Effect of Sodium Chloride Concentration on Adriamycin and N-Trifluoroacetyladriamycin-14-valerate (AD32)-induced Cell Killing and DNA Damage in Chinese Hamster V79 Cells

George Iliakis and Wendy Lazar

Thomas Jefferson University Hospital, Department of Radiation Therapy and Nuclear Medicine, Laboratory of Experimental Radiation Oncology, Philadelphia, Pennsylvania 19107

ABSTRACT

Exponentially growing Chinese hamster V79 cells were exposed to Adriamycin either in phosphate buffered saline (PBS) or fresh growth medium (F-med) supplemented with various amounts of NaCl in the range between 50–1000 mM and survival was measured by the colony forming assay. Compared to the survival obtained after exposure of cells to isotonic (140 mM NaCl) PBS (D0 = 0.16 µg/ml, D1 = 0.49 µg/ml) a potentiation in cell killing was observed after treatment in hypotonic (50 mM NaCl) PBS (D0 = 0.08 µg/ml, D1 = 0.19 µg/ml) and a reduction in cell killing after treatment in hypertonic (500 mM NaCl) PBS (D0 = 0.36 µg/ml, D1 = 0.55 µg/ml). Cells exposed to Adriamycin in F-med were more sensitive to Adriamycin (D0 = 0.1 µg/ml, D1 = 0.27 µg/ml) than cells exposed to Adriamycin in PBS, but cell killing was reduced when the medium was made hypertonic by the addition of NaCl (500 mM NaCl) (D0 = 0.23 µg/ml, D1 = 0.45 µg/ml). The amount of Adriamycin accumulated in the cells during treatment was measured in a spectrophotofluorometer and was found to vary as a function of the treatment medium and NaCl concentration. Cells exposed to Adriamycin in PBS (isotonic) were accumulating three to four times less drug than cells exposed to Adriamycin in F-med. Less Adriamycin (two to three times) was also accumulated in cells treated in hypertonic (500 mM NaCl) F-med. Compared to the Adriamycin accumulation observed after exposure to cells in isotonic PBS, an increase was observed after exposure in hypotonic PBS (2.3 times) but no change after exposure in hypertonic PBS (500 mM NaCl). Adriamycin-induced DNA damage was assayed with the alkaline filter elution technique and it was found to increase after treatment in hypotonic PBS and to decrease after treatment in hypertonic PBS. The modification in the survival curve slope and DNA damage induction observed after exposure in hypotonic PBS was quantitatively similar to the modification in intracellular drug concentration (factor of 2.3, comparison based on the results obtained in isotonic PBS). However, after exposure of cells to Adriamycin in hypertonic PBS, a reduction by a factor of 20 was observed in the induction of DNA damage but a reduction only by a factor of 2.3 was observed in cell killing with no modification of intracellular Adriamycin concentration. Exposure of cells to the Adriamycin analogue N-trifluoroacetyladriamycin-14-valerate (AD32) resulted in a dose- and time-dependent cell killing which was enhanced after incubation in hypotonic or hypertonic PBS. The importance of Adriamycin binding to DNA for cytotoxicity and its modification by theionic strength of the medium used for the treatment are considered for the interpretation of the results obtained.

INTRODUCTION

Anthracyclines, in particular Adriamycin, are antitumor antibiotics extensively used in the treatment of a great variety of human tumors (see Ref. 1 for a review). The mechanism of their action at the molecular level is not exactly known, and it has been the subject of numerous investigations. Identification of the determinants responsible for the cytotoxic effects of these compounds is expected to help devise improved treatment protocols for their clinical application and design new analogues with improved action characteristics. Among the interactions of anthracyclines with biological macromolecules the interaction with DNA is of particular interest, since the biological activity of these compounds is thought to reside in their ability to bind to DNA (2). The high affinity mode of binding to nucleic acids was shown to involve intercalations of the planar chromophore (3) between DNA bases. It is thought that this DNA-drug association is a cause for the observed inhibition of DNA and RNA synthesis (4), which is also observed at very low drug concentrations (5).

Although a cause-effect relationship between molecular damage induced by Adriamycin and observed cytotoxicity has not been established yet conclusively, it is usually assumed that there is a correlation between affinity of binding to the DNA and observed cell killing (6). As expected for charged ligands, the association constants for the interaction of these drugs with DNA were found to be dependent on the ionic strength (7–9). It was of interest, therefore, to examine whether the observed modifications in the affinity of Adriamycin binding to DNA as a function of the ionic strength of the solution were also reflected by modifications in the killing efficacy and in the efficacy of induction of DNA damage, when treatment was carried out in solutions supplemented with various amounts of NaCl. Despite the potential importance such studies may have in elucidating the in vivo mechanism of Adriamycin action, no information is available on the possible modulation of cell killing and DNA damage induction after exposure to anthracyclines of cells incubated in solutions of various tonicities.

Brief changes in tonicity of the medium introduced during or shortly after exposure of cells to ionizing radiations were found to result in a significant increase in radiosensitivity (10–13). Since Adriamycin, and anthracyclines in general, are radiomimetic drugs, it was of additional interest to compare the effect of solutions of variousionic strength on drug or radiation-induced cell killing.

In the present paper, we report experiments designed to study the modulation of Adriamycin-induced cytotoxicity and DNA damage as a function of NaCl concentration in the medium used during treatment. The effect of an analogue, AD32, known to be cytotoxic without binding to DNA (14, 15), was also studied, and the results obtained compared to those observed after treatment with Adriamycin.

MATERIALS AND METHODS

For experiments, a line of Chinese hamster V79 cells (S171) was used (16). Cells grew in a humidified atmosphere of 5% CO2 in MEM supplemented with 15% fetal bovine serum (Hazelton-Dutchland, Den-
**NaCl Modulation of Cellular Effects of Anthracyclines**

**RESULTS**

The surviving fraction of cells treated with 0.1 μg/ml Adriamycin (30 min) in phosphate buffered saline containing various amounts of NaCl (50–1000 mM) are shown in Fig. 1. Treatment in hypotonic PBS (NaCl concentrations lower than 140 mM) considerably enhanced, in a concentration dependent way, Adriamycin-induced cell killing. Surviving fraction was reduced to 0.05 when the treatment was carried out in PBS containing 100 mM NaCl and to 0.007 after treatment in PBS containing 50 mM NaCl. On the other hand, a reduction in killing was observed when cells were treated with Adriamycin in hypotonic PBS (NaCl concentration higher than 140 mM). Increase in NaCl concentration to 250 and 500 mM resulted in an increase in surviving fraction to 0.6 and to 0.93, respectively. There was no toxicity observed after incubation of cells in salt solutions in the range between 50 and 500 mM NaCl. However, some killing was observed (30–40% decrease in plating efficiency) at NaCl concentrations higher than 700 mM, probably responsible for the enhancement in killing observed in Fig. 1 at NaCl concentrations higher than 500 mM.

The effect of toxicity on the shape of the Adriamycin dose-effect curve was studied for NaCl concentrations of 50 and 500 mM and was compared to that obtained after exposure to isotonic PBS. The results obtained are shown in Fig. 2. Treatment of cells with various doses of Adriamycin in isotonic PBS resulted in a shoulder-type survival curve (C) with \( D_0 = 0.16 \mu g/ml \) and \( D_q = 0.49 \mu g/ml \). Treatment of cells with Adriamycin in hypotonic PBS (50 mM NaCl) enhanced cell killing and resulted in a survival curve (D) with \( D_0 = 0.08 \mu g/ml \) and \( D_q = 0.19 \mu g/ml \). On the other hand, treatment of cells in hypotonic PBS reduced Adriamycin-induced killing and resulted in a survival curve with \( D_0 = 0.36 \mu g/ml \) and \( D_q = 0.55 \mu g/ml \).

A similar reduction in Adriamycin-induced killing was observed in cells exposed in growth medium made hypertonic by the addition of NaCl. As shown in Fig. 3, exposure of cells to Adriamycin in isotonic fresh medium caused more killing than after exposure in isotonic PBS (Fig. 1) and resulted in a survival curve with \( D_0 = 0.10 \mu g/ml \) and \( D_q = 0.27 \mu g/ml \). Treatment in hypertonic (500 mM NaCl) medium reduced cell killing under these conditions as well and resulted in a survival curve with \( D_0 \) of 12.9. Fractions (90 min) were collected and weighed. Aliquots from each fraction were neutralized with 0.5 N HCl and counted in a scintillation counter. The fraction of the total activity remaining on the filter was plotted as a function of the elution time, after each particular treatment. To facilitate comparison of the results obtained under various experimental conditions, the slope of the elution curve was calculated using linear regression analysis and is given expressed as \( h^{-1} \).
NaCl MODULATION OF CELLULAR EFFECTS OF ANTHRACYCLINES

Fig. 2. Survival curves of cells exposed to various concentrations of Adriamycin for 30 min in isotonic PBS (O), hypotonic PBS (△), or hypertonic PBS (●).

Fig. 3. Survival curves of cells exposed to various concentrations of Adriamycin for 30 min in isotonic fresh growth medium (O) or in medium that was made hypertonic by the addition of NaCl.

= 0.23 µg/ml and $D_0 = 0.45$ µg/ml. The modification observed in $D_0$ was similar (increase by a factor of 2.3 after treatment in PBS and a factor of 2.4 after treatment in growth medium) for exposure in hypertonic PBS or growth medium, but the modification in $D_0$ was larger in growth medium (increase by a factor of 1.7 ± 0.1 compared to 1.1 ± 0.1 after treatment in PBS).

To study the extent to which the observed modification in Adriamycin-induced cell killing as a function of NaCl concentration reflected modifications in the intracellular drug accumulation, cells were treated in PBS or growth medium of various tonicities and the intracellular drug content was measured as described in “Materials and Methods.” The results obtained are shown in Fig. 4. Treatment of cells in isotonic PBS (O) resulted in a concentration-dependent increase in the amount of drug accumulated in the cells. Reduction in the amount of NaCl caused an increase in intracellular drug accumulation (●); 2.3 times higher Adriamycin concentrations were found to be needed after treatment in isotonic solution to reach intracellular drug levels similar to those measured after treatment in hypotonic solution. This value is similar to the values obtained for the modification of the survival curve parameters in Fig. 2 where modification in $D_0$ and $D_1$ by a factor of 2.3 and 2.5, respectively, was observed. When Adriamycin treatment was carried out in hypertonic PBS (V) no modification in intracellular drug accumulation was observed.

Cells treated with Adriamycin in isotonic growth medium (△) were found to accumulate larger amounts of Adriamycin than cells treated in isotonic PBS (increase by 3.4 ± 0.4, Fig. 4). However, contrary to the observations in PBS, treatment in hypertonic growth medium caused a reduction in the intracellular drug accumulation (●). The Adriamycin dose in the medium required to achieve similar intracellular drug accumulation increased with dose and it was higher by a factor of 2 and 3 for an accumulation of 0.2 and 0.5 µg/10^6 cells, respectively.

The effect of NaCl concentration on the induction of DNA damage by Adriamycin, as assayed by the alkaline elution technique (see “Materials and Methods”), is shown in Fig. 5. Cells were treated in hypotonic (50 mM NaCl; Fig. 5, left) PBS with Adriamycin concentration resulting in approximately equal cell killing (see Fig. 2) and analyzed immediately thereafter. Treatment with increasing doses of Adriamycin in isotonic PBS increased the DNA elution rate. This indicates induction by Adriamycin of DNA damage, which, under the experimental conditions employed, mainly comprised DNA breaks and alkali labile sites. Treatment in hypotonic PBS resulted in an increase and treatment in hypertonic PBS in a decrease in the DNA damage induced for the same Adriamycin concentrations. In Fig. 6 the slopes of the linear part of the elution curves as shown in Fig. 5 are plotted as a function of the Adriamycin concentration. A large increase in the induction of DNA damage was observed as a function of Adriamycin dose when treatment was carried out in hypotonic PBS compared to that obtained after treatment in isotonic PBS and there was
only a small amount of DNA damage induced after treatment in hypertonic PBS. Adriamycin concentrations higher by a factor of 2.8 ± 0.2 were required after treatment in isotonic PBS to produce the same amount of damage as that observed after treatment in hypotonie PBS. This value correlates well with the modification observed in the survival curve slope (factor of 2) and the modification observed in the intracellular accumulation of Adriamycin (factor of 2.3). On the other hand, concentrations of Adriamycin higher by more than a factor of 20 were required after treatment in hypertonic PBS to produce the same amount of DNA damage as after exposure in isotonic PBS. This value far exceeds the modifications observed in the $D_0$ (factor of 2.3) and does not correlate with the modification in intracellular Adriamycin content, which was found to be essentially the same after treatment in hypertonic or isotonic PBS.

To study whether the effect of NaCl on Adriamycin-induced cell killing was related to the ability of Adriamycin to bind on DNA, experiments similar to those shown in Fig. 2 were performed with the Adriamycin analogue AD32, which is known to be cytotoxic without binding to DNA (14, 15). Modifications in AD32 cytotoxicity were measured after exposure in fresh growth medium for various times or in PBS containing various amounts of NaCl (30 min). The results obtained are summarized in Fig. 7. For cells incubated in fresh growth medium an increase in cell killing was observed with increasing drug exposure time between 0.5 and 3 h (Fig. 7, top). Contrary to the observations after exposure to Adriamycin (see Figs. 2 and 3), exposure of cells to AD32 in isotonic PBS (Fig. 7, Δ, lower) resulted in more killing than that observed after treatment in growth medium. Cell killing was further enhanced when treatment was carried out in hypotonie PBS (50 mM NaCl) (Fig. 7, △, bottom). Treatment of cells with AD32 in PBS containing 500 mM NaCl (Fig. 7, Δ) caused additional sensitization and resulted in survival levels lower than those obtained after treatment in isotonic or hypertonic PBS.

**DISCUSSION**

The results presented in the previous section indicate a strong dependence of Adriamycin-induced cytotoxicity and DNA damage on the ionic strength (tonicity) of the medium used during treatment. Enhancement of killing, expressed as a decrease in $D_0$, was observed when NaCl concentration in PBS was reduced to 50 mM, and a reduction in cell killing expressed as an increase in $D_0$ was observed when NaCl concentration in PBS was increased to 500 mM. A similar reduction in cytotoxicity was obtained when treatment was carried out in fresh growth medium supplemented with NaCl to give a final concentration of 500 mM. These results are different from those obtained after exposure of cells to AD32 in PBS supplemented with various amounts of NaCl (Fig. 7). In this case, an enhancement of cell killing was observed after treatment in hypertonic (500 mM) or hypotonie (50 mM) PBS, a result similar to that obtained after exposure to X-rays and subsequent treatment in hypertonic or hypotonie solution (12). The modulation observed in Adriamycin-induced cell killing as a function of NaCl concentration was accompanied by similar, although not always analogous, modifications in the induction of DNA damage. This observation suggests a possible cause-effect relationship between Adriamycin-induced damage in the DNA and observed cell killing. It should be pointed out that similar results were also obtained after a 1 h treatment with Adriamycin despite the relatively high salt-induced cytotoxicity. This finding indicates that the observed effect is not confined to short treatment times and, therefore, to conditions where a steady state of drug uptake may not have been reached.

The difference in the modulation by NaCl of Adriamycin and AD32-induced cell killing suggests that alterations in the binding affinity of Adriamycin to DNA under the various conditions of treatment used may have caused the modifications in cell killing and DNA damage induction observed. Because AD32 is cytotoxic without binding to DNA, it is assumed that variations in salt concentration, in this case, affects repair and fixation of induced lesions, in a way similar to that observed after exposure to X-rays, rather than the induction of lesions per se. As indicated by the results shown in Figs. 2 and 4, the enhancement in cell killing and DNA damage induction observed after treatment in PBS containing 50 mM NaCl was comparable to the increase observed in the intracellular Adriamycin accumulation under similar conditions. Also, the reduction in cell killing observed after treatment in growth medium containing 500 mM NaCl correlated well with the reduction observed in intracellular Adriamycin accumulation after treatment under similar conditions. The apparent lack of correlation between Adriamycin-induced cell killing or DNA damage and intracellular...
NaCl MODULATION OF CELLULAR EFFECTS OF ANTHRACYCLINES

drug accumulation as observed after treatment in hypertonic PBS will be discussed later.

It is possible, therefore, that the observed modifications in the intracellular accumulation of Adriamycin as a function of NaCl concentration are related to modifications by NaCl in the affinity of Adriamycin binding to DNA. It has been reported (7–9) that the binding of Adriamycin to chromatin in vitro is strongly dependent upon the ionic strength of the medium, decreasing with increasing concentration of salt. Reduction in the affinity of binding to chromatin is expected to reduce the diffusion gradient and thus the amount of drug accumulated in the cells per unit time, if passive transport is assumed as the prevalent transport mechanism of anthracyclines through cell membrane (19). On the other hand, incubation in hypertonic or hypotonic solutions may also affect the energy household of the cell, due to the increased requirements in energy for the maintenance of membrane potentials, affecting thus indirectly active drug transport through cell membrane (20).

Similar consideration can also be invoked to explain the reduction in cell killing observed after treatment of cells in isotonic PBS compared to that observed after treatment in isotonic growth medium (compare results of Figs. 2 and 3). As indicated in Fig. 4, this reduction in cell killing (increase in $D_0$ by a factor of 1.6) is accompanied by a reduction in intracellular drug accumulation by a factor of 3 to 4. This reduction in intracellular Adriamycin accumulation may be related to a reduction in the energy supply of the cells, due to the fact that PBS does not contain glucose. However, the reduction in intracellular drug content is disproportionately large compared to the increase in $D_0$ and it is possible that other molecular alterations also contribute to the observed reduction in killing. For example, it is possible that alterations in chromatin structure mediated by the reduction in the metabolic activity of the cells in PBS, prevent Adriamycin binding to DNA, thus affecting cytotoxicity. This possibility is also supported by the results obtained with cells exposed to AD32 either in fresh medium or in isotonic PBS (Fig. 7). In this case, treatment in PBS was found to cause more cell killing than treatment in fresh medium, thus indicating a correlation between drug binding to DNA and reduction in cytotoxicity after incubation in PBS.

Similar conclusions can also be drawn based on the results obtained after treatment of cells with Adriamycin in hypertonic PBS. Since the intracellular drug accumulation was not significantly modified under these conditions, the reduction in cell killing and DNA damage induction can be attributed to salt-mediated alterations in chromatin structure, which either prevents binding of Adriamycin to DNA or alters in efficiency of damage induction in the DNA by bound Adriamycin.

It is interesting to mention that the reduction in the induction of DNA damage observed after treatment in hypertonic PBS was significantly larger than the reduction in cell killing (see “Results”), a result that can be interpreted as indicating the existence of cellular targets other than DNA for Adriamycin-induced killing (21), or a potentiation of killing (fixation of DNA damage) after treatment in hypertonic PBS. The former hypothesis is supported by the observation that Adriamycin can be cytotoxic without entering the cell (21), a result suggesting that other cellular structures, as for example the cell membrane, can be a target for Adriamycin-induced killing. It is possible, therefore, that at high NaCl concentrations, where the induction of DNA damage is significantly reduced, effect of Adriamycin on other cellular targets may be the reason for the observed cytotoxicity. The latter hypothesis is based on the killing potentiation observed after incubation in hypertonic PBS of cells exposed to ionizing radiations (10–13). If a similar potentiation mechanism is active also for Adriamycin-induced DNA damage, it is likely that more killing will be observed after incubation in hypertonic PBS at the same level of DNA damage. The observation that treatment in hypertonic PBS enhanced AD32-induced killing (Fig. 2) suggests that hypertonic salt solutions may act on anthracyclines and ionizing radiation-induced damage in a similar way, the effect of which may be, however, masked in cases where ionic-strength dependent drug binding to DNA is involved in the inactivation mechanism. More experiments are required to distinguish between these two alternative interpretations.

Microscopy studies showed that hypertonic or hypotonic treatment of cultured mammalian cells results in a dispersion of condensation of cellular chromatin (10, 22, 23). These alterations in chromatin state are likely to affect the rate of Adriamycin binding to DNA and, therefore, the rate of induction of DNA damage. Furthermore, it is possible that modifications in the state of chromatin shortly after the induction of damage in the DNA affect the course of its repair and promote its fixation. The results obtained with Adriamycin at the DNA and cell survival level indicate that both mechanisms may act simultaneously resulting in the observed response.

Recent studies have indicated that the nuclear enzyme type II DNA topoisomerase may be a mediator of DNA damage produced by diverse groups of antitumor drugs including anthracyclines (24, 27). Modifications in the activity or the DNA association of the enzyme as a function of the ionicity of the medium is expected, therefore, to also affect the observed cytotoxicity. Thus, to the extent that the observed cytotoxicity was correlated to topoisomerase II-mediated DNA damage, the observed modifications in cell killing for PBS versus fresh growth medium incubation or for various NaCl concentrations may be associated to an effect on this enzyme.

The possibility that the observed reduction in Adriamycin cytotoxicity might be due to self association of the compound at high salt concentrations (28) seems unlikely for the following reasons: (a) any significant self association of Adriamycin would affect the drug transport into the cell. However, as the results in Fig. 4 show, Adriamycin enters cells at the same rate under hypertonic and isotonic conditions (PBS treatment); (b) intracellular aggregation of Adriamycin is unlikely due to the high affinity of binding to DNA; (c) it has been shown that self association of daunomycin in solutions containing up to 200 mm salt does not affect its binding to DNA even at concentrations significantly higher than those used in this work (28). As shown in Fig. 1 the effect of ionic strength on Adriamycin cytotoxicity has nearly reached a maximum at 200 mm.

In summary, a significant reduction in Adriamycin cytotoxicity was observed with increasing ionic strength. It is postulated that this phenomenon may be due to: (a) a reduction in the binding affinity of the drug to DNA at high salt concentrations, which reduces the driving force for the drug diffusion into the cell and caused the observed reduction in intracellular drug accumulation; and (b) to a reduction in the amount of DNA damage induced by inhibition of the damage-induction process, for example, by inhibiting topoisomerase II activity. More experiments are required for an elucidation of these processes.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Mervyn Israel for kindly supplying samples of AD32, and for fruitful discussions on the manuscript; to Suzanne Bobyock for preparation of the artwork; and to Susan Dou-
NaCl MODULATION OF CELLULAR EFFECTS OF ANTHRACYCLINES

thart for typing the manuscript. Special thanks go to Dr. Dennis B. Leeper for useful suggestions in the preparation of the manuscript, and to Dr. P. Mojaverian from the Department of Clinical Pharmacology at Thomas Jefferson University for his support in the measurements involving the use of a spectralfluorometer.

REFERENCES

6. Neidle, S., and Taylor, G. L. Nucleic acid binding drugs. Some «informa...
Effect of Sodium Chloride Concentration on Adriamycin and N-Trifluorooacetyladiamycin-14-valerate (AD32)-induced Cell Killing and DNA Damage in Chinese Hamster V79 Cells

George Iliakis and Wendy Lazar

Cancer Res 1987;47:1853-1858.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/7/1853

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.