences. The first one includes the problems of CDDP utility as an antitumor agent. The therapeutic effect, obtained only with some tumors, development of secondary resistance to the drug and significant toxic side effects (O'Dwyer *et al*., 1997) have prompted the search for CDDP derivatives and other metal complexes with antitumor activity (reviewed by Kratz & Schütte, 1998; Dabrowski, 1999).

The comprehensive literature of the subject concerns basic studies, including problems characterizing the effect of CDDP on animal, bacterial, and plant cells and viruses. Some literature data have been collected in a monograph form (Lippert, 1999).

The molecular mechanism of CDDP activity, leading to its cytotoxic effect, has not been fully elucidated (Villani *et al*., 1999), despite the use of sophisticated techniques and various tumor cell lines. The studies of the cytotoxic effect on *Escherichia coli* (Richmond *et al*., 1977) and *Bacillus megaterium* (Richmond & Powers, 1976) demonstrated considerable enhancement of the effect of cisplatin after exposure of the bacterial suspensions to ionizing radiation, which led to the classification of cisplatin as a radiosensitizer, especially active towards cells in hypoxia (Douple & Richmond, 1978). Subsequent laboratory tests using tumor cell lines and clinical studies demonstrated enhanced cytotoxicity of irradiation-modified cisplatin (Dewit, 1987; Coughlin & Richmond, 1989). In this context, the possibility of CDDP reactions with water radiolysis products (e_{aq}, $OH^{\bullet}$) and emergence of a highly reactive form of this chemical compound is not considered to be fully proven (Dewit, 1987).

The literature contains only a few reports concerning the sensitivity of bacteriophages to cisplatin (Shooter *et al*., 1972; Drobnik *et al*., 1975; Filipski *et al*., 1980; Tanaka *et al*., 1998). So far, no experimental models using bacteriophages to determine the combined effect of CDDP and ionizing radiation, have been applied.

Taking into consideration the cited observations, our study was carried out with the following aims: (i) to determine Vi bacteriophage III sensitivity to CDDP and (^{60}Co) radiation, (ii) to determine Vi bacteriophage III sensitivity to irradiated cisplatin, (iii) to check the possibility of enhanced Vi bacteriophage III inactivation under combined exposure to cisplatin and γ radiation.

Abbreviations: CDDP, cisplatin; m.o.i., multiplicity of infection; pfu, plaque forming units; PBS, phosphate-buffered saline.

It was also intended to collect preliminary data for a simple model illustrating the molecular mechanisms of reaction with cisplatin, which determines its lethal effect. The bicomponent structure of Vi phage III (protein, double-stranded DNA) as well as the static state of the virus outside the cell (which eliminates the direct repair effect) pointed to the usefulness of determination of phage infectiveness within the biological test system.

It is believed that cisplatin may considerably modify the survival curve of irradiated cells because of its direct cytotoxicity against tumor cells and inhibition of DNA repair. The phage model allows one to eliminate the effects resulting from radiation damage repair, which are typical in cellular models and interfere with the outcome of examinations. Many reported clinical data concerning concurrent chemoradiotherapy have been focused on the analysis of the effects of increasing anti-tumor activity of cisplatin without any serious attempts at explaining this phenomenon. This explanation is of great importance for the improvement of the clinical benefits of concurrent therapy.

MATERIALS AND METHODS

Propagation and purification of phage was carried out according to the method described in previous studies (Kwiatkowski *et al.*, 1975; 1998; Tucholski *et al.*, 1999). *Citrobacter* Ci 23 Vi^+ culture in modified Stockes-Bayne (mSB) liquid medium, after reaching the optical density — $OD^1_{660} = 0.5$, was inoculated with the phage with m.o.i. = 0.1. The onset of marked lytic effect was observed after 30 min. The lowest culture OD value was obtained 2 h after phage addition. The lysate after preliminary „debris" centrifugation (2000 × *g*, 20 min) with the titer of about 1×10^{10} pfu/ml, was stored in the cooler over chloroform. Precipitation of viruses from the lysate was obtained after adding NaCl up to 0.5 M and polyethyleneglycol 6000 up to 10% concentration. After overnight storage in the cooler, the lysate was centrifuged again (2000 × *g*, 20 min). After centrifugation, the precipitate was suspended in a small (0.01 of lysate volume) amount of PBS of pH = 7.4. Final purification of the suspension was carried out by isopycnotic centrifugation in a cesium chloride linear gradient of 1.13–1.64 g/cm^3 (150 min, 60 000 × *g*). The band containing bacteriophages, after collection from the tube, was dialysed against PBS. The obtained suspension demonstrated a titer of about 10^{12} pfu/ml and the value of $OD_{260}/OD_{280} = 1.63$. It was stored in the cooler over chloroform.

Cisplatin was used in the form of a preparation for clinical use — Cisplatin (Ebewe, Austria), i.e. a solution in 0.9% NaCl.

Irradiation was performed using a γ radiation source (^{60}Co, Gammatron 3, Siemens) with various doses (1 Gy/min) in darkness at room temperature. The absorbed dose was determined with a Frick dosimeter (Bray, 1960; Broszkiewicz, 1971).

For pH determinations, a model 350 pH-meter (Orion, Boston, USA) with a combined ROSS type electrode were used. Only a slight pH increase as a result of irradiation was observed for the cisplatin solution (ΔpH < 0.1 unit).

Phage suspensions in PBS were titrated using the double agar layer method (Adams, 1959; Kwiatkowski *et al.*, 1975). Series of dilutions of an individual sample were prepared each time so as to obtain eventually from 100 to 300 plaques with halos on the plate. Titration results were obtained from counts carried out for at least three plates of the same dilution series. Taking into consideration that the highest m.o.i. value in titration amounts to 0.01 and using Poisson distribution, it was calculated that 99% of the plaques with halos resulted from single infections of bacterial cells. The count error level was higher than the error level resulting from multiple infections. However, for the confidence coefficient of 0.95, the relative titration error did not exceed 10%. Approximation of the N = f(D) function was accomplished using the minimum chi-square method, determining the correlation coefficients and their errors. LD_{37} values are given at 0.95 confidence level.

RESULTS

Inactivation of bacteriophages by cisplatin

Preliminary studies were carried out to determine the sensitivity of Vi bacteriophages III to cisplatin and the conditions of phage suspension incubation in CDDP-containing PBS medium. Phage suspension containing no CDDP served as a control for these determinations. The results of suspension titration after incubation (30 min, 37°C) with various cisplatin concentrations show an exponential correlation (data not shown). In the medium containing the highest concentration of CDDP (20 μg/ml), the inactivation rate reaches 97.9%. The corresponding calculated LD_{37} value amounts to 4.815 μg/ml. The phage titer in the control sample (N_o) was 2.8×10^5 pfu/ml and was not changed during incubation of the suspension in PBS medium.

In further experiments, by reducing the scope of the determinations to specific incubation conditions, it was intended to determine the magnitude of inactivation with respect to suspensions with various baseline phage titers. For that purpose, suspensions with baseline phage titers from 10^3 to 10^9

pfu/ml were incubated with 10 μg/ml cisplatin. Final determination results are presented in Fig. 1.

An increase of phage concentration by 6 orders of magnitude (10^3 to 10^9 pfu/ml) reduces the effect of inactivation from 91% to 83.1% and increases the LD_{37} value from 4.15 μg/ml to 5.62 μg/ml. The above may indicate a decrease of the biological availability of cisplatin with increasing phage concentration. Taking into consideration the results of preliminary experiments, further procedure involved use of cisplatin concentrations in the 1–10 μg/ml range of CDDP concentrations and use of phage suspensions with specific baseline titers of 10^4 or 10^8 pfu/ml.

The next stage of the study involved determination of phage activity changes as a function of incubation time. At that stage, phage suspensions were incubated with 10 μg/ml CDDP at 37°C or 0°C. In the suspension incubated at 0°C, 60% activity was observed after 60 min of incubation, whereas in that kept at 37°C the phage activity decreased by nearly two orders of magnitude. The calculated LD_{37} values after 60 min incubation at 37°C and 0°C are 2.8 and 16.54 μg/ml, respectively.

Inactivation of bacteriophages after incubation with irradiated cisplatin

The previously specified conditions used for the determination of the inactivation effect caused by CDDP alone were then used in experiments determining the effect of irradiated cisplatin solution. Each experiment involved control determination series carried out on non-irradiated solution (D = 0) and determinations carried out on solutions irradiated with doses increasing up to about 150 Gy. Elimination of the stable product of water radiolysis, hydrogen peroxide, which causes post-radiation phage inactivation, was obtained by preincubation of the irradiated cisplatin solution with catalase or by its 100-fold dilution. Table 1 lists the data from these determinations.

If cisplatin irradiated with a 105.25 Gy dose is used, LD_{37} is reduced to 0.92 μg/ml, whereas for non-irradiated cisplatin the LD_{37} value is 2.52 μg/ml. In terms of phage inactivation effect, it means an over 2.5-fold increase of cisplatin efficiency.

Compared with the inactivation effect of non-irradiated cisplatin as a reference value, the use of irradiated cisplatin reduces the relative phage survival (Fig. 2). Cisplatin solution irradiated with a 105.25 Gy dose increases inactivation of Vi bacteriophages III over 30-fold.

Inactivation of bacteriophages in suspension exposed to irradiation

The methodology of determination of radiobiological effect on the investigated Vi bacteriophage III suspensions included their inactivation by non-irradiated cisplatin and the post-radiation effect. Phages was added to the suspensions immediately after their exposure to irradiation. Elimination of post-radiation effect caused by hydrogen peroxide was achieved by 100-fold dilution of irradiated suspensions with buffer. Such treatment of cisplatin-containing media reduced the effect of CDDP only until the moment of sample irradiation.

The use of appropriate cisplatin concentrations in PBS medium was also an important methodological problem faced in the course of the determinations. As a result of multiple preliminary experiments (not shown), each series of determinations was carried out simultaneously in three different

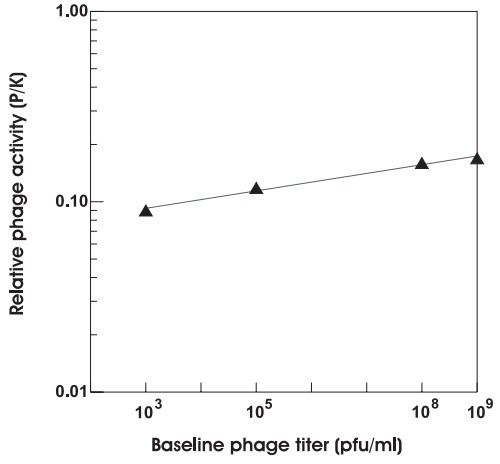

Figure 1. Relative Vi phage III activity as a function of baseline phage titer after 30 min incubation at 37°C with 10 μg/ml CDDP.

Abbreviations: P, phage titer in CDDP assay; K, phage titer in control assay. For baseline phage titers 10^3, 10^5, 10^8 and 10^9 pfu/ml, the respective LD_{37} values are: 4.15, 4.68, 5.45 and 5.62 μg/ml.

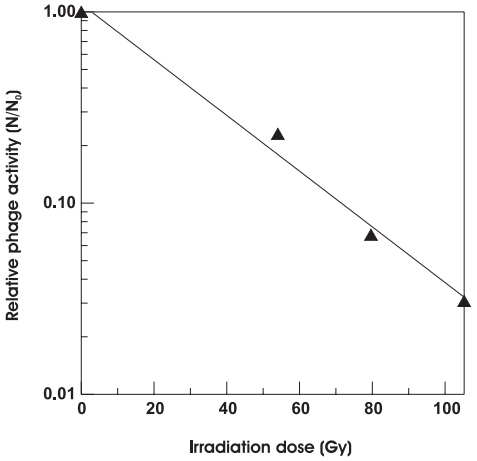

Figure 2. Relative Vi phage III activity after incubation (1 h, 37°C) with 5 μg/ml cisplatin irradiated with a given dose.

Abbreviations: N, phage titer in irradiated assay; N_o, phage titer in non-irradiated assay.

Table 1. Inactivation of phages by irradiated cisplatin.

Irradiation dose [Gy]	Relative phage activity	LD_{37} [μg/ml]
0	1	2.52
54.04	0.23	1.45
79.65	0.068	1.07
105.25	0.031	0.92

Incubation was performed for 1 h at 37°C in irradiated PBS medium containing 5 μg/ml CDDP.

media: PBS, PBS containing 1 μg/ml cisplatin and PBS containing 5 μg/ml cisplatin. The radiobiological effect obtained in PBS medium was treated as the reference and control. Samples containing 1 μg/ml cisplatin were the reference for determinations of phage inactivation by irradiated CDDP (LD_{37} = 0.92 μg/ml), whereas those with 5 μg/ml cisplatin were compared with non-irradiated CDDP (LD_{37} = 5.45 μg/ml).

The results of one complex determination series are presented in Fig. 3.

The relative phage activity (N/N_o) demonstrates, for all the experiments, an exponential dependence on the radiation dose. The presence of cisplatin in the medium enhances the inactivation effect. Under the highest irradiation dose (82.81 Gy) conditions used for the medium containing 1 μg/ml CDDP, inactivation increases almost 100 times, and for the medium with 5 μg/ml CDDP — almost 10^5-fold in comparison with the effect obtained for PBS medium. In comparison with control determinations (N_o), inactivation in the latter case reduced phage survival 7-fold only. The LD_{37} values for individual assays, calculated on the basis of detailed data (N/N_o, D) are as follows: PBS medium (without CDDP) — 55.54 Gy; PBS medium (1 μg/ml CDDP) — 18.03 Gy; PBS medium (5 μg/ml CDDP) — 7.07 Gy.

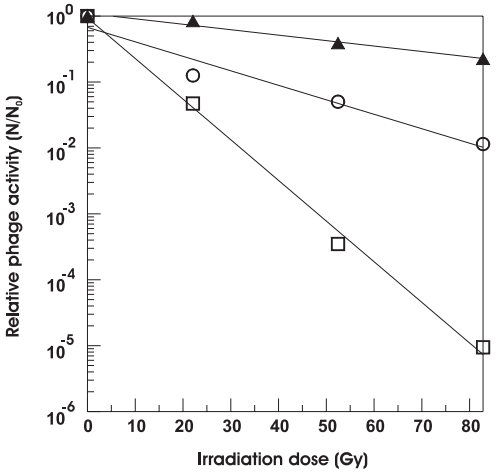

Figure 3. Effect of absorbed γ radiation dose on the relative activity of Vi phages III suspended in PBS with 1 or 5 μg/ml cisplatin.

CDDP was added immediately before irradiation. Assay symbols: ▲, phage suspension; ○, phage suspension with 1 μg/ml CDDP; □, phage suspension with 5 μg/ml CDDP.

DISCUSSION

An important aim of this study was to determine the effect of inactivation of Vi phages III by native and irradiation-modified cisplatin. Besides other reasons presented in the introduction, another justification for undertaking the study was the small number of reports concerning inactivation of bacteriophages by cisplatin (Shooter *et al.*, 1972; Drobnik *et al.*, 1975; Filipski *et al.*, 1980). The available literature contains no data concerning phage inactivation under combined exposure to ionizing radiation and cisplatin or the toxic effect of radiation-modified cisplatin. This also makes it difficult to compare and analyze own results in the context of literature data.

In coordination chemistry, cisplatin (II) and other compounds of this type are regarded as "soft" Lewis acids, demonstrating high affinity for sulphur and their affinity for nitrogen as a donor is higher than that for oxygen (Wang *et al.*, 1996). This explains the binding of Pt(II) with S and N atoms as donors in peptides and proteins, as well as in DNA and with the polar portion of lipids. With respect to proteins, reaction with native cisplatin takes place, whereas other biological compounds react with the products of cisplatin hydrolysis (Wang *et al.*, 1996), primarily with $[Pt(NH_3)_2Cl(H_2O)]^+$. This and other hydrolysis products appear spontaneously in the cytoplasm due to reduced chloride ion concentration (Dewit, 1987). Recently, Yachnin *et al.* (1998) obtained unequivocal experimental evidence confirming this hypothesis with respect to the U-1285 tumor cell line. It has been demonstrated that the use of monohydroxy form potentiates the toxic effect in comparison with that obtained with native cisplatin. The increased cytotoxic effect is considered to be related more to the changes in the cell membrane than to adduct formation in DNA (Wang *et al.*, 1996; Yachnin *et al.*, 1998). However, the hypothesis that inside the cell native cisplatin is rapidly transformed into ionic forms at pH ≈ 7.4 and 37°C is still valid (Eastman, 1990). Products of CDDP hydrolysis interact with cytoplasmatic macromolecules, but it is chromosomal DNA damage that determines directly the cytotoxicity of the compound (Villani *et al.*, 1999). An increased content of reactive CDDP forms in the medium does not eliminate reactions with cytoplasmatic elements, but it enhances only the reactions with cell membrane components.

Some other aspects of the reaction are emphasized with respect to bacteriophages. Drobnik *et al.* (1975), on the basis of survival determinations carried out for phages T2, T4, T4 BO1, T3 and λ in media containing cisplatin and products of its hydro-

lysis, observed two-stage inactivation under incubation conditions: a slow stage lasting 40–90 min and the following stage when inactivation occurs in an exponential manner. The slow stage is prolonged in the case of osmotically sensitive bacteriophages (T2, T4), which is associated with low capsid permeability. Inactivation of phages by the product of hydrolysis is 40–80-fold higher than that induced by the native compound. The authors conclude that inactivation is caused by the cisplatin hydrolysis product formed inside the phage capsid.

The experimental data presented in this paper indicate high sensitivity and rapid inactivation of Vi bacteriophage III, with an exponential dependence on the time of incubation with cisplatin at different temperartures (0°C, 37°C). This is very similar to the literature data (Drobnik *et al*., 1975). Incubation at 37°C for 30 min requires cisplatin concentration of 4.815 μg/ml to obtain survival reduction to 37%, and after 60 min at incubation the same effect is obtained with 2.8 μg/ml cisplatin. Inactivation of Vi bacteriophage III, expressed in terms of relative activity (N/N_o), depends on their baseline titer N_o (Fig. 1). This may indicate a decrease of reagent bioavailability. In view of the relatively high concentration of cisplatin $[(NH_3)_2PtCl_2]^o$ used in our experiments, such a decrease of bioavailability may be related to its ion forms, such as $[(NH_3)_2Pt(H_2O)]^+$ or $[(NH_3)_2Pt(H_2O)_2]^{2+}$. This would be consistent with the hypothesis presented by Wang *et al.* (1996) that even in the presence of high concentrations of Cl^- ions, a fraction of cisplatin, although very small, undergoes hydrolysis. It seems that the hydrated forms of cisplatin are primarily responsible for the effect of Vi phage III inactivation during incubation with native cisplatin.

The pH values for irradiated cisplatin solutions ($\Delta pH < 0.1$) were determined with high precision, in view of the reports by Laurencot and Kennedy (1995) and Yachnin *et al.* (1998) that pH may be an important factor, even more important than chloride concentration, for reactions with cisplatin.

Incubation of Vi phage III (1 h, 37°C) with irradiated cisplatin solution demonstrates enhanced inactivation. LD_{37} of the control sample (D = 0) in these determinations was 2.52 μg/ml, whereas for the solution irradiated with the highest dose used (D = 105.25 Gy) it was only 0.92 μg/ml. This solution increases Vi phage III inactivation over 30-fold. This seems to confirm the hypothesis that Vi phage III inactivation is caused primarily by the products of cisplatin hydrolysis present in the medium. It is worth emphasizing that enhanced inactivation takes place in a medium containing high concentrations of Cl^-. A low value of LD_{37} (below 1 μg/ml) was also obtained by Shooter *et al.* (1972) in survival tests of T7 bacteriophage, which may be regarded as a structural counterpart of Vi phage III, exposed to cisplatin. The determinations were carried out in 0.02 M phosphate buffer (pH = 7), which generates the substitution of Cl^- by H_2O in the native cisplatin molecule. According to the calculations presented in the cited paper, the mean lethal dose for the phage corresponds to 5 Pt molecules, but 76% of Pt binds with DNA. On the basis of analysis of own and literature data, it seems justifiable to conclude that inactivation of Vi phage III is due, first of all, to the presence of ionic forms of cisplatin in the medium. Capsid permeability, reaction with proteins and generation of hydrated forms inside the phage head may additionally modify the inactivation effect.

Changes of relative Vi phage III activity under combined exposure to cisplatin and ionizing radiation demonstrate exponential dependence in the function of dose (Fig. 3). They were obtained for the concentrations of 1 and 5 μg/ml cisplatin in PBS. The radiobiological effect occurring in the buffer (without cisplatin added) after irradiation of Vi phage III suspension was treated as a reference. Relative phage activity (N/N_o) demonstrates, for all the experiments, exponential dependence on the radiation dose. The presence of cisplatin in the medium enhances the inactivation effect. Under the highest irradiation dose (82.81 Gy) used for the medium containing 1 μg/ml CDDP, inactivation increases almost 100 times, and for the medium with 5 μg/ml CDDP concentration – almost 10^5 times in comparison with the effect obtained for PBS medium. In comparison with control determinations (N_o), inactivation in the latter case reduced phage survival only 7-fold.

Taking into consideration the studies by Zaremba *et al.* (1989; 1990), it should be assumed that Vi phage III inactivation in the foregoing experiments is the result of indirect inactivation, i.e. one caused by water radiolysis products (control samples) and/or the products of their reaction with cisplatin. A considerable element of uncertainty is associated with the reactivity of intermediate products of free radical reactions with cisplatin (Dewit, 1987). The radiation doses corresponding to LD_{37} are markedly lower for media containing cisplatin. A low concentration of cisplatin in the medium (1 μg/ml) reduces LD_{37} over three-fold in comparison with the effect obtained for the control sample (18.03 Gy and 55.54 Gy, respectively). A five-fold increase of cisplatin concentration (5 μg/ml) causes a further decrease of this value, but only 2.5-fold (LD_{37} = 7.07 Gy). This relative reduction of the inactivation effect may result from potential free radical — cisplatin reaction under specific radiation intensity conditions.

In summary, combined exposure to ionizing radiation and cisplatin results in an increased extent of Vi bacteriophage III inactivation as compared with the additive effect of the two factors independ-

ent of each other, which means the cisplatin is a factor potentiating the effect of ionizing radiation.

The presented results and conclusion can be treated only as a preliminary stage of studies carried out with the aim to elucidate the mechanism of molecular reaction with cisplatin. Numerous problems in this field remain to be explained.

Acknowledgments

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