THE MODE OF ACTION OF A NEW ANTITUMOR ANTIBIOTIC, SPORAMYCIN

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Sporamycin, an antitumor antibiotic, primarily inhibited DNA synthesis, while RNA and protein synthesis were not significantly affected in HeLa S3 cells. The antibiotic also caused strand scission of cellular DNA. However, the effects were not observed when the cells were incubated at 0°C before washing and subsequently incubated at 37°C. The *Tm* of calf thymus DNA decreased when incubated with sporamycin *in vitro*. Sporamycin did not affect DNA synthesis *in vitro* catalyzed by partially purified DNA polymerase α, β, and γ derived from EHRLICH ascites cells.

Sporamycin[1,2], a polypeptide antitumor antibiotic isolated from the culture filtrate of *Streptosporangium pseudovulagare*, strain No. PO-357, inhibits both bacterial and mammalian cell growth[3], and shows antitumor activity against certain experimental tumors such as leukemia P-388 and L-1210, and sarcoma-180. The objective of this work was to examine the effect of sporamycin mainly on DNA synthesis and on strand scission of cellular DNA.

Materials and Methods

[^{3}H]-Thymidine (27 Ci/mmol), [^{14}C]-thymidine (59 mCi/mmol), [^{3}H]-leucine (57 Ci/mmol), and [^{3}H]-uridine (28 Ci/mmol) were obtained from the Radiochemical Center, Amersham, U.K., [^{3}H]-TTP (17 Ci/mmol) from New England Nuclear, Mass., poly(rA) and oligo (dT12-18) from Boehringer Mannheim, Germany.

Cell line

HeLa cells were cultured in monolayers in EAGLE's minimum essential medium (MEM) supplemented with 10% bovine serum and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin) at 37°C in 95% air-5% CO_2 atmosphere.

Sporamycin was purified according to the procedure as previously reported[2], and the antibiotic gave a single SCHLIEREN peak during sedimentation in a Spinco model E ultracentrifuge.

Determination of the synthesis of cellular macromolecules

HeLa S3 cells (3×10^4) were plated into 30-mm Petri dishes, each inserted with 18-mm cover glass with 2 ml of MEM and allowed to reach exponential growth. Triplicate specimen were treated with ^{3}H-thymidine (TdR, 0.5 μCi/ml), ^{3}H-uridine (UR, 0.5 μCi/ml) or ^{3}H-leucine (Leu, 1 μCi/ml) for 30 minutes at 4 hours after the addition of sporamycin. Then the cells were rinsed 3 times with ice-cold HANKS' solution and 2 times with ice-cold 5% trichloroacetic acid. The radioactiviy of acid-insoluble fraction was determined by the Aloka gas-flow counter.

Effect of temperature on the activity of sporamycin

At 30 minutes after the incubation of HeLa cells with 10 μg/ml of sporamycin at 0°C or 37°C, the cells were rinsed with fresh MEM, and placed in two culture flasks. One flask was added with 0.5 μCi of ^{3}H-TdR, and this mixture was incubated for 30 minutes at 37°C to determine the ratio of

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DNA synthesis. The other culture flask was incubated further for 3 days at 37°C, and the number of cells were counted by the Coulter counter.

Preparation of DNA polymerase

DNA polymerases were partially purified from EHRLICH ascites cells by the method of MATSUKAGE[4]. Briefly, EHRLICH ascites cells were extracted with a buffer containing 0.5 mM KCl, 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol and 1 mM EDTA. DNA polymerases were fractionated with 25~65% saturated ammonium sulfate. DNA polymerase α was purified by column chromatography over DEAE-cellulose, P-cellulose, and hydroxyapatite. DNA polymerase β and γ were applied to column chromatography over DEAE-cellulose and P-cellulose. Terminal deoxynucleotidyl transferase from calf thymus was obtained from Boeringer Mannheim, Yamanouchi K.K.

Assay of DNA synthesis

DNA polymerase α: The reaction mixture (total volume, 25 μl) contained 50 mM Tris-HCl (pH 7.9), 6 mM $(CH_3COO)_2Mg$, 1 mM dithiothreitol, 0.1 mM each of deoxynucleoside triphosphates (dTTP carried a tritium label of 2 Ci/mmol), 16% glycerol, 400 μg/ml bovine serum albumin, 2 μg/ml calf thymus-activated DNA, and DNA polymerase (0.1~1 μg protein).

DNA polymerase β or γ: The reaction mixture (total volume, 25 μl) contained 50 mM Tris-HCl (pH 7.9), 0.5 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM ^{3}H-dTTP (2 Ci/mmol), 16% glycerol, 400 μg/ml bovine serum albumin, 80 mM KCl, 2 μg poly(rA), 0.4 μg oligo (dT 12–18), and DNA polymerase (1~5 μg protein).

Terminal deoxynucleotidyl transferase: The reaction mixture (total volume, 25 μl) contained 50 mM Tris-HCl (pH 7.5), 0.5 mM $MnCl_2$, 80 mM KCl, 1 mM dithiothreitol, 400 μg/ml bovine serum albumin, 0.1 mM ^{3}H-dTTP (2 Ci/mmol), 2 μg calf thymus-activated DNA, and the enzyme. After incubation of this reaction mixture at 37°C for 30 minutes, the acid-insoluble radioactivity was determined.

Determination of strand scission of cellular DNA

HeLa cells were incubated with ^{14}C-TdR (0.2 μCi/ml) or ^{3}H-TdR (1 μCi/ml) for 16 hours. HeLa cells labeled with ^{3}H-TdR were treated with sporamycin at 37°C for 4 hours. At the end of incubation period, equal number of non-treated HeLa cells prelabeled with ^{14}C-TdR and sporamycin-treated HeLa cells were mixed. The cells were placed on the top of 5~20% sucrose gradient consisting of 0.7 M NaCl, 0.001 M EDTA and 0.3 M NaOH overlayerd with the lysis solution (2% SDS). The preparation was sedimented in an RPS 40 rotor at 20,000 rpm for 30 minutes in a Hitachi ultracentrifuge.

Thermal profile of DNA

Calf thymus DNA was dissolved in 1/100 SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and incubated with sporamycin at 37°C for 30 minutes. After the incubation the temperature of the reaction mixture was raised at the rate of 2°C/minute with a linear temperature programmer. The thermal denaturation profile of DNA was automatically recorded, using Hitachi Model 124 spectrophotometer.

Results

Effect of Sporamycin on the Synthesis of Cellular Macromolecules

HeLa cells were incubated with ^{3}H-TdR, ^{3}H-UR or ^{3}H-Leu for 30 minutes after the cells were preincubated for 4 hours in the presence of sporamycin, and the incorporation ratio was compared with that of non-treated cells. As shown in Fig. 1, the incorporation of ^{3}H-TdR into DNA was strongly inhibited at a concentration of 0.3 μg/ml of the antibiotic in spite of very slight inhibition of the incorporation of ^{3}H-UR into RNA and ^{3}H-Leu into protein. When HeLa cells were incubated with various concentrations of sporamycin, the incorporation of ^{3}H-TdR into DNA was markedly inhibited at concentrations of more than 0.1 μg/ml for 60 minutes.

Effect of Temperature on the Activity of Sporamycin

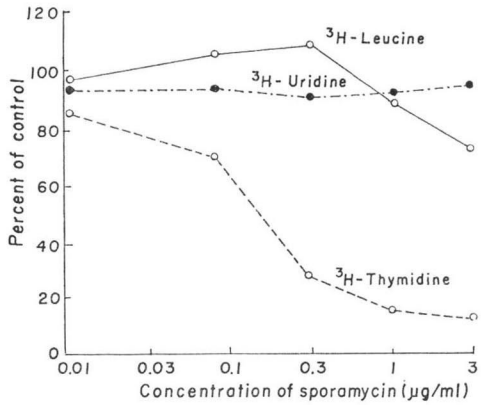
HeLa cells were incubated with sporamycin at 37°C or 0°C for 30 minutes, rinsed, and incorporation of ^{3}H-TdR into DNA and their cell growth were measured. As shown in Table 1, the incorporation of ^{3}H-TdR and cell number decreased markedly when the cells were treated at 37°C. On the other hand, neither inhibition of DNA synthesis nor cell growth *in vitro* was observed when the cells were treated at 0°C.

Effect of Sporamycin on DNA Polymerase

The effect of sporamycin on DNA synthesis was studied using various kinds of DNA polymerase extracted from EHRLICH ascites cells. As shown in Table 2, sporamycin did not inhibit DNA polymerase α, β or γ, and terminal deoxynucleotidyl transferase. Although data are not shown, even when DNA template was preincubated with sporamycin before the addition of DNA polymerase, no significant inhibition was observed.

Fig. 1. Effect of sporamycin on the synthesis of macromolecules in HeLa cells.

After 4-hour incubation of HeLa cells with various concentrations of sporamycin, the cells were treated with 0.5 μCi/ml of ^{3}H-TdR, 0.5 μCi/ml of ^{3}H-UR, or 1 μCi/ml of ^{3}H-Leu for 30 minutes.



Effect of Sporamycin on the Size of Cellular DNA

HeLa cells were incubated with sporamycin for 4 hours, and the size of cellular DNA was analyzed by alkaline sucrose gradient. Sedimentation pattern of DNA treated with 0.1 μg/ml of the

Table 1. Effect of temperature on sporamycin inhibition of HeLa-S3 cell growth and DNA synthesis.

Sporamycin (μg/ml)	Incubation condition	^{3}H-TdR incorporation	Cell count ($\times 10^{3}$) at 72 hours
0	37°C	3,630 (100)	328 (100)
10	37°C	2,282 (67)	6 (2)
10	37°C, 30 min.→washed→37°C	2,426 (67)	17 (5)
0	0°C, 30 min.→washed→37°C	3,605 (99)	317 (100)
10	0°C, 30 min.→37°C	2,464 (68)	9 (3)
10	0°C, 30 min.→washed→37°C	3,786 (104)	321 (101)

( ); percent of control.

Table 2. Effect of sporamycin on DNA polymerizing enzymes from EHRLICH ascites carcinoma.

Sporamycin (μg/ml)	[^{3}H]-TMP incorporated			
	Polymerase α	Polymerase β	Polymerase γ	TDT*
0	15,296 (100)	3,120 (100)	4,997 (100)	4,982 (100)
1	15,131 (99)	3,010 (96)	5,303 (107)	4,600 (92)
10	15,700 (103)	3,119 (100)	5,082 (102)	4,443 (89)
100	20,439 (135)	3,111 (100)	5,638 (113)	4,469 (90)

* terminal deoxynucleotidyl transferase from calf thymus.

Fig. 2. Alkaline sedimentation patterns of cellular DNA from HeLa cells treated with sporamycin.

HeLa cells prelabeled with ^{3}H-TdR were incubated with 0.1, 1, or 10 μCi/ml of sporamycin (closed circles). The cells prelabeled with ^{14}C-TdR served as non-treated cells (open circles).

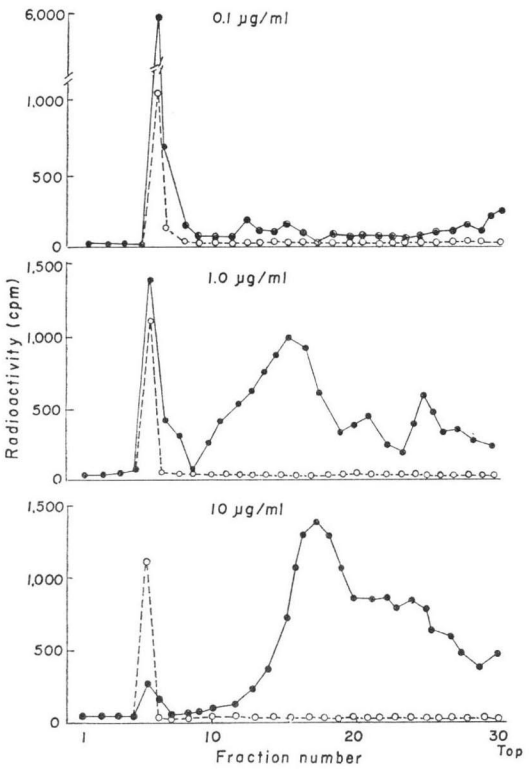
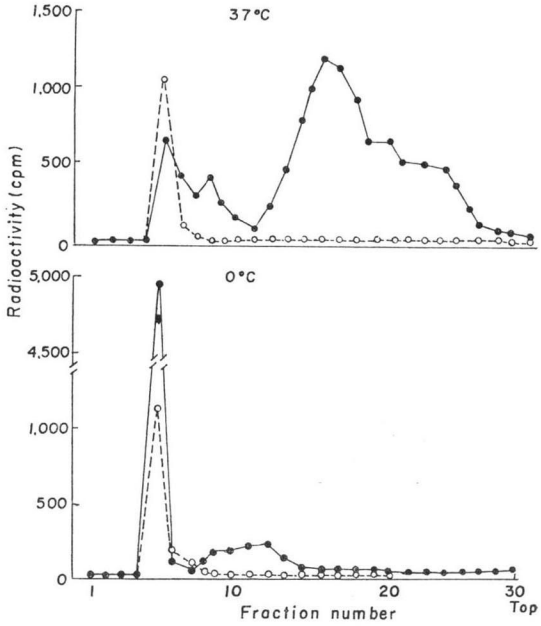


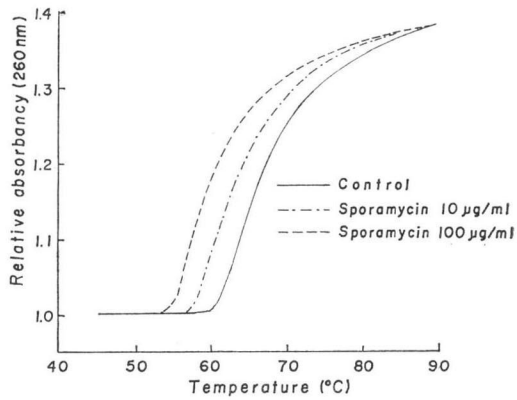
Fig. 3. Alkaline sedimentation patterns of cellular DNA of HeLa cells prelabeled with ^{14}C-TdR for non-treated cells (open circles) or ^{3}H-TdR for sporamycin-treated cells (closed circles) were incubated with 10 μg/ml of sporamycin for 30 minutes at 37°C or 0°C.



antibiotic at 37°C for 4 hours was similar to the non-treated one, but the change of sedimentation pattern of DNA was remarkable when treated with 1 or 10 μg/ml of sporamycin, and seemed to be dose dependent (Fig. 2). These data suggested that single-strand scission occurred in DNA strand of HeLa cells incubated with sporamycin. When HeLa cells were treated with the antibiotic at 0°C for 30 minutes, the change of sedimentation pattern was not observed (Fig. 3).

Fig. 4. Effect of sporamycin on the shift in the *Tm* of calf thymus DNA.

Calf thymus DNA was incubated with 10 μg/ml and 100 μg/ml of sporamycin, or without the antibiotic.



Effect of Sporamycin on *Tm* of the Calf Thymus DNA

The *Tm* of calf thymus DNA in 1/100 SSC was 67°C. When DNA was incubated with 10 or 100 μg/ml of sporamycin at 37°C for 30 minutes, the *Tm* decreased to 64°C or 60.5°C, respectively. However sporamycin did not affect hyperchromicity (Fig. 4).

Discussion

The present series of experiments showed that sporamycin inhibited DNA synthesis, while RNA and protein synthesis were not significantly affected in HeLa cells. Moreover sporamycin directly or indirectly caused single-strand scission in cellular DNA at relatively low concentration of the antibiotic. When the cells were treated with sporamycin at 0°C and incubated in fresh MEM at 37°C, inhibition of the cell growth, DNA synthesis, and strand scission of cellular DNA was not observed. This mechanism is still obscure, but sporamycin dose not bind effectively to cells or physiological temperature is essential to the activation of this antibiotic.

We found that sporamycin induced a decrease of *Tm* of calf thymus DNA *in vitro*, but did not affect its hyperchromicity. This result suggests that sporamycin affects the hydrogen bonds of double-stranded DNA. Similar phenomenon was observed in bleomycin[5], neocarzinostatin[6], DNA unwinding protein of *Escherichia coli*[7], and the DNA helix-destabilizing protein[8]. The former two antibiotics caused inhibition of DNA polymerase activity[9~12], while the latter two proteins stimulate the activity of homologous DNA polymerase *in vitro*. However, in contrast to these agents, sporamycin did not affect DNA polymerase activity *in vitro*. This indicated that the DNA template and primer in polymerase assay mixture at 37°C was unaffected by sporamycin.

Macromomycin, another macromolecular antibiotic, is also known to cause strand scission in cellular DNA[13], but did not affect the DNA polymerase activity[14], or rather, stimulated it in an isolated nucleus system[15]. The cytotoxicity of macromomycin could be recovered by removal of the antibiotic from the binding locus by a treatment with trypsin[13]. Although data are not shown, the same phenomenon was not observed in sporamycin. Recently, SUZUKI *et al.*[15] reported that when L-5178Y cells were treated with macromomycin, the growth of the cells did not recover by trypsin treatment.

The mode of action of sporamycin seems to be complicated, but it is considered that it has a cytocidal activity due to its primary effect on cellular DNA.

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