ABSTRACT

The mechanism of action of auromomycin, a new tumor-inhibitory antibiotic, was studied in a growing culture of mouse lymphoblastoma L5178Y cells and with isolated viral DNA. Auromomycin prevented growth of L5178Y cells completely and irreversibly at antibiotic concentrations higher than 0.03 μg/ml. DNA synthesis was preferentially inhibited by the antibiotic, whereas RNA and protein syntheses were not significantly affected. In synchronous cultures of L5178Y cells, resulted indicated that limited auromomycin-induced inhibition of DNA synthesis may occur independently of a much stronger inhibition of mitosis. In a short incubation period, a marked strand scission in cellular DNA of auromomycin-treated L5178Y cells was observed by an analysis of alkaline sucrose gradient centrifugation. In vitro, the antibiotic also induced strand breaks in linear duplex T-7 phage DNA and in the supercoiled circular duplex of SV40 DNA. 2-Mercaptoethanol neither enhanced nor was required for strand scission of isolated DNA by auromomycin. These data indicate that the mechanism of the antitumor activity of auromomycin is different from that of bleomycin, neocarzinostatin, or macromycin.

INTRODUCTION

A new antitumor antibiotic, auromomycin, has been isolated from culture broths of Streptomyces macromyceticus, a macromomycin-producing organism (12). The antibiotic is a light yellow crystalline acidic polypeptide with a molecular weight of 12,500 and an isoelectric point of pH 5.4. It exhibits a significant antitumor activity against Ehrlich ascites carcinoma, ascites Sarcoma 180, L1210 leukemia, and Lewis lung carcinoma in mice, as well as an antibacterial activity against Gram-positive and Gram-negative bacteria. Auromomycin is different from macromycin in its UV absorption spectrum, optical rotatory dispersion spectrum, and antimicrobial spectrum (12).

We have studied the mode of action of auromomycin in inhibiting the growth of cultured mammalian tumor cells and found that the antibiotic inhibits DNA synthesis preferentially to RNA and protein syntheses and causes strand scission of cellular DNA. DNA strand breaks have also been observed in vitro, using isolated T-7 phage or SV40 DNA. It is suggested by the current experiments that DNA strand scission may be responsible for the tumor-inhibitory activity of auromomycin.

MATERIALS AND METHODS

[^3H]Thymidine (55.2 Ci/mmole), [^3H]Juridine (44.9 Ci/mmole), and [^14C]Leucine (354 mCi/mmole) were purchased from New England Nuclear, Boston, Mass. [^3H]Thymidine-labeled SV40 DNA, containing 77% superhelical DNA and 23% open circular DNA, was kindly given by Dr. H. Asakura, Institute of Microbial Chemistry, Tokyo, Japan. The T-7 phage DNA was a gift from Dr. Y. Masamune of the Institute of Applied Microbiology. Both DNA's were dissolved in solutions of 0.1 x standard saline citrate (0.15 M NaCl and 0.015 M sodium citrate) containing 2 mM EDTA. Auromomycin (Lot 11-1), generously provided by K. Watanabe, Kanegafuchi Chemical Industry Co., Takasago, Hyogo-ken, Japan, was dissolved in phosphate-buffered saline (in g/liter: NaCl, 8.0; KCl, 0.2; Na₂HPO₄, 1.15; and KH₂PO₄, 0.2) for cultured cells or in water for isolated DNA just prior to use.

Growth and Macromolecular Syntheses of Mouse Lymphoblastoma L5178Y Cells. Mouse lymphoblastoma L5178Y cells were grown in Fischer's medium, supplemented with 10% horse serum, in suspension culture, and numbers of cells were determined by a Coulter counter. Syntheses of nucleic acids and protein in L5178Y cells were assayed by the procedure described previously (6), in which radioactive precursors and auromomycin were simultaneously introduced into the culture.

Synchronous Culture of L5178Y Cells. By an excess thymidine method, L5178Y cells, grown to the logarithmic phase in a 100-ml medium bottle, were treated with 2 mM thymidine at 37° for 5 hr. The cells were washed with phosphate-buffered saline and suspended in a fresh medium. The 2-mI cell suspension cultures in short test tubes were incubated immediately in a water bath at 37°. DNA synthesis started promptly and continued for about 5 hr. The increase of cell numbers began at about 6 hr and continued for 2 hr. By Colcemid treatment, the metaphase arrest of L5178Y cells by Colcemid followed the method described previously (9). By the method used, the generation time was approximately 10 hr: S phase was approximately 5 hr; and M phase was approximately 2 hr.

Alkaline Sucrose Gradient Centrifugation Analysis of DNA Strand Scission in Vivo and in Vitro. DNA strand scission in intact L5178Y cells, incubated with auromomycin, was examined by the procedure described previously (9), except that L5178Y cells were used instead of HeLa cells. DNA strand scission of isolated DNA by the antibiotic was performed as follows. Reaction mixtures contained 50 mM Tris-Cl (pH 7.6) and the indicated concentrations of auromomycin, [^3H]thymidine-labeled SV40 DNA, or T-7 phage DNA. The total volume was 100 μl for SV40 DNA and 200 μl for T-7 DNA, in which 4 × 10⁻³ A₂₆₀ unit (approximately 1000 cpm) and 50 μg of DNA were contained, respectively. The incubation was carried out at 37° for an indicated period and chilled quickly. The samples were gently applied on the top of a 5 to 20% alkaline sucrose gradient (4.6 ml, containing the ingredients as used for intact cells), upon which 0.2 ml of 0.5 N NaOH solution had been gently applied on the top of a 5 to 20% alkaline sucrose gradient (4.6 ml, containing the ingredients as used for intact cells), upon which 0.2 ml of 0.5 N NaOH solution had been layered. The centrifugation was performed in a Beckman SW50L rotor at 40,000 rpm for 3.5 hr at 4° for SV40 DNA or at 45,000 rpm for 3 hr at 20° for T-7 DNA. After centrifugation, the absorbance of T-7 DNA in the gradient was measured at...
254 nm, using an ISCO Model UA-5 absorbance monitor. The distribution of the radioactivities of SV40 DNA in the gradient was determined as described previously (9), and the total radioactivity in the 53S peak (Form 1) in each treatment was summed up. The ratio of DNA strand scission (conversion of Form 1 to Form 2) was expressed as the percentage of radioactivity remaining in the 53S peak.

RESULTS

Growth Inhibition of L5178Y Cells by Auromomycin. Auromomycin at various concentrations was introduced to the culture of L5178Y cells at Day 0, and the effect on the growth was examined for 3 days by counting cell numbers in a Coulter counter. The antibiotic prevented growth of L5178Y cells completely at concentrations higher than 0.03 µg/ml, and approximately 50% growth inhibition was observed at a concentration of 0.004 µg/ml (Chart 1). A simple washing or trypsin treatment of the cells did not recover the growth after a short period of incubation with auromomycin (0.008 µg/ml at 37°C for 10 min), indicating that the lethal damage was induced in the cells by the antibiotic promptly and irreversibly (data are not shown).

Effects of Auromomycin on Macromolecular Syntheses in Intact L5178Y Cells. Auromomycin was observed to inhibit [3H]thymidine incorporation into the cold trichloroacetic acid-insoluble fraction of L5178Y cells at concentrations higher than 0.004 µg/ml, where [3H]uridine and [14C]leucine uptakes were not prevented. The results suggested that the antibiotic preferentially blocked DNA synthesis rather than RNA or protein synthesis. The inhibition of DNA synthesis by auromomycin was incomplete even at high antibiotic concentrations (Table 1).

Effect of Auromomycin on DNA Synthesis and Cell Division in Synchronized L5178Y Cells. The inhibition of DNA synthesis and cell mitosis by auromomycin was examined in 2 different stages of synchronous L5178Y cells to determine whether these occurred independently. First, the cells grown to the logarithmic phase were synchronized by an excess thymidine method. After being transferred to a fresh medium, control cells started DNA synthesis immediately and continued for approximately 5 hr. Mitosis occurred at approximately 6 hr. When auromomycin at a concentration of 0.2 µg/ml was added to the synchronized culture at 0 hr, the incorporation of [3H]thymidine was partially inhibited, whereas cell division was completely prevented (Chart 2). The complete inhibition of [3H]thymidine uptake in the second cycle seemed to be due to the arrest of the cell cycle at M phase by the antibiotic. The results suggested that M phase was more sensitive to auromomycin than was S phase in the cell cycle. Another synchronization method was also used to examine the effect of the drug, especially on mitosis. Chart 3 shows that control cells,
arrested at metaphase by Colcemid, resumed mitosis, after a 0.5-hr lag period, with removal of the Colcemid and that DNA synthesis in control cells started at 1.5 hr and reached a maximum at approximately 3.5 hr after Colcemid removal. Auromomycin at a concentration of 0.06 μg/ml prevented cell mitosis strongly before DNA synthesis began, suggesting that the inhibition of cell division by auromomycin was not a consequence of the inhibition of DNA synthesis.

DNA Strand Scission by Auromomycin in Intact L5178Y Cells. The fragmentation of cellular DNA by auromomycin was observed by alkaline sucrose density gradient centrifugation analysis. DNA from the cells, treated with the antibiotic for 10 min at 37°, appeared at different positions in the gradient depending on the drug concentrations, while DNA of control cells was pelleted to the bottom of the tube because of its very large size (Chart 4).

DNA Strand Scission by Auromomycin in Isolated DNA. The direct action of auromomycin of cutting the DNA strand was confirmed by a shift in the molecular size of isolated DNA, treated with the drug, as demonstrated by alkaline sucrose density gradient centrifugation. The peak of the linear duplex strand of T-7 DNA (37S in alkaline) was decreased after incubation with auromomycin at 37° for 2 hr with a concomitant increase of absorbance in smaller fractions. The peak at 37S shifted to 30S after incubation with the drug at a concentration of 100 μg/ml (Chart 5). A more sensitive and quantitative experiment for production of a single-strand break in DNA was performed, using [3H]thymidine-labeled SV40 DNA. Native SV40 DNA is double stranded, covalently closed circular, and superhelical and sediments with a 53S value at alkaline pH. If a single nick is produced on either strand of the native form, the products sediment at 18S (single stranded and closed circular DNA) and at 16S (single stranded and linear DNA). Our sample of [3H]thymidine-labeled SV40 DNA contained 77% of Form 1 and 23% of Form 2, and the degree of DNA strand breaks was calculated from the percentage of the decrease in radioactivity at 53S peak. When SV40 DNA was incubated with auromomycin at 37° for 10 min, about 15 and 35% of Form 1 converted to Form 2 at concentrations of 1 and 10 μg/ml, respectively (Chart 6). The 2 experiments described in Charts 5 and 6 were carried out in the presence of 3 mM 2-mercaptoethanol, because a sulfhydryl compound is required for or enhances the activities of bleomycin and neocarzinostatin in causing DNA strand scission in vitro. The effect of 2-mercaptoethanol on auromomycin activity of DNA strand scission was examined in vitro at 37° for 10 min. The degree of conversion of Form 1 to Form 2 by auromomycin did not significantly change in the presence or absence of 3 mM 2-mercaptoethanol (Chart 7). In another experiment, the addition of 10 mM 2-mercaptoethanol stimulated DNA strand scission by auromomycin (10 μg/ml) only by approximately 10% after 60 min incubation at 37° (data are not shown). In this respect, the mechanism of action of auromomycin seemed to be different from that of bleomycin or neocarzinostatin. No significant acid-soluble degradation of SV40 DNA was observed after incubation with auromomycin at indicated concentrations for 10 min at 37°, and DNA profiles were analyzed on alkaline sucrose gradients. Sedimentation was carried out at 30,000 rpm for 90 min at 20° in a Beckman SW 50L rotor and recorded from left to right. The radioactivity recovered from the bottom of tubes was 15,300 cpm (85% of total radioactivity), 848 cpm (4.7%), 143 cpm (0.7%), and 130 cpm (0.7%) at auromomycin concentrations of 0 (C), 0.06 (×), 0.25 (○), and 1 (△) μg/ml, respectively.

![Chart 3](image-url)

**Chart 3.** Effects of auromomycin on cell division in a synchronized culture of L5178Y cells (Colcemid treatment). Auromomycin at 0.06 μg/ml was added to the culture at 0 and 1 hr (arrows) after removal of the Colcemid, and the cell number at each period was determined by a Coulter counter. The activity of DNA synthesis in control cells was measured by [3H]thymidine uptake for 60 min (— — — —). △, control; ○, auromomycin added at 0 hr; ×, auromomycin added at 1 hr.

![Chart 4](image-url)

**Chart 4.** DNA sedimentation profiles of auromomycin-treated L5178Y cells. The cells (25,000 cells/ml), prelabeled with [3H]thymidine, were incubated with auromomycin at indicated concentrations for 10 min at 37°, and DNA profiles were analyzed on alkaline sucrose gradients. Sedimentation was carried out at 30,000 rpm for 90 min at 20° in a Beckman SW 50L rotor and recorded from left to right. The radioactivity recovered from the bottom of tubes was 15,300 cpm (85% of total radioactivity), 848 cpm (4.7%), 143 cpm (0.7%), and 130 cpm (0.7%) at auromomycin concentrations of 0 (C), 0.06 (×), 0.25 (○), and 1 (△) μg/ml, respectively.

![Chart 5](image-url)

**Chart 5.** Effects of auromomycin on sedimentation profile of T-7 phage DNA. Auromomycin at 10 (— — — —) and 100 (— — — —) μg/ml was incubated with T-7 phage DNA (4 A260 units/ml) in a total volume of 200 μl in the presence of 3 mM 2-mercaptoethanol at 37° for 2 hr and applied on the gradient. Centrifugation was done at 45,000 rpm for 3 hr at 20°. △, control.
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Chart 6. Effects of auromomycin on sedimentation profile of [3H]thymidine-labeled SV40 DNA. Auromomycin at indicated final concentrations and [3H]thymidine-labeled SV40 DNA were incubated at 37° for 10 min in the presence of 3 mM 2-mercaptoethanol. Inset, radioactivity remaining at 53S peak (Form 1) as a percentage of control. ○, 0 μg/ml; ×, 1 μg/ml; ●, 10 μg/ml.

Chart 7. Influence of 2-mercaptoethanol on strand scission of SV40 DNA by auromomycin. Auromomycin at 10 μg/ml was incubated with [3H]thymidine-labeled SV40 DNA in the presence and in the absence of 3 mM 2-mercaptoethanol at 37° for 10 min. ○, control; ×, auromomycin without 2-mercaptoethanol; ●, auromomycin with 2-mercaptoethanol.

DISCUSSION

In the current experiments, auromomycin has been observed to block DNA synthesis and mitosis independently, with a majority of the inhibition of DNA synthesis probably resulting from the strong mitotic inhibition observed. Auromomycin was also found to cause DNA strand scission both in vitro and in vivo, suggesting that the chemoreceptor of the antibiotic may be DNA. The small amount of inhibition of DNA synthesis seen when exposure occurs during the G2-S and S phases may result from DNA strand breaks. However, the interrelationships among the effects of auromomycin on DNA synthesis, mitosis, and DNA breaks and the precise mechanism of the interaction of auromomycin with DNA remain to be determined. More than 1 or 10 μg/ml were necessary to produce a significant shift in the SV40 or T-7 DNA distribution, while 0.06 μg/ml was all that was required for the L5178Y cells. The reason for the difference of in vivo and in vitro effects may be partly due to the size of the DNA's.

The mode of action of auromomycin is similar to that of neocarzinostatin, another antitumor protein antibiotic (2-4). However, the effects of sulfhydryl compounds on DNA strand scission by the 2 antibiotics are different. 2-Mercaptoethanol is required for neocarzinostatin, but not for auromomycin, to induce DNA strand scission (3, 5). Sulfhydryl compounds also enhance the direct action of bleomycin on DNA (7, 8, 11). It has been suggested recently that the free iron produced when the ferrous bleomycin complex binds to oxygen is reduced by sulfhydryl compounds (10). Macromomycin causes strand scission in cellular DNA of cultured tumor cells but fails to induce strand breaks in isolated DNA, even at very high antibiotic concentrations, under the same conditions as in the current experiments (1, 9). Thus, the mechanism of action of auromomycin seems to be different from that of bleomycin, neocarzinostatin, or macromomycin.

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DNA Strand Scission *in Vivo* and *in Vitro* by Auromomycin

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