Effect of Deoxyribonuclease on Adriamycin-Polynucleotide Complexes

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SUMMARY

Recent interest in the use of adriamycin-DNA complex as an approach to improve the therapeutic effectiveness and to reduce toxicity of adriamycin for cancer chemotherapy requires an in-depth understanding of the physicochemical and biochemical properties of such complexes. The interactions of adriamycin with single-strand polydeoxyribonucleotides, double-strand DNA, and double-strand ribodeoxyribopolynucleotide hybrids were therefore investigated. Association constants ($K_{assoc}$) of adriamycin and polynucleotides were obtained. These data showed that the inherent variable in such complex lies in the composition of the polynucleotides. Alternate deoxyguanylate (dG)-deoxycytidylate (dC) sequence binds 7-fold better than alternate deoxyadenylate (dA)-deoxythymidylate (dT) sequence. Comparative studies of the hydrolysis of DNA duplexes by deoxyribonucleases I and II with and without adriamycin were also carried out. The rate of hydrolysis decreased in the order poly(dA-dT) > calf thymus DNA > poly(dG-dC) > poly(dA)·poly(dT) > poly(dG)·poly(dC) for DNase I and poly(dA-dT) > calf thymus DNA > poly(dG-dC) > poly(dA)·poly(dT) > poly(dG)·poly(dC) for DNase II. Interception of adriamycin to deoxyribopolynucleotide duplex resulted in inhibition of DNase II two to three times more than that of DNase I. On the other hand, interception of adriamycin to homodeoxypolynucleotide duplex poly(dA)·poly(dT) and poly(dG)·poly(dC) enhanced the DNase I hydrolysis. If DNase I activity could be related to serum DNase and DNase II related to tumor lysosomal DNase as in the endocytosis mechanism proposed by Trouet et al. (Cancer Chemotherapy Rept., 59: 260, 1975), the best adriamycin carrier suggested by this investigation could be poly(dA)·poly(dT) and poly(dG-dC). It is also suggested in this study that adriamycin–DNA–RNA hybrid could be of interest as an antiviral agent by a similar release mechanism via RNase H, an enzyme associated with viral reverse transcriptase.

INTRODUCTION

While the release of a cytotoxic moiety via the enzymatic activation principle has interested many researchers in cancer chemotherapy (22, 27, 28), the recent work of Trouet et al. (5, 24, 25) using a daunomycin-DNA complex activated by DNase is an important example. In particular, the novelty of this approach is not in the enzymatic release mechanism but in the endocytosis mechanism proposed by DeDuve et al. (6).

The high endocytotic activity, characteristic of many tumor cells, allows the transport of the macromolecular complex into the target cell preferentially (20). Once inside the tumor cell, the complex would most probably be taken up by the lysosomes, the carrier DNA would then be digested by the enzyme DNase, and the drug would be released. The beneficial effect of using such a complex over using the drug alone depends on the fact that the complex is less toxic than the drug. Indeed, Langslet et al. (13) recently reported that the cardiotoxicity of daunomycin and adriamycin was reduced by complexing with DNA. Similar studies with ethidium bromide have been reported by Heinen et al. (10). More recently, Marks and Venditti (18) also studied the effect on adriamycin and actinomycin D in vivo. These last authors pointed out the necessity of considering the effect of DNA as an immunoenhancing agent in this work, based on the fact that DNA could be given either prior to or after the drug was given to achieve similar improvement. Atassi and Tagnon (1) were unable to demonstrate differences in activity between adriamycin and its DNA complex in DBF hybrid mice. There was also a difference in opinion on the efficacy of the DNA-adriamycin complex prepared in different laboratories (11). In addition, controversy exists as to the origin and method of preparation of the DNA complex (24). Therefore, the work carried out in our laboratory relating to the enzymatic basis of this approach should be of interest.

Wroblewski and Bodansky (31) and Kurnick (12) found that the serum enzyme hydrolysis of double-strand DNA yields oligonucleotides with 5’-nucleotide terminals and that this enzyme resembles the DNase I of bovine pancreas (12). Since the stability of the DNA complex before reaching the target tumor where endocytosis occurs merits some attention, a model study with bovine pancreas DNase I may provide this information (7). Subsequent to endocytosis, the lysosomal DNase II activity will dictate the rate of release of adriamycin. Spleen acid DNase II is a well-known example of this lysosomal activity. This work therefore examines the rate of hydrolysis of DNA, adriamycin-DNA complex, and adriamycin-synthetic polynucleotide complexes by these 2 model enzymes. The results of this investigation should serve as a useful basis to a better understanding of the value...
and the limitation of "lysosomotropic" drugs for cancer chemotherapy (6).

MATERIALS AND METHODS

Adriamycin was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and was a preparation from Farmitalia, Milan, Italy. Calf thymus DNA (type V) was purchased from Sigma Chemical Co., St. Louis, Mo. Synthetic polynucleotides were purchased from P-L Biochemicals, Milwaukee, Wis. The molecular weights of the polynucleotides were: poly(dA-dT), 3.15 x 10^6; poly(dA)-poly(dT), 5.1 x 10^6; poly(dG)-poly(dC), 5.2 x 10^6; poly(dG-dC), 6.3 x 10^6; poly(dT), 3.7 x 10^6; poly(dA), 2.05 x 10^6; poly(dG), 6.7 x 10^6. A poly(dA-dT) sample with a molecular weight of 1.0 x 10^6 from Boehringer-Mannheim, Mannheim, Germany, was also used. The small difference in molecular weight does not affect the enzyme study.

Bovine pancreas DNase I was purchased from Worthington Biochemical Corp., Freehold, N. J., and acid spleen DNase II was purchased from Miles Laboratories, Elkhart, Ind.

Fluorescence study was carried out with an Aminco-Bowman fluorescence spectrophotometer. Polarization study (29) was done with a set of Glen prisms on the same instrument.

Hydrolysis of DNA complexes with DNase were studied on a Gilford instrument attached to a Beckman DU spectrophotometer and equipped with a 10-inch Honeywell recorder, which was adjusted to full scale of 0.2 A and sensitivity of ±0.0002 A.

Titrations of adriamycin with nucleic acid were performed at concentrations of 2.3 x 10^-4 M nucleotide in 0.95 M sodium cacodylate-0.15 M NaCl, pH 7.0, at 30°. The fluorescent intensity was measured at 575 nm and excited at 480 nm, after the adriamycin and polynucleotide were thoroughly mixed. The titration curves were plotted in terms of percentage of fluorescence remaining compared to the same concentrations of solution without the nucleic acid versus mole ratio of nucleotides to added adriamycin (R). From such titration data, Scatchard plots (23) were obtained, where r is the ratio of bound adriamycin to nucleotides, and C is the molar concentration of unbound adriamycin. The apparent association constant in moles^-1 (K_app) and the apparent number of binding sites per nucleotide (B_app) were determined from the linear regression analysis of the Scatchard plots with the aid of a Hewlett-Packard Calculator 9810A stat Pac.

Under the conditions of our experiments for calf thymus DNA, 0.90 and 2.70 x 10^6 M^-1 were found for B_app and K_app, respectively. Zunino et al. (32) reported values of 0.20 and 2.8 x 10^6 M^-1 measured by equilibrium dialysis. The difference could be explained by the fact that our fluorescence measurements were performed at 30° in the presence of 0.15 M NaCl. The values of 0.41 and 0.19 x 10^6 M^-1 were reported by Trouet et al. (26) for herring sperm DNA.

The indirect hyperchromic shift at 260 nm (2) was used to follow the enzymatic degradation of the nucleic acid with and without inhibitors. Since the concentration of adriamycin used in the inhibition study was much lower than that of the nucleic acid, the absorption due to adriamycin was negligible. The assay medium for DNase I consisted of 55 μmoles of sodium cacodylate (pH 7.0), 165 μmoles of NaCl, 5 μmoles of MgCl₂, 100 to 180 nmoles of nucleotide unit of nucleic acid, and 2.5 to 25 μg of bovine pancreas DNase I in 1.1 ml of solution at 27°. The assay medium for DNase II consisted of 480 μmoles of sodium acetate, 0.5 μmoles of MgCl₂, 100 to 150 nmoles of nucleotide unit nucleic acid, and 200 to 500 μg of spleen DNase II in 1.1 ml of solution at 27°. For the inhibition study, appropriate amounts of adriamycin or other intercalating agents were added to the assay systems. The initial increase in 260 nm absorption was recorded, and rates were compared.

RESULTS

The reason for choosing bovine pancreas DNase I as a model for the serum enzyme and spleen acid DNase II as a model for the lysosomal enzyme has been given under "Introduction."

As expected, calf thymus DNA was found to be a very good substrate for both DNase I and DNase II. Addition of adriamycin (Chart 1) markedly prolonged the time required for hydrolysis by either DNase I or DNase II. Adriamycin effectively inhibited DNase II more than DNase I. Fifty % inhibition of DNase II was obtained at a level of 65 nucleotides per adriamycin. The same degree of inhibition for DNase I was achieved only at a level of 25 nucleotides/adriamycin (12.5 base pair). Using the data in Chart 1 and analyzing by Reinem's method (19), one could also show that adriamycin, similar to other intercalating agents (8, 15), competed with DNase for binding sites on DNA. When the concentration of the total inhibition (I_o) was plotted against a function of fractional inhibition, the concentration of substrate units protected by adriamycin (S) could be determined (Chart 2). The average number of nucleotide pairs protected per adriamycin was thus found to be 11.7 for DNase I and 15 for DNase II (Table 1).

The effect of polynucleotide on the fluorescence and

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3 Polynucleotide abbreviations are in accordance with the recommendations of the Commission on Biochemical Nomenclature (IUPAC-IUB).
there was no evidence of intercalation of adniamycin, be
tively.) S,, see text. Points are derived from Chart 1.

Single-strand homopolynucleotides on the mononucleotide must
be due to the ionic interaction of adniamycin and the phos-
Pcm. Neither of them was affected by an increase of the
quenched the fluorescence of adniamycin. Mononucleotide
on the fluorescence of adniamycin. Poly(A) slightly
shown in Chart 3, poly(dT) and poly(U) had very little effect
fluorescence of adniamycin was then investi-
crease in polarization was used as an indicator of intercal-
complex). The ionic interaction and intercalation can be
strands, however, was poly(dG), which efficiently quenched
of the freedom of motion of adniamycin by polymers. The
the fluorescence of adniamycin and is definitely affected by
ratio of polynucleotide/adniamycin. At R = 200, only
5 of the fluorescence of adniamycin remained. Fluores-
cent polarization gave a value of 0.20, which also indicated
the strong interaction between poly(dG) and adniamycin
(Chart 4). These data suggested that there was intercalation
of poly(dG) by adniamycin. It is probably due to the known
fact that poly(dG) could form a multistrand complex with
itself, through hydrogen bonding of the guanine bases.
Similar types of intercalation between other dyes and
poly(dG) were also reported by Weisblum (30). The smaller
association constant of the poly(dG)-adniamycin complex,
when compared to other double-helical adniamycin com-
exes, suggested that this complex could be thermody-
namically less stable.

Double-strand poly(dA), poly(dT), alternate copolymer
poly(dA-dT), and hybrid double-strand poly(A)-poly(dT)
were then investigated. These polymers were all found to
quench the adniamycin fluorescence, and the quenching
increased with the increase of the amount of polynucleo-
tides. At R = 200, about 40% of the original fluorescence of
adniamycin remained. On the other hand, fluorescence po-
larization increased with the increase in R, and the value
reached 0.3 to 0.35 at R = 200.

In order to provide information on the tightness of the
binding of the dye to the polynucleotides, Scatchard plots
of these polymer/adniamycin complexes were made (Charts
5 to 7). The results are summarized in Table 2. Among the 3
polymers, the association constant (K_{av}) was highest for
the RNA-DNA hybrid, while the largest amount of adniamy-
cin incorporation into the duplex was found for
poly(dA):poly(dT) (B_{av} = 8.9 \times 10^{-4}). However, K_{av}
and B_{av} were lower for all 3 synthetic polymers than for calf
thymus DNA.

Multistrand copolymers poly(dG)-poly(dC), poly(C)-
the binding of adriamycin to poly(dA-dT). r, ratio of bound adriamycin to nucleotides; C, molar concentration of unbound adriamycin.

poly(dG), and poly(dG-dC) were also studied. Similar quenching of the adriamycin fluorescence was observed for the 1st 2 polymers (Charts 8 and 9). At R = 200, only 6 to 8% of adriamycin fluorescence remained, while the polarization increased to 0.24. As shown in Table 2, these 2 complexes had higher B_{max} values or more adriamycin bound than did calf thymus DNA, although the association constant is lower than that of calf thymus DNA. Of particular importance was the finding that the alternate double-strand poly(dG-dC) complex of adriamycin had both higher association constant and adriamycin incorporation than did calf thymus DNA. It quenched significantly at R = 200; only 2% of the fluorescence remained, and the fluorescence polarization increased to 0.33. This polymer therefore intercalated very effectively with adriamycin (Chart 10).

With the above basic understanding of the interaction of adriamycin-DNA and polynucleotides complex available, one could then study their enzymatic hydrolysis.

Using the conditions suggested by Bollum (3), we were able to observe the degradation of the double-strand deoxy-polynucleotides poly(dA-dT), poly(dA)·poly(dT), and poly(dG-dC), but we were unable to demonstrate any hydrolysis of poly(dG)·poly(dC) by the bovine pancreas DNase I. The rate of hydrolysis varied significantly from polymer to polymer (Table 3). Poly(dA-dT) hydrolyzed 20% faster than did calf thymus DNA. This result agrees with the known data that dApdT linkage is sensitive to DNase I, and the product analysis of DNase I usually showed high 3'-dA-containing oligomers (14). Poly(dG-dC) hydrolyzed at only 5% of the rate of calf thymus DNA. Poly(dA)·poly(dT) was hydrolyzed even slower, at only 0.8% of the rate of DNA.

The adriamycin-polynucleotide complex, however, was clearly inhibitory to DNase I hydrolysis with the exception of poly(dA)·poly(dT). Poly(dA-dT) and DNA complexes were similar in their degree of reduction in rate of hydrolysis, and poly(dG-dC) complex was only slightly reduced. The introduction of adriamycin into poly(dA)·poly(dT), however, has resulted in an increase in the rate of hydrolysis by DNase I. Subsequent study of this interesting finding not only has confirmed this result but also has shown clearly that, as R reached 20, an increase in rate of almost 300% was observed. The hydrolysis of poly(dG)·poly(dC)-adriamycin complex could not be demonstrated by the spectroscopic method but could be detected by the release of adriamycin fluorescence after the complex was treated with DNase I. This result suggested that the resultant alteration in secondary structure after adriamycin binding is different in a double-strand homopolymer and in a double-strand alternate copolymer. The effect of adriamycin to the alternate copolymer may be similar to other intercalating agents, while that of the homopolymer may be similar to the effect of Mn^{2+} or Ca^{2+} ions. These ions have been reported (14) to promote DNase I activity towards poly(dG)·poly(C). However, this enhancing effect seems quite unique for adriamycin, since similar intercalating complexes of quinacrine and ethidium bromide with poly(dA)·poly(dT) were found to be inhibitory towards DNase I (Table 4). DNase II was also found to hydrolyze poly(dA-dT) slightly more effectively than did DNA (Table 5). In contrast to the result with DNase I, poly(dA)·poly(dT) and poly(dG-dC) were also hydrolyzed fairly rapidly by this enzyme. The only polymer resistant to hydrolysis by DNase II was found to be poly(dG)·poly(dC). Intercalation by adriamycin to the duplexes resulted in inhibitory effect similar to that found in DNA. As indicated by the smaller number of protected base pairs per adriamycin molecule (Table 1), none of these complexes was found to be a better inhibitor than the DNA-adriamycin complex. No enhancement of hydrolysis rate was observed in any of the complexes. Also the inhibitory effects of such complexes on DNase activity were generally 2 to 3 times more effective in the DNase II system than in the DNase I system.

DISCUSSION

The effectiveness of the use of adriamycin-DNA complex in cancer chemotherapy as suggested by Trouet et al. (25) should depend on many factors. The present work represented a model study as to how long the complex is stable in the circulatory system before reaching the target tissue. The most important factor determining the stability of this complex is the interaction of the complex with DNase I in
Adriamycin-Polynucleotide Complexes and DNase

Chart 6. A, fluorescence (—) and polarization (- - - -) of adriamycin to poly(dA)-poly(dT) at various nucleotide/adriamycin ratios (R). B, Scatchard plot of the binding of adriamycin to poly(dA)-poly(dT). r, ratio of bound adriamycin to nucleotides; C, molar concentration of unbound adriamycin.

Chart 7. A, fluorescence (—) and polarization (- - - -) of adriamycin to poly(A) poly(dT) at various nucleotide/adriamycin ratios (R). B, Scatchard plot of the binding of adriamycin to poly(A) poly(dT). r, ratio of bound adriamycin to nucleotides; C, molar concentration of unbound adriamycin.

Chart 8. A, fluorescence (—) and polarization (- - - -) of adriamycin to poly(dG)-poly(dC) at various nucleotide/adriamycin ratios (R). B, Scatchard plot of the binding of adriamycin to poly(dG)-poly(dC). r, ratio of bound adriamycin to nucleotides; C, molar concentration of unbound adriamycin.

Chart 9. A, fluorescence (—) and polarization (- - - -) of adriamycin to poly(C)-poly(dG) at various nucleotide/adriamycin ratios (R). B, Scatchard plot of the binding of adriamycin to poly(C)-poly(dG). r, ratio of bound adriamycin to nucleotides; C, molar concentration of unbound adriamycin.

Chart 10. A, fluorescence (—) and polarization (- - - -) of adriamycin to poly(dG-dC) at various nucleotide/adriamycin ratios (R). B, Scatchard plot of the binding of adriamycin to poly(dG-dC). r, ratio of bound adriamycin to nucleotides; C, molar concentration of unbound adriamycin.

Table 2

Interaction of adriamycin and DNA's

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>$K_{\text{app}}$ ($\times 10^4$ M$^{-1}$)</th>
<th>$B_{\text{app}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus DNA</td>
<td>2.70</td>
<td>0.90</td>
</tr>
<tr>
<td>Poly(dA-dT)</td>
<td>0.71</td>
<td>0.073</td>
</tr>
<tr>
<td>Poly(dA)-poly(dT)</td>
<td>0.45</td>
<td>0.089</td>
</tr>
<tr>
<td>Poly(A)-poly(dT)</td>
<td>1.39</td>
<td>0.063</td>
</tr>
<tr>
<td>Poly(dG)</td>
<td>0.41</td>
<td>0.133</td>
</tr>
<tr>
<td>Poly(dG)-poly(dC)</td>
<td>0.47</td>
<td>0.114</td>
</tr>
<tr>
<td>Poly(dG)-poly(C)</td>
<td>0.55</td>
<td>0.148</td>
</tr>
<tr>
<td>Poly(dG-dC)</td>
<td>4.91</td>
<td>0.115</td>
</tr>
</tbody>
</table>

The activity of this enzyme is known to vary from species to species and possibly from individual to individual in humans. It is also known that, in some cancer patients, variable levels of this enzyme have been found (12). Therefore, a meaningful pharmacokinetic study of the effect of such a complex compared to the drug alone should take into consideration the interaction of the complex with DNase I in serum. Our biochemical in vitro model study could therefore be used as a reference for the in vivo application of such drugs to humans. In other words, the DNase I level in serum of cancer patients should be determined prior to therapy, and cancer patients with low serum DNase I levels might be expected to benefit more from the complex than from adriamycin alone.

DNA-adriamycin complex was found to have very low...
The exploitation of these toxins can be attributed to the free adriamycin before reaching the target tumor. If we could slow down the degradation of the complex in the serum, we may be able to reduce the side effects and increase the effectiveness of the treatment. Administration of the DNA complex with DNase I inhibitor simultaneously should therefore increase the effectiveness of the drug. Although no known effective synthetic inhibitor now exists, potent natural inhibitor for this enzyme has been isolated from spleen (16) and thymus (17). The exploitation of these inhibitors with this form of therapy should be a fruitful area of clinical research. Improvement in efficacy could also be expected from other DNA-like carriers that are less susceptible to DNase I attack. All readily available double-strand synthetic deoxyribonucleotides complexed with adriamycin. The association constant was, however, lower than that of native DNA for most of the duplexes, with the exception of poly(dG-dC). In addition, the maximum amount of adriamycin carried by dG-dC-type polymer is higher than that by dA-dT-type polymer. With the exception of poly(dA-dT), all polymers tested resisted DNase I hydrolysis better than did calf thymus DNA. Although poly(dG)·poly(dC) was completely resistant to DNase I, its resistance to DNase II at the same time makes it a less desirable carrier, since DNase II degradation is required to release the drug from the complex after entering the target cell. The intercalation of adriamycin in the polymers [except poly(dA)·poly(dT)] increased the resistance of degradation by DNase I. At R = 20 (the most commonly used DNA/adriamycin ratio), poly(dG-dC) complex was hydrolyzed at one-tenth the rate of the DNA complex. While DNase I degradation of poly(dA)·poly(dT) was enhanced by the intercalation of adriamycin, the overall rate (at R = 20) was only one-twentieth that of the DNA complex (an even slower rate can be achieved if the R value was increased). Hence, adriamycin poly(dA)·poly(dT) or adriamycin poly(dG-dC) complexes should stay intact in circulation longer than does DNA-adriamycin complex. The rates of hydrolysis by DNase II for poly(dG-dC) complex and DNA complex were found to be similar, and that of poly(dA)·poly(dT) is about 50% of the DNA. Furthermore, the association constant for poly(dG-dC) and adriamycin was higher than that for DNA. Less dissociation of the complex could be expected if poly(dG-dC) was used as the carrier. The in vivo study of poly(dG-dC) and poly(dA)·poly(dT)-adriamycin complex should therefore be carried out in the future. The sensitivity of the rate of hydrolysis by DNase I to the primary structure of the polynucleotide may also explain the different chemotherapeutic effect by using different sources of DNA for complexing. The DNA that contains more and longer homonucleotide segments should be more resistant to the serum DNase I degradation. This type of DNA should give better protection to adriamycin and greater therapeutic effectiveness. Our result suggested that DNA would be degraded in the serum, but the chance of the longer homonucleotide segment surviving the degradation should be better than that of the DNA segment that contains nucleotides with random sequence. The residue homonucleotide segments in the serum could intercalate with adriamycin and may be responsible for the synergistic effect observed by Mark and Venditti (18). These authors reported that DNA injected in a period of time prior to adriamycin injection exhibited effects similar to those of DNA and adriamycin injected simultaneously. Adriamycin also intercalated ribodeoxyribopolynucleotide hybrid duplexes very effectively. The adriamycin-hybrid complex should be a low toxic compound, and the adriamycin could be released only by the action of RNase H, a nuclease that has been found to associate with RNA-dependent DNA polymerase related to viral infection (9). Because adriamycin had been found to be a potent inhibitor for virus RNA-dependent DNA polymerase (21), the antiviral effect of this type of complex warrants further study in the future.

ACKNOWLEDGMENTS

We acknowledge Dr. T. Marks and Dr. J. Venditti, for discussions of their data, and Dr. Harry Wood, for providing adriamycin for this work.

REFERENCES

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