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DNA–Anthracycline Complexes. I. Toxicity in Mice and Chemotherapeutic Activity Against L1210 Leukemia of Daunorubicin–DNA and Adriamycin–DNA*

D. DEPREZ-DE CAMPENEERE† and A. TROUET

International Institute of Cellular and Molecular Pathology and Université Catholique de Louvain, Bruxelles, Belgium

Abstract—*We report here comparative studies performed in mice on the toxic and therapeutic effects of free and DNA bound anthracyclines: daunorubicin (DNR) and adriamycin or doxorubicin (ADR).*

DNR–DNA is less toxic than DNR when given i.p., while a similar overall toxicity is observed when the drugs are given i.v. In contrast, ADR–DNA is significantly less toxic than ADR, both by the i.p. or the i.v. route.

The better therapeutic effectiveness of DNR–DNA on L1210 leukemia compared with free DNR is demonstrated; however, the activities of both DNR and DNR–DNA are dependent on the schedule of administration.

The most striking chemotherapic effect is always obtained with ADR–DNA, due to the combination of a higher activity and a lower toxicity when the drug is associated to high mol. wt DNA. The results of the toxicologic and chemotherapic studies are discussed in view of the distinctive pharmacologic properties of free and DNA bound drugs.

INTRODUCTION

WE HAVE shown previously [1, 2] that the chemotherapeutic properties of daunorubicin (DNR) and of adriamycin (or doxorubicin) (ADR) can be improved by administering them in association with high mol. wt DNA.

To gain a better insight in the properties and the mechanism of action of anthracycline–DNA complexes we have conducted a series of comparative studies on the toxic and chemotherapeutic properties of DNR–DNA and ADR–DNA. We report, in this paper, the results obtained during toxic and chemotherapeutic studies in mice. A second paper deals with the compared toxicity of ADR and ADR–DNA, as studied by histopathologic techniques [3].

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†Mailing address: D. Deprez-de Campeneere, I.C.P. 74, avenue Hippocrate, 74, B-1200 Bruxelles, Belgium.

MATERIALS AND METHODS

Daunorubicin hydrochloride was provided by Rhône–Poulenc (Paris, France) and ADR hydrochloride was purchased from Farmitalia (Milan, Italy). DNA extracted from herring sperm (type VII) was purchased from Sigma Chemicals (St. Louis, Missouri, U.S.A.). This DNA has an average mol. wt of about 5×10^5.

The drug–DNA complexes were prepared as described previously [2]. A nucleotide/drug molar ratio of 20 was used. In a few experiments a DNA of smaller mol. wt ($\pm$70,000) was used. This DNA, extracted from milt, was kindly provided by Rhône–Poulenc (Paris, France).

The LD_{50} of free and DNA bound drugs were determined in female NMRI mice (Proefdierencentrum, Heverlee, Belgium) and DBA_2 mice (Charles River, St.-Aubin-les-Elbeuf, France). The drugs were given i.p. or i.v., using at least 5 different dosages per drug form, for 5 or 2 consecutive days. The LD_{50} was calculated after linear regression and using a probit scale. The percentage of survivors were determined after 30 days of obser-

vation and an average number of 10 mice was used per dosage.

In order to assess the statistical significance of this toxicity study a χ^2 test was performed for each dosage on the number of dead animals on day 30.

Chemotherapeutic tests were performed on female DBA_2 mice, inoculated on day 0 with L1210 leukemic cells harvested from a 6–8-day old ascitic form of L1210 leukemia. This L1210 cell line was kindly provided by Dr. C. Gosse and J. Morizet at Villejuif (France) and is very sensitive to both DNR and ADR. The L1210 cells were inoculated either i.v., i.p. or s.c. in the right flank of the animal. The treatment was started on day 1 following the cell inoculation and was administered either i.p. or i.v.

The chemotherapeutic results are expressed by the mean survival time, by the percentage of increase in life span (ILS) calculated on the basis of the median survival time, and by the number of animals surviving 45 days after inoculation of leukemic cells. The statistical significance of the results was estimated by analysis of variance (Fisher's test) of the mean values.

RESULTS

Toxicity studies

The LD_{50} values of free and DNA bound drugs after i.p. or i.v. administration are reported in Table 1. When given i.p. to NMRI mice both DNA complexes are significantly less toxic than the corresponding free drug. After i.v. administration however the toxicity of ADR only and not that of DNR is reduced when the drugs are administered as DNA complexes. The LD_{50} of ADR–DNA is about 1.7 times higher than that of ADR when given on 5 consecutive days. These results are confirmed in DBA_2 mice when drugs are administered i.v. on 2 consecutive days.

Chemotherapy studies

When cells and drugs are injected i.p. on 5 consecutive days into DBA_2 mice (Table 2), DNR–DNA is as active as DNR, at a non-toxic dose for DNR of 1.5 mg/kg. At 2.5 mg/kg however, DNR–DNA, being less toxic, induces significantly longer survival times than DNR. At a non-toxic dose for ADR, ADR–DNA is, at least, as active. DNA has no effect on the survival of the leukemic mice.

If both L1210 cells and drugs are administered i.v. for 5 consecutive days (Table 3) DNR–DNA is significantly more active than DNR at 5 mg/kg and not significantly different at the toxic dose of 6 mg/kg. At the non-toxic ADR doses of 2.5 and 4 mg/kg, the difference in activity between ADR and ADR–DNA is small but significant; when the dosage is increased up to 5 and 6 mg/kg, the superiority of ADR–DNA complex becomes very striking with an ILS reaching 500% and nearly 50% of the animals surviving at 45 days. Here again DNA by itself has no effect on the evolution of the L1210 leukemia.

As seen from Table 4, when cells and drugs are given i.v., significantly better therapeutic results are obtained when using ADR bound to high mol. wt DNA.

In an attempt to improve the relatively poor therapeutic results obtained with DNR and DNR–DNA we have varied the inoculation schedule from 2 to 5 consecutive daily injections while administering a total non toxic dosage varying between 24 and 28 mg/kg. As can be concluded from the data of Table 5, a 3-day administration schedule of free DNR gives better results inducing an ILS of 144% and 2 survivors out of 12 at 45 days. The superiority of the DNR–DNA complex is revealed by using a 2 consecutive day schedule, with an ILS of 301% and ±40% of long term survivors.

DISCUSSION

Confirming our earlier results, DNR–DNA is less toxic than DNR in NMRI mice when given i.p. during 5 consecutive days. No decrease in toxicity, however, is observed when DNR–DNA is injected i.v. in NMRI or DBA_2 mice for 5 or 2 consecutive days. This result is in contrast with the decrease in toxicity observed when ADR is administered as a DNA complex according to the same schedule and via the same route. It is important to notice that toxicity tests were performed on two different mouse strains using a 2 or 5 daily consecutive schedule. Since the LD_{50} was determined after an observation period of 30 days, this decrease in toxicity of the DNA complexes seems not to be due to a delayed effect of the drug when bound to DNA. Moreover, we have shown previously [4] that association of DNR and ADR with DNA reduces their uptake by such tissues as kidney, lung, heart and gastrointestinal tract.

Table 1. Compared toxicity of free and DNA *bound drugs in mice:* LD_{50} *study*

Drug	LD_{50}* (mg/kg per day)		
	i.p. days 1–5 (NMRI mice)	i.v. days 1–5 (NMRI mice)	i.v. days 1 and 2 (DBA_2 mice)
DNR	2.9	6.8	19.7
DNR–DNA	4.3	6.8	20.1
Ratio complex/free	*1.48*†	*1.0*	*1.02*
ADR	2.7	4.8	14.2
ADR–DNA	5.2	8.4	23.7
Ratio complex/free	*1.93*‡	*1.75*§	*1.67*‖

LD_{50} = dose which induces 50% lethality in mice after 30 days of observation.
†$P<0.01$ for 4 mg and $P<0.05$ for 3 mg.
‡$P<0.01$ *for* 3.5 and 4.5 mg.
§$P<0.01$ for 4, 5, 6 mg and $P<0.02$ for 7 mg.
‖$P<0.01$ for 15 and 19 mg.

Table 2. Chemotherapeutic activity of free and DNA *bound drugs administered i.p. into* DBA_2 *mice inoculated i.p. with* L1210 *leukemia*

Drug	Doses (mg/kg per day)	Mean survival time (days)	Increase in life span (%)	No. of survivors on day 45 / Total No. of mice
Control	—	9.2	0	0/15
DNA	29*	9.0	0	0/15
DNR	1.5	22.3 †	44	1/7
DNR–DNA		21.3	83	0/6
DNR	2.5	13.3 ‡	44	0/7
DNR–DNA		30.8	266	2/6
ADR	1.0	25.5 †	100	3/10
ADR–DNA		28.5	177	3/10

10^4 L1210 cells were given i.p. on day 0. The drugs were administered i.p. on 5 consecutive days, following the cells inoculation.
*This dose corresponds to a drug concentration of 2.5 mg/kg per day (on the basis of the nucleotide/drug molar ratio of 20).
†$P>0.05$.
‡$P<0.01$.

The decreased toxicity of ADR–DNA has been confirmed by other groups in rats [5], in NMRI mice [6] and in BDF_1 or DBA_2 mice [7]. A detailed histopathologic study on the toxicity observed in mice after administration of ADR and ADR–DNA is reported in a companion paper [3].

Given i.p. against the i.p. form of L1210 leukemia, DNR–DNA is more active than DNR mainly because of its lower toxicity (Table 2); these results confirm our previous data [1] and are in agreement with data from Ohnuma *et al.* [8], while Marks *et al.* [9] obtained less significant results. The discrep-

Table 3. Chemotherapeutic activity of free and DNA *bound drugs administered i.v. on 5 consecutive days into* DBA_2 *mice inoculated i.v. with* L1210 *leukemia*

Drug	Doses (mg/kg per day)	Mean survival time (days)	Increase in life span (%)	No. of survivors on day 45 / Total No. of mice
Control	—	8.5	0	0/30
DNA	58*	8.4	0	0/12
DNR	5.0	14.5 ‡‡	69	0/51
DNR–DNA		17.2	124	0/58
DNR	6.0	14.0 †	100	0/7
DNR–DNA		13.6	129	0/7
ADR	2.5	12.5 ‡‡	62	0/15
ADR–DNA		14.0	87	0/15
ADR	4.0	16.8 §	94	0/13
ADR–DNA		23.1	131	2/14
ADR	5.0	15.1 ‡‡	81	0/14
ADR–DNA		27.1	194	4/15
ADR	6.0	16.7 ‡‡	87	4/43
ADR–DNA		34.7	519	21/44

10^4 L1210 cells were given i.v. on day 0. The drugs were administered i.v. on days 1–5.
*This dose corresponds to a drug concentration of 5 mg/kg per day (on the basis of the nucleotide/drug molar ratio of 20).
†$P > 0.05$.
‡‡$P < 0.01$.
§$P < 0.05$.

Table 4. Chemotherapeutic activity of free and DNA *bound drugs on the i.v. inoculated* L1210 *leukemia: influence of the* DNA *mol. wt*

Drug	Doses (mg/kg per day)	Mean survival time (days)	Increase in life span (%)	No. of survivors on day 45 / Total No. of mice
DNR	5.0	11.3	57	0/9
DNR–DNA (mol. wt ± 70,000)	5.0	13.2	100	0/9
DNR–DNA (mol. wt ± 500,00)	5.0	14.6	121	0/9
ADR	6.0	17.7	136	0/9
ADR–DNA (mol. wt ± 70,000)	6.0	32.8	271	2/6
ADR–DNA (mol. wt ± 500,000)	6.0	39.9	614	6/7

10^4 L1210 cells were given i.v. on day 0 into DBA_2 mice. The drugs were administered i.v. on days 1–5.
$P < 0.01$ for DNR and DNR–DNA (±500,000).
$P > 0.05$ for DNR and DNR–DNA (±70,000); for DNR–DNA (±70,000) and (±500,000).
$P < 0.01$ for ADR and ADR–DNA (±70,000); for ADR and ADR–DNA (±500,000); for ADR–DNA (±70,000) and (±500,000).

Table 5. Chemotherapeutic activity of free and DNA *bound daunorubicin on the i.v. inoculated* L1210 *leukemia: influence of the treatment schedule*

Drug	Treatment schedule	Doses (mg/kg per day)	Mean survival time (days)	Increase in life span (%)	No. of survivors on day 45 / Total No. of mice
Control	—	—	7.9	0	0/96
DNR	days 1–5	5.0	13.1 †	70	0/27
DNR–DNA			16.1	123	0/27
DNR	days 1–4	7.0	14.9 †	87	0/10
DNR–DNA			31.5	225	3/12
DNR	days 1–3	9.0	24.1 *	144	2/12
DNR–DNA			27.8	181	3/12
DNR	days 1 and 2	12.0	18.9 †	110	
DNR–DNA			31.8	301	

10^4 L1210 cells were given on day 0 into DBA_2 mice.
The drug treatment was administered i.v. according a different schedule.
*$P > 0.05$.
†$P < 0.01$.

ancies between our results and these of Ohnuma and Marks are most probably due to differences in sensitivity towards anthracyclines between different L1210 cell lines as described by Atassi *et al.* [7].

We have not extended our studies using the i.p. route of administration and have rather studied the more realistic route of administering cells and drugs i.v. trying in this way to approach as closely as possible some aspects of the treatment of human hematologic neoplasia.

When given i.v. on 5 consecutive days DNR–DNA is moderately more active than DNR in conditions where both drug forms are equally toxic. However, the results of Table 5 show that the effectiveness of both DNR and DNR–DNA is very much dependent on the schedule of administration. For a similar global dosage DNR is indeed much more active when administered on 3 consecutive days while the superiority of DNR–DNA over DNR is much more significant after administration on 2 consecutive days. These results clearly indicate that by combining DNR to DNA it is possible to enhance its chemotherapic activity.

The chemotherapic results obtained with ADR–DNA on a 5 days schedule are the most spectacular, since at 6 mg/kg/day the life span is increased by 500% and $\pm 50\%$ of the animals survive on day 45. These results can be explained by the combination of a higher chemotherapic activity and a lower overall toxicity of ADR–DNA. Our results indicate very clearly that both DNR and ADR, when bound to DNA and administered i.v. have a significantly higher activity against the i.v. form of L1210 leukemia. This can be explained by the pharmacokinetic properties of the complexes. Indeed, when the drug is associated with DNA, the plasma levels reached *in vivo*, after i.v. administration, are strikingly increased with regard to the corresponding free drugs [4]. According to the data of Table 4, complexes of ADR with DNA of high mol. wt, close to 500,000, give better results against the i.v. inoculated L1210 leukemia than complexes obtained with DNA of a lower mol. wt close to 70,000. This also reflects a different rate of plasma disappearance between the two kinds of complexes, the latter one being characterized by lower plasma levels, with regard to the former one (unpublished results).

Finally, the differences observed between the toxic and chemotherapic activities of DNR–DNA and ADR–DNA can be correlated with their respective pharmacokinetics. The DNR–DNA complex, indeed, behaves after i.v. injection into mice much more like a prodrug, slowly released in the bloodstream, as indicated by the rapid dissociation of DNA and DNR in the plasma [4]. However, the increased plasma concentrations reached with DNR–DNA, as compared with free DNR, are responsible for the better therapeutic effect obtained with the complex. On the other

hand, the lower tissue concentrations of DNR found after administration of the DNA complex [4] may explain why the overall toxicity of DNR–DNA is not increased. In contrast, ADR–DNA remains much more stable in the bloodstream [4], with a very similar rate of disappearance for both drug and carrier. In these conditions the ADR–DNA complex will reach only those tissues which are accessible to high mol. wt DNA and will act on those cells in which or in the vicinity of which ADR can be released from its carrier. The lower toxicity and higher chemotherapic activity of ADR–DNA suggest therefore that several normal tissues and cells are less accessible to the complex, in contrast with the leukemic cells which dissociate the ADR–DNA complex very actively. The exact mechanism of this activation is still unclear but could occur by endocytosis of the complex and its intralysosomal digestion.

It is necessary to stress finally that the association of DNR and ADR to DNA does not allow to overcome the resistance of tumor cells towards the anthracyclines. As shown by Atassi *et al.* [7] the chemotherapeutic activity of ADR bound to DNA can only be demonstrated on a L1210 subline which is sensitive to the free drugs.

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