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CELLULAR DNA DAMAGE BY THE ANTITUMOR PROTEIN MACROMOMYCIN AND ITS RELATIONSHIP TO CELL GROWTH INHIBITION

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SUMMARY

Macromomycin, an antitumor protein, after purification inhibited HeLa S_3 cell growth at low nanomolar concentrations. DNA synthesis was inhibited at drug levels that left RNA and protein synthesis unaffected. Incubation of macromomycin with HeLa S_3 cells resulted in a rapid fragmentation of cellular DNA that was detectable at low nanomolar drug levels. Heat denaturation of macromomycin showed that both its ability to inhibit cell growth and to fragment cellular DNA were lost at similar rates.

INTRODUCTION

Macromomycin is a 12,000 dalton protein antibiotic (1) isolated from Streptomyces macromomycetius (2). The drug has demonstrated good antitumor activity in animal systems such as mice bearing L1210 and P388 leukemias, B16 melanoma, and animals with s.c. implanted Lewis lung tumor (3). Non-purified macromomycin inhibited cell growth in tissue culture systems such as Yoshida sarcoma and HeLa cells also inhibited DNA synthesis (4). Interestingly, the growth inhibitory effect of the drug could be overcome by incubation of drug-treated cells with trypsin (4). This was seen as evidence that the drug exerts its cytostatic effect on cells at the outer cell membrane. A recent review on antitumor drugs describes macromomycin as being able to cause damage to cellular DNA although data was not presented (5). Yamashita et al. have now published a drug purification procedure which yields pure macromomycin as a single homogeneous protein (1).

This investigation was undertaken to show how purified macromomycin affects cell growth and cellular DNA structure and function. The drug's effect on DNA structure, that is DNA strand scission, is shown to be related to the drug's ability to inhibit cell growth. Cellular DNA damage in macromomycin-treated cells occurs very

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rapidly after drug addition and at low nanomolar drug concentrations. It would appear that macromomycin induced damage of cellular DNA is an important aspect of the drug's mechanism of action.

MATERIALS AND METHODS

[Me-^{3}H]-Thymidine, [Me-^{14}C]-thymidine were obtained from Amersham Corporation. All tissue culture media and sera were from Grand Island Biological. HeLa S_3 cells, stock CCL2.2, were from American Type Culture Collection. Crude macromomycin NSC #170105 was provided by the Developmental Therapeutics Program, Chemotherapy, of the National Cancer Institute. This macromomycin was purified according to the procedure of Yamashita et al. (1).

Maintenance of HeLa S_3 cultures

HeLa S_3 spinner cultures were maintained as previously described (6). HeLa S_3 plate cultures were maintained in Basal Medium (Eagle) with 10% fetal calf serum at 37°C under 5% CO_2 and at densities between 2 x 10^4 cells/ml and 8 x 10^5 cells/ml.

Measurement of DNA synthesis in HeLa S_3 cells

DNA synthesis was measured in HeLa S_3 cells grown in spinner culture by treating cell suspensions (3 x 10^5 - 4 x 10^5 cells/ml) at 37°C with [^{3}H]-thymidine (50 Ci/mmol, 0.4 μCi/ml). Samples (10 ml) were then centrifuged at 3,000 rev./min. for 5 min. at 24°C and resuspended in 2 ml of phosphate-buffered saline (0.17 M NaCl, 0.27 mM KCl, 8.1 mM Na_2HPO_4, 0.21 mM KH_2PO_4, pH 7.2). This was followed by three additional centrifugations in which the pellets were resuspended in 2 ml of cold 0.4 M perchloric acid and 0.005 M sodium pyrophosphate. The washed pellet was taken up in 0.5 ml of 0.4 M perchloric acid and the suspension was added to 10 ml of scintillation fluid (ACS-Amersham). The tubes were then rinsed with 0.5 ml of distilled water and the rinse was added to the scintillation fluid for counting.

Measurement of HeLa S_3 cell growth

HeLa S_3 cells were grown in 35 mm tissue culture dishes maintained at 37°C in 5% CO_2 and containing 2 ml of Basal Medium (Eagle) with 10% fetal calf serum. Cell suspensions (2.5 x 10^4 cells/ml) were added to tissue culture dishes and were later treated with drug. After 3-4 days of growth, the cell density was measured by washing the plates with phosphate-buffered saline and by then determining the protein content with a Lowry assay (7).

Alkaline sucrose gradient analysis of strand scission of cellular HeLa S_3 DNA

Cell suspensions (2 x 10^5 - 3 x 10^5 cells/ml) were prelabelled for 16-18 hours with [^{14}C]-thymidine (50 mCi/mmole, 0.05 μCi/ml) and then treated with drug at 37°C. Cells were then washed 3 times in phosphate-buffered saline before placing 100 μl of cells at 4 x 10^6 - 6 x 10^6 cells/ml onto a 5-20% sucrose gradient consisting of 0.7 M NaCl, 0.3 M NaOH and 0.01 M EDTA overlayed with 0.5 ml of lysis solution (0.3% sarkosyl and 2.5% alkaline sucrose). After 10 minutes at 20°C the preparation was sedimented in a Spinco SW 50.1 rotor for 60 minutes at 25,000 rpm at 20°C. Following centrifugation, 200 μl fractions were collected from the top of the gradient, neutralized and counted in ACS scintillation fluid. Radioactivity that appeared in the tube bottom was also counted. When the time course of DNA scission by macromomycin was measured, cells were labelled with higher levels of [^{14}C]-thymidine (50 mCi/mmol, 0.25 μCi/ml) and after the incubation with drug, 100 μl

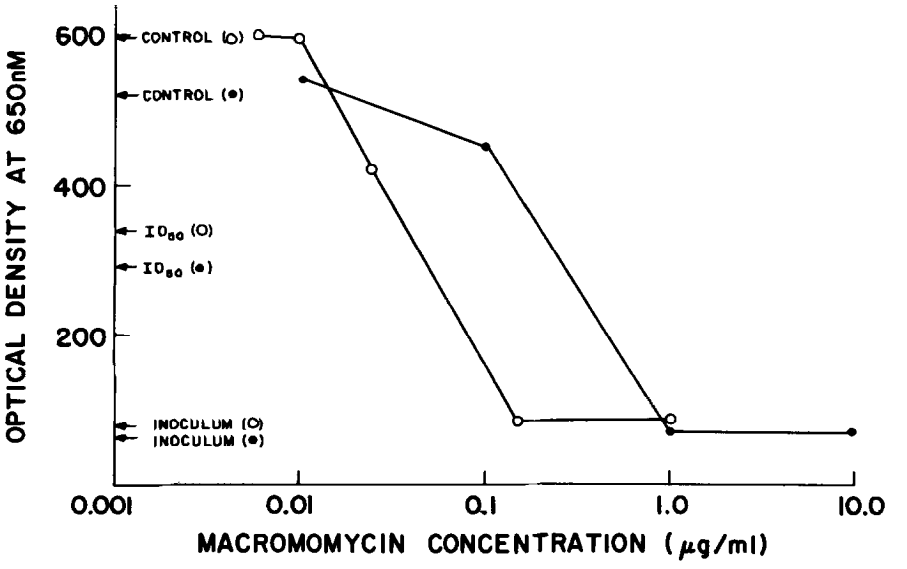


Figure 1

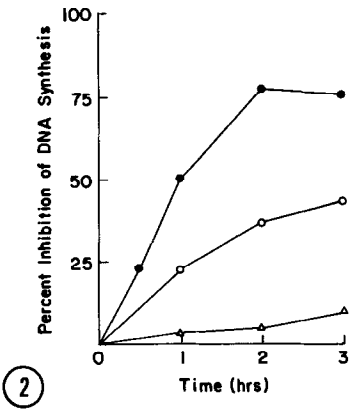
Cell growth of HeLa S_3 cells treated with varying concentrations of crude (•) and purified (o) macromomycin. See Materials and Methods for assay conditions.

of suspension at 4 x 10^5 - 6 x 10^5 cells/ml was placed directly on the alkaline sucrose gradient.

RESULTS AND DISCUSSION

The procedure of Yamashita et al (1) was used to purify macromomycin to a homogeneous protein that represented roughly 2 percent of the starting-crude material. The protein on isoelectric focusing gave a single band at a pH of 5.54, a value that was in close agreement with that obtained by Yamashita et al. (1). The potency of macromomycin was measured during the purification procedure by determination of the drug's ID_{50} level with HeLa S_3 cells. The ID_{50} level of the crude starting compound and pure macromomycin were 0.28 and 0.031 μg/ml respectively (Fig. 1). Removal of the drug after a 30 minute incubation period did not alter the final level of cell growth inhibition.

The effect of macromomycin on DNA synthesis in HeLa S_3 cells is shown in Fig. 2. At the highest level of drug studied, (2.0 μg/ml) inhibition of DNA synthesis is seen early on after the addition of drug. Even at drug levels of 0.04 μg/ml there are still detectable levels of inhibition. RNA and protein synthesis were unaffected



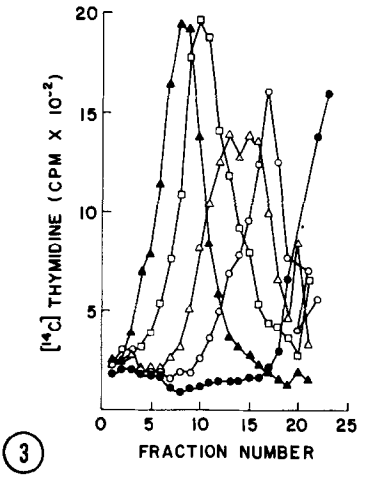


Figure 2

Inhibition of DNA synthesis in HeLa S_3 cells as a function of macromomycin concentration. HeLa S_3 cell suspensions were treated with [^{3}H]-thymidine (50 Ci/mMole, 0.4 μCi/ml) and macromomycin, as indicated below. At the indicated times, DNA synthesis was measured as described in Materials and Methods. The results are shown as the percent inhibition of DNA synthesis by comparing the incorporation of drug treated samples to control values. Generally, control cells incorporated 1,000 cpm per 10 ml of cells in one hour. Macromomycin levels were as follows: Δ——Δ, 0.04 μg/ml; o——o, 0.2 μg/ml; and ●——●, 2.0 μg/ml.

Figure 3

Sedimentation on alkaline sucrose of cellular DNA from macromomycin-treated HeLa S_3 cells prelabelled with [^{14}C]-thymidine. Cells were treated with macromomycin at ●——●, control; o——o, 0.01 μg/ml, Δ——Δ, 0.04 μg/ml; □——□, 0.2 μg/ml; and ▲——▲, 1.0 μg/ml for 30 minutes at 37°C. Fractionation was from top to bottom. Recovery of the DNA was generally greater than 90 percent. Radioactivity that was pelleted does not appear on the Figure.

by concentrations of macromomycin that caused substantial inhibition of DNA synthesis.

Neocarzinostatin, another protein antitumor agent that inhibits cell growth and DNA synthesis, is also known to cause strand scissions in cellular DNA (6, 8 and 9). Macromomycin was tested to see if it also damaged cellular DNA. Analysis of the effects of drug on cellular DNA can be seen in the alkaline sucrose gradient profile of DNA from drug-treated cells (Fig. 3). DNA damage in macromomycin-treated HeLa S_3 cells is first detected at a drug level (between 0.01 and 0.04 μg/ml) close to that of the drug's ID_{50} value (0.031 μg/ml). Higher macromomycin levels cause a progressive increase in DNA damage. A time course study (Fig. 4) demonstrates the rapidity of macromomycin damage to cellular DNA. Measurable breakdown of

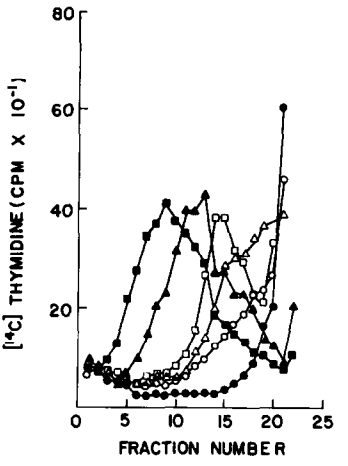


Figure 4

Sedimentation on alkaline sucrose of cellular DNA from HeLa S_3 cells treated with macromomycin for varying time periods. Macromomycin at 1.0 μg/ml was given to HeLa S_3 cells at 37° for the following time periods; ●——●, no drug; o——o, 0 min.; Δ——Δ, 1 min.; □——□, 2.5 min.; ▲——▲, 10 min.; and ■——■, 30 min. See Fig. 3 and Materials and Methods for details of alkaline sucrose gradient conditions.

DNA is seen as early as 1 - 2.5 minutes after the addition of drug to cells. The increase in DNA damage seen with time of incubation with drug, indicates that DNA breakdown is occurring prior to cell lysis. The pH of the cell lysis conditions (> 12.0) was found to completely inactivate the drug, insuring that DNA breakdown is not occurring during the period of cell lysis. When non-labelled cells were treated with drug and lysed, followed immediately by the addition of [^{14}C]-thymidine labelled cells, no DNA damage was seen in the labelled cells. There was also no shift in cellular DNA molecular weight if untreated cells were added to macromomycin that was already present in the lysis solution, again indicating that DNA damage occurs prior to cell lysis (data not shown). In a study done with crude macromomycin, roughly 10 times more drug was required to see levels of DNA damage comparable to those obtained with pure drug.

The appearance of cellular DNA damage as an early effect of the drug-cell interaction would suggest that the drug's effect on DNA structure is a primary drug activity. DNA damage has already been found to occur at drug levels where cell

TABLE I

A COMPARISON OF THE LOSS OF CELL GROWTH INHIBITION AND DNA STRAND SCISSION ACTIVITY OF MACROMOMYCIN SUBJECTED TO THERMAL DENATURATION

Time of heat Inactivation of Macromomycin (min.)	Sedimentation distance of cellular DNA	Percent Inhibition of HeLa S_3 Cell Growth
Control (no drug)	18	0
Drug standard	10.5	100
0	11.0	91.4
5	12.5	84.4
10	13.5	76.8
20	14.5	66.0
40	15.5	49.2
80	16.0	24.4

* The drug designated as 0 time was obtained by bringing macromomycin to the appropriate dilution using diluent at 50°C. The sample was quickly mixed and an aliquot withdrawn and cooled. The drug standard designation is an equivalent concentration of drug that has received no treatment.

Macromomycin at 100 μg/ml was heated at 50°C for the periods of time indicated and then assayed for its ability to inhibit HeLa S_3 cell growth and to induce strand scissions in HeLa S_3 cellular DNA at a concentration of 0.1 μg/ml (see Materials and Methods). Loss of cell growth inhibition activity represents a change of activity using untreated macromomycin as the criterium for complete activity. The sedimentation distances represent the peak positions of cellular DNA bands as determined from alkaline sucrose gradients (see Fig. 3).

growth inhibition takes place. Heat denatured macromomycin revealed that the ability of the drug to fragment cellular DNA and to inhibit cell growth was lost at roughly comparable rates (Table I). Loss of both activities are first detected after 5 minutes of heat treatment while by 80 minutes both activities are much diminished. This type of data suggests that macromomycin-induced cell growth inhibition is accompanied by damage to cellular DNA. Whether DNA damage is also related to the inhibition of DNA synthesis, as is the case with neocarzinostatin, remains to be studied (10).

Preliminary data indicates that, unlike neocarzinostatin which damages both cellular and cell free DNA (6, 8 and 11), macromomycin does not cause DNA strand scissions in a cell free system using linear duplex λ DNA. Of course, finding DNA damage may be a matter of obtaining proper reaction conditions. A recent report (12), indicated that macromomycin does cause strand scissions in both cellular and cell free DNA systems.

Additional studies are needed to determine if and how DNA damage is related to the drug's growth inhibitory effect. The relationship between cellular DNA damage and cell growth inhibition for a drug thought to act at the cell surface is an intriguing question. It will be important to understand how DNA reactive antitumor drugs such as neocarzinostatin, bleomycin and now macromomycin, all of which damage cellular DNA (5), are able to translate this effect into an inhibition of cell growth.

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