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Effects of Adriamycin on the Reverse Transcriptase and the Production of Murine Leukemia Virus[1]

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SUMMARY

Adriamycin inhibited the endogenous RNA-, poly(A)·$d(T)_{12}$-, and calf thymus DNA-catalyzed reaction of reverse transcriptase from AKR mouse murine leukemia virus (AKR-MLV). This inhibition was found at the reaction levels of endogenous RNA-directed and subsequent DNA-directed DNA synthesis. Although adriamycin and actinomycin D significantly reduced the growth of AKR mouse cells (K3b), the treatment with adriamycin could not inhibit the AKR-MLV production in these cells. Actinomycin D inhibited AKR-MLV production completely in the same experimental condition. In adriamycin-resistant K3b/Am cells, which were isolated by intermittent treatment of K3b cells with adriamycin, persistence of AKR-MLV was demonstrated. K3b/Am cells showed some altered characteristics such as reduced growth rate and tumorigenicity.

INTRODUCTION

Adriamycin is a glycoside antibiotic with a strucutre similar to that of daunomycin (19). It has been under clinical investigation and has been found to be useful as an antineoplastic drug for a fairly wide range of human leukemia and solid tumors (3, 4). Its mode of action is based on binding to cellular DNA and inhibiting DNA and RNA synthesis (10, 11, 21). These antibiotics have also been reported to inhibit reverse transcriptase of Rauscher murine leukemia virus and murine sarcoma virus (5, 8, 16, 22). However, the inhibition steps of DNA synthesis by adriamycin and the effect on the production of biologically active virus have not been fully analyzed.

In previous papers (13, 14) we have reported on the characteristics of AKR mouse cells transformed by RSV,[2] the K3b cells. This cell line releases AKR-MLV, but not RSV. Persistence of RSV genome in this line was also demonstrated after treatment with chromomycin A_3 and actinomycin D (13, 14). We report in this paper on adriamycin inhibition of reverse transcriptase and especially on the production of biologically active AKR-MLV from K3b cells after treatment with adriamycin.

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[2] The abbreviations used are: RSV, Rous sarcoma virus; AKR-MLV, AKR mouse murine leukemia virus.

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MATERIALS AND METHODS

Cells and Viruses. The K3b cell line was derived from a sarcoma induced in an AKR mouse by inoculating duck-grown sarcoma cell suspension infected with Schmidt-Ruppin strain RSV. Sarcoma cells were transferred to *in vitro* culture in 1966 and, since then, have been maintained continuously in our laboratory (13). This cell line did not produce any infectious RSV, but the presence of RSV genome was demonstrated by injecting dense cell suspension into wing webs of chicks and by subsequent induction of RSV-producing sarcomas. K3b cells release murine leukemia virus into culture medium as described before (13).

XC cells and NIH/3T3 cells were kindly supplied by T. Odaka, Institute of Medical Science, Tokyo, and by Y. Ikawa, Cancer Institute, Tokyo, Japan, respectively.

Cultivation of Tumor Cells. K3b cells were seeded into Falcon plastic dishes of 6 or 10 cm diameter and cultivated at 37° in modified Eagle's minimum essential medium, containing 10% calf serum and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml). The culture medium was changed at 24-hr intervals.

Purification of AKR-MLV. Pooled K3b cell culture medium or [^{3}H]uridine-labeled virus suspension stored at −75° was thawed and centrifuged at 8,000 × *g* for 10 min to remove cell debris. The virus suspension was then pelleted by centrifugation at 25,000 rpm for 90 min in a Spinco SW 27 rotor. The pellet was resuspended in 0.01 M Tris-HCl, pH 7.4, containing 0.1 M NaCl and 0.001 M EDTA. The crude virus suspension was placed on a layer of 20% sucrose in 0.01 M Tris-HCl, pH 7.4, containing 0.1 M NaCl and 0.001 M EDTA and centrifuged at 25,000 rpm in a SW 27 rotor for 90 min on the cushion of 50% sucrose. The banded virus was then sedimented further to equilibrium in a 15 to 50% sucrose density gradient at 25,000 rpm for 2 hr. The sample banding at a density of 1.15 to 1.17 g/ml was used as purified virus.

DNA Polymerase Assay. Reverse transcriptase was assayed by using exogenous template unless otherwise stated. Fifty μl of the sample were added to 50 μl of the standard reaction mixture containing 5 mM Tris-HCl, pH 8.0; 5 mM dithiothreitol; 30 mM NaCl; 1 mM MnCl; 100 μM each of dATP, dGTP, and dCTP; 2 μCi [^{3}H]dTTP; 5 μg poly(A)·$d(T)_{12}$; and 50 μg of bovine serum albumin. After incubation at 37° for 60 min, 80-μl aliquots were withdrawn, collected onto Whatman No. 3MM filter paper discs, and washed consecutively each time for 15 min, once in 10% trichloroacetic acid containing 1% sodium pyrophosphate, twice in 5% trichloroacetic acid, once in 95% ethanol, and

dried. The acid-insoluble radioactivity on the filter was counted in 10 ml toluene scintillant using a Beckman LS235 liquid scintillation counter.

XC Cell Assay. The XC cell assay was done essentially as described by Rowe *et al.* (18). Each virus dilution in 0.5 ml was adsorbed for 2 hr onto an NIH/3T3 monolayer containing 8×10^4 cells/6-cm culture dish. Five days after inoculation the cells were treated with UV irradiation and overlaid with 10^6 XC cells. Two to 3 days later, cells were fixed with methanol and stained with Giemsa, and the plaques were counted.

Transplantability Test. Cultured cells were treated with trypsin solution (Difco Laboratories, Detroit, Mich.; 1:250), and single-cell suspensions were prepared. After cells were counted with a hemocytometer, cell suspensions were serially diluted 10-fold with culture medium, and 0.2 ml of each diluted material was injected into the groins of AKR mice. Growth of tumors was followed for about 2 months. AKR mice were bred in our laboratory.

Chemical Materials. Adriamycin was kindly supplied by Kyowa Hakko Co., Ltd., Tokyo, Japan. Actinomycin D, poly(A)·d(T)$_{12}$, dATP, dGTP, and dCTP were purchased from Boehringer Mannheim Corp., Mannheim, Germany, and [^{3}H]dTTP (30 to 49 μCi/mmole) was purchased from Radiochemical Center, Amersham, England. Nonidet P-40 was purchased from Nakarai Chemicals Co., Ltd., Kyoto, Japan, and calf thymus DNA was purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan.

RESULTS

Characteristics of AKR-MLV from K3b Cells. AKR-MLV released in the culture medium of K3b cells was concentrated and analyzed with sucrose density gradient centrifugation as described in "Materials and Methods." The virus showed a density of 1.16 g/ml, and the presence of virion-associated reverse transcriptase and 70 S RNA was verified as reported in other oncornaviruses (20). The reverse transcriptase required 1 mM manganese ion, and it was not replaced with magnesium ion.

Inhibition of Reverse Transcriptase by Adriamycin. The effect of adriamycin on reverse transcriptase in AKR-MLV was examined *in vitro* with the templates poly(A)·d(T)$_{12}$, heat-denatured calf thymus DNA, and viral endogenous RNA. As shown in Chart 1, *A* and *B*, adriamycin inhibited the enzyme activity catalyzed by each template. This inhibition was most effective on the reaction catalyzed by heat-denatured calf thymus DNA, whereas a high concentration (100 μg/ml) of adriamycin completely inhibited the total endogenous RNA- and poly(A)·d(T)$_{12}$-catalyzed activity (Chart 1*C*). These results suggest that adriamycin inhibits both RNA-directed and subsequent DNA-directed DNA synthesis of AKR-MLV reverse transcriptase. To ascertain further that adriamycin inhibited this reverse transcriptase at the level of RNA-directed DNA synthesis, inhibitory action of adriamycin was compared with that of actinomycin D. Actinomycin D has been reported to have little effect on RNA-directed DNA synthesis of RNA tumor viruses, but it inhibits subsequent DNA-directed DNA synthesis (15). As shown in Chart 1*B*, 100 μg actinomycin D per ml reduced the endogenous

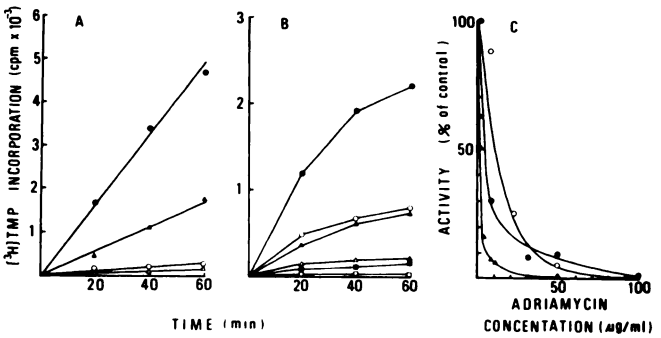

Chart 1. Inhibition of reverse transcriptase by adriamycin. *A*, time course of poly(A)·d(T)$_{12}$- and heat-denatured calf thymus DNA-directed DNA polymerase activity. The enzyme assay was done, adding 25 μl of antibiotic to 100 μl of standard reaction mixture that contained the template, at a dose of 5 μg/ml. Tenfold concentrated enzyme was used for the assay of heat-denatured calf thymus DNA-directed activity. Poly(A)·d(T)$_{12}$: ●, no adriamycin; ○, 30 μg adriamycin per ml. Heat-denatured calf thymus DNA: ▲, no adriamycin; △, 10 μg adriamycin per ml. *B*, time course of endogenous RNA-directed DNA polymerase activity. The reaction mixture contained 12 μCi [^{3}H]dTTP, 10-fold concentrated enzyme, and 25 μl of antibiotic. ●, no antibiotic; ○, 100 μg actinomycin D per ml; ▲, 10 μg adriamycin per ml; △, 100 μg actinomycin D per ml plus 10 μg adriamycin per ml; ■, 100 μg actinomycin D per ml plus 15 μg adriamycin per ml; □, 100 μg actinomycin per ml plus 20 μg adriamycin per ml. *C*, dose dependence of reverse transcriptase inhibition on adriamycin. The enzyme assay was done as described in *A* and *B*. ○, poly(A)·d(T)$_{12}$; ▲, heat-denatured calf thymus DNA; ●, endogenous RNA.

RNA-directed DNA synthesis less than 30% of control. About 60% of the residual actinomycin D-resistant enzymic activity was inhibited by the addition of 10 μg adriamycin per ml, and 20 μg adriamycin per ml inhibited this activity completely. These results confirm the above experiments.

Comparison of Inhibitory Effect of Adriamycin and Actinomycin D on AKR-MLV Production. The inhibitory effect of adriamycin on cell growth and virus production was compared with that of actinomycin D in the dose range of 0.1 to 1.0 μg/ml. We performed the following 2 experiments. First, the cells were cultured for 18 hr in the presence of adriamycin or actinomycin D, and the amount of virus released in the medium was estimated by reverse transcriptase and [^{3}H]uridine incorporation into the virus particles. Second, the cells were pretreated with adriamycin or actinomycin D for 8 hr, then washed with fresh medium, and cultured again in the presence of adriamycin or actinomycin D for 16 hr; the viruses, which were produced later in 16 hr, were estimated as above. As shown in Chart 2, *A* and *B*, in the 1st experiment, adriamycin and actinomycin D reduced the cell growth significantly, but the reverse transcriptase activity was about 1.5×10^3 cpm/10^6 cells, and it was almost equal in the absence or presence (0.1 to 1.0 μg/ml) of adriamycin. On the other hand, actinomycin D greatly reduced this value to 0.25 to 0.76×10^3 cpm/10^6 cells. In the 2nd experiment, the difference in the inhibitory effect of adriamycin and actinomycin D on virus production was demonstrated more clearly by the pretreatment of cells with adriamycin or actinomycin D (Chart 2, *C* and *D*). Namely, actinomycin D treatment caused complete inhibition of virus production, but adriamycin failed to do so, and the values were more than 1.30×10^3 cpm/10^6 cells. The same results were further demonstrated by [^{3}H]uridine incorporation into the virus particles (data not shown). Considering the fact that actinomycin D binds to DNA and inhibits mRNA synthesis in cells, these results suggest that, unlike actinomycin D, inhi-

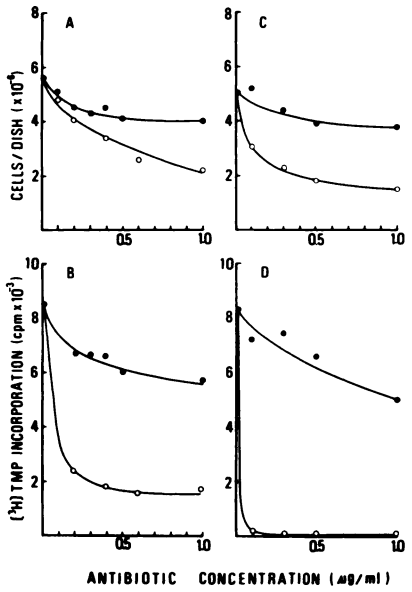

Chart 2. Effects of adriamycin and actinomycin D on K3b cell growth and AKR-MLV production. A K3b cell culture was set up 24 hr before the addition of antibiotics. *A*. K3b cells were grown for 18 hr in the presence (0.2 to 1.0 μg/ml) of adriamycin (●) or actinomycin D (○). The cells were harvested with 0.1% trypsin and counted with a hemocytometer. *B*. Twelve ml of each medium in Experiment A were centrifuged at 8,000 × *g* for 10 min. The supernatant was then centrifuged through 10 ml of 20% glycerin at 25,000 rpm in a Spinco SW 27 rotor for 90 min. The pellet was suspended in 300 μl of 0.01 M Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl, 0.001 M EDTA, and 0.02% Nonidet P-40. Fifty-μl aliquots were assayed for reverse transcriptase. ●, adriamycin; ○, actinomycin D. *C*. After K3b cells were grown for 8 hr in the presence (0.1 to 1.0 μg/ml) of adriamycin (●) or actinomycin D (○), the cells were washed with fresh medium and cultured for 16 hr in the presence of the same concentration of adriamycin or actinomycin D. Then, the cells were counted as described in *A*. *D*. Reverse transcriptase in 12 ml of each medium (later 16-hr culture) in Experiment C was estimated as described in *B*. ●, adriamycin; ○, actinomycin D.

bition of adriamycin on mRNA synthesis is less effective than the DNA synthesis in our K3b cells, so the virus production is not stopped in a limited experimental period (24 hr). These results were confirmed by XC cell assay. As shown in Table 1, adriamycin treatment failed to stop the virus production, but actinomycin D reduced the plaque-forming titer less than 0.4% of control.

Isolation and Characterization of Adriamycin-resistant Cells. An adriamycin-resistant strain was isolated by intermittent treatment of K3b cells with 0.2 μg adriamycin per ml and then increasing the adriamycin dose and was named K3b/Am cells. The K3b/Am cells were maintained in drug-free medium, and at the time of passages 1.0 μg adriamycin was routinely added to maintain the drug resistance. As shown in Chart 3, in the absence of the drug, the parent strain showed exponential growth with a doubling time of about 30 hr, whereas the growth rate of K3b/Am cells was slightly reduced. Furthermore, 0.2 μg adriamycin remarkably suppressed the cell multiplication of the parent strain, but K3b/Am cells were unaffected by the presence of 0.5 μg adriamycin per ml, and the growth reduction was observed only in the presence of 1.0 μg or more of adriamycin per ml.

Tumorigenicity of K3b/Am cells was compared with that of K3b cells in AKR mice (Table 2). The K3b/Am cells showed lower tumorigenicity than did the parent cells. When mouse-grown K3b/Am cells were cultivated again *in vitro*, cells showed resistance to 1.0 μg adriamycin as before. On the other hand, the presence of RSV genome in the K3b and K3b/Am cells was tested. Both cell strains induced sarcoma in the wing webs of chicks after about 10^6 cells were inoculated, and the presence of RSV genome was verified in the K3b/Am cells even after extensive adriamycin treatment.

Persistence of AKR-MLV in K3b/Am Cells. Production of AKR-MLV in K3b/Am cells was followed after treatment with 1.0 to 2.0 μg adriamycin per ml. Although changes of K3b cells from an adriamycin-sensitive to an adriamycin-resistant state resulted in some decrease of virus production, a considerable amount of reverse transcriptase (about one-third of K3b cells) and the incorporation of [³H]uridine into

Table 1

XC plaque-forming activity of AKR-MLV from K3b cells after treatment with adriamycin or actinomycin D

K3b cells were treated with 1.0 μg adriamycin or actinomycin D per ml for 18 hr as described in Chart 2*B*; then the cells were washed with fresh medium and cultured for 2 hr in the absence of the antibiotics. The medium from the 2-hr culture was used for XC cell assay.

Antibiotics	Virus titer (plaque-forming units/ml)
None	3×10^4
Adriamycin	3×10^4
Actinomycin D	$<10^2$

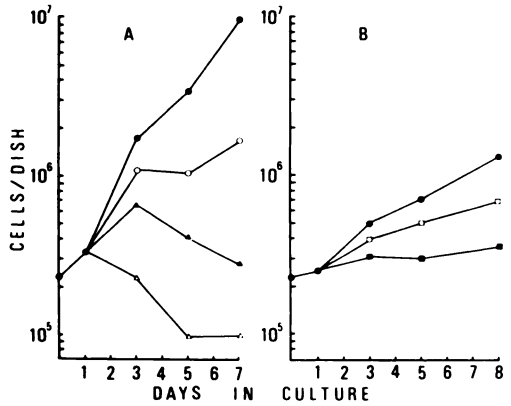

Chart 3. Effect of adriamycin on the growth of K3b and K3b/Am cells. *A*. K3b cells were cultured for 7 days in the presence of adriamycin. The medium was changed at 24-hr intervals, and the cells were harvested at the indicated time with 0.1% trypsin and counted with a hemocytometer. ●, control; ○, 0.1 μg/ml; ▲, 0.2 μg/ml; △, 0.5 μg/ml. *B*. K3b/Am cells were cultured for 8 days in the absence and presence of adriamycin. The cells were counted at the indicated time. ●, control; □, 1 μg/ml; ■, 2 μg/ml.

Table 2

Tumorigenicity of K3b cells and adriamycin-resistant K3b/Am cells

Cell lines	No. of cells inoculated	No. of mice with tumor/no. inoculated
K3b	3.8×10^6	4/4
	3.8×10^5	4/4
	3.8×10^4	2/4
	3.8×10^3	1/4
K3b/Am	1.1×10^6	1/4
	1.1×10^5	1/4
	1.1×10^4	0/4

virus particles were demonstrated. Moreover, biological activity of these viruses was verified by the XC cell test.

DISCUSSION

Adriamycin and daunomycin have recently been reported to be effective growth inhibitors of various human solid tumors and leukemic cells, and the inhibitory action seems to result from its ability to bind to DNA and to inhibit DNA and RNA synthesis (3, 4, 10, 11, 21). These antibiotics have also been reported to inhibit reverse transcriptase activity of Rauscher murine leukemia and murine sarcoma viruses. The results presented in this study indicated that K3b cells produced AKR-MLV, and the reverse transcriptase activity in this virus was inhibited by adriamycin *in vitro*. A considerably high concentration of adriamycin inhibited this endogenous RNA; poly(A)·d(T)$_{12}$-, and heat-denatured calf thymus DNA-catalyzed reaction almost completely. These results suggest that adriamycin inhibits not only DNA-directed, but also RNA-directed DNA polymerase activity. Then, to ascertain whether adriamycin inhibited at the level of RNA-directed DNA synthesis, this inhibitory action was compared with that of actinomycin D, which had been reported to inhibit DNA-directed DNA synthesis but not RNA-directed DNA synthesis of RSV reverse transcriptase (15). In our experiments, 100 μg actimonycin D per ml caused about 70% reduction of DNA synthesis, and the residual actinomycin D-resistant enzymic activity, which was thought to be the reaction of endogenous RNA-directed DNA synthesis, was inhibited completely by 15 to 20 μg adriamycin per ml. These results confirmed that adriamycin inhibited at the levels of both RNA-directed and subsequent DNA-directed DNA synthesis of AKR-MLV reverse transcriptase. It has been reported that actinomycin D treatment of cells infected with RNA tumor viruses causes a rapid cessation of viral RNA synthesis and a marked decrease in virus production as determined by assay of incorporation of [^{3}H]uridine into virus RNA (2). Accordingly, the inhibitory effect of adriamycin on virus production was compared with that of actinomycin D. It was evident from the present experiments that, although adriamycin reduced K3b cell growth significantly, AKR-MLV production was not inhibited by short- or long-term treatment with adriamycin, and the released viruses showed infectivity to NIH/3T3 cells when assayed with XC cells. On the other hand, actinomycin D inhibited virus production completely in the same experimental conditions. Considering these observations, it would appear that adriamycin bound to DNA and reduced the cell viability as the result of inhibition of DNA-dependent DNA polymerase of cells, but it could not inhibit viral mRNA synthesis as vigorously as did actinomycin D. Therefore, for virus production and release from cells chronically infected with RNA tumor viruses, RNA synthesis is essential; however, DNA-directed and RNA-directed DNA synthesis are not required in a limited experimental period. These findings are consistent with other observations that some inhibitors of RNA-directed DNA polymerase from murine leukemia and sarcoma viruses (7, 17) inhibit infection by exogenous viruses but fail to suppress the formation of endogenous leukemia and sarcoma viruses in transformed cells.

Treatment of transformed cells with antineoplastic drugs often causes the appearance of cells resistant to these drugs, and such cells have been shown to have some altered biological characteristics (1, 6, 9, 12). In our experiments, changes from adriamycin-sensitive to adriamycin-resistant cells (K3b/Am cells) showed concomitant reduction in growth rate, tumorigenicity, and virus production. These changes may be explained as the results of alteration in cell membrane, but for exact analysis of drug resistance further experiments are required.

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