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Inhibition of DNA Replication in HeLa S_3 Cells by Macromomycin-I, an Antitumor Protein Antibiotic

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SUMMARY

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Macromomycin-I, a chromophore-containing form of an antitumor protein antibiotic macromomycin, was found to be a potent inhibitor of [^{3}H]thymidine incorporation into DNA in intact nonsynchronous and G_1/S-synchronized HeLa S_3 cells. This inhibitory effect depended on the drug dose and duration of treatment, but was essentially independent of the cell cycle position, within the G_1 through S phases. A parallel inhibition of DNA synthesis was observed in lysates and purified nuclei from drug-treated cells (as measured by incorporation of radioactive dTTP into DNA), indicating that the drug interferes with DNA biosynthesis after formation of deoxynucleoside triphosphates. The addition of a cytoplasmic fraction from untreated cells to purified nuclei from drug-treated cells failed to overcome the inhibition of DNA synthesis, while a cytoplasmic fraction from drug-treated cells was able to stimulate efficiently DNA synthesis in purified nuclei from untreated cells. Moreover, the inhibition of DNA synthesis in nuclei from drug-treated cells could be overcome by the addition of exogeneous DNA. These data demonstrate that macromomycin-I acts on DNA replication by interfering with the template and probably does not affect DNA polymerase or other factors involved in replication. In addition, macromomycin-I was found to enhance the incorporation of [^{3}H]thymidine into DNA in HeLa S_3 cells in the presence of hydroxyurea; thus, the drug seems to stimulate DNA repair. Both findings, inhibition of DNA replication and stimulation of repair synthesis, probably arise from the drug's damage to DNA and support the assumption that this damage is a primary event in the cytotoxic action of macromomycin.

INTRODUCTION

Macromomycin (MCR)[2] is a protein antibiotic isolated from *Streptomyces macromomycetius* with a molecular weight of about 12,000 (1, 2). The drug was demonstrated to be active against several experimental tumors *in vivo* and cytotoxic to cultured tumor cells (3, 4). MCR also has antibacterial activity (4). The drug was reported to inhibit the incorporation of thymidine into DNA in tissue cultures (4), and this inhibition has been shown to parallel the inhibition of cell growth (5). Incorporation of RNA and protein precursors was affected by MCR, but only at higher drug levels than are needed to inhibit thymidine incorporation (6). It has also been demonstrated that the drug damages cellular DNA, and this effect seems to be related to cell growth inhibition (7, 8). Besides damage to cellular DNA, MCR also causes breaks in cell-free DNA (6, 9).

Nevertheless, the mechanism of biological action of MCR is still not clearly understood. The decrease in thymidine incorporation by MCR was interpreted as an inhibition of DNA synthesis (4–6, 8, 10). Though this is the most likely explanation, the observed effect might also result from other reasons, such as impaired transport or phosphorylation of thymine in drug-treated cells. So far, no attempt has been made to exclude unequivocally these possibilities and to localize the site of the drug's action on the pathway of DNA biosynthesis. Furthermore, previous studies were performed on the antibiotic purified to various degrees by several different procedures. Recently, it has become clear that the drug exists in two forms of identical molecular weight and isoelectric

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[2] Abbreviations used: MCR, macromomycin; PCA, perchloric acid; EDTA, ethylenediaminetetraacetic acid; EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; Tris, tris(hydroxymethyl)aminomethane; HU, hydroxyurea.

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point, but that one contains, in addition, a 350 nm-absorbing chromophore (11, 12). Using our own purification scheme (13), we have been able to achieve almost complete separation of these two forms of the drug and have designated the more active chromophore-containing substance as macromomycin-I (MCR-I). This is probably the same substance that has been designated auromomycin by another group (11, 12).

In this paper, a possible relationship between the drug's ability to damage DNA and its effect on DNA synthesis is explored. We have investigated the effects of MCR-I on the incorporation of radioactive thymidine into DNA in nonsynchronous and synchronous HeLa S_3 cultures, as well as the effects of the drug on DNA synthesis in cell lysates and purified nuclei from MCR-I-treated cells. The results demonstrate that MCR-I does inhibit DNA synthesis and this effect arises from the drug's action on the DNA template rather than on DNA polymerase or biosynthesis of DNA precursors. The drug also seems able to stimulate DNA repair synthesis in intact cells. These data support the assumption that direct DNA damage is a primary event in the antitumor action of MCR.

MATERIALS AND METHODS

Macromomycin-I. Crude MCR (NSC No. 170105) was provided by the Developmental Program, Chemotherapy, of the National Cancer Institute. MCR-I was isolated from the crude material by chromatography on DEAE-Sephacel as described elsewhere (13). Briefly, crude MCR (1.5 g) was fractionated at 5°C in the dark on a column (3 × 80 cm) eluted with 10 mM Tris, pH 7.9, followed by elution with a two-step linear gradient of NaCl concentration (0–10 mM NaCl in 250 ml and 10–20 mM NaCl in 2000 ml, respectively). MCR-I was eluted at about 17 mM NaCl, slightly ahead of the chromophore-lacking MCR, producing a distinct shoulder on the MCR peak in the elution profile. The pooled fractions were concentrated by ultrafiltration and stored at −20°C. The MCR-I preparations used throughout this work consisted of about 80% of the chromophore-containing drug [as estimated from uv spectra assuming extinction coefficients reported by others for auromomycin and chromophore-lacking MCR (3)]. The total amount of protein calculated from uv spectra of MCR-I preparations was similar to that determined by the Lowry method (14) using bovine serum albumin for a calibration curve. The ID_{50} of MCR-I preparations against HeLa cells, determined as described elsewhere (7), was 2.9 ng/ml. Due to the sensitivity of macromomycin to light, all operations with the drug were done in partial or complete darkness. Exposure of MCR-I to normal laboratory light for 3 h resulted in a 15–20% decrease in its activity even if the samples were kept in ice, while the drug stored for 6 months at −20°C in the dark retained about 40–50% of its original activity.

Radiochemicals. [*Methyl*-^{3}H]thymidine (2 Ci/mmol), [*methyl*-^{3}H]dTTP (42 Ci/mmol), 5′-[α-^{32}P]dTTP (200 mCi/mmol), and [^{3}H]dGTP (14 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England.

Tissue culture. HeLa S_3 cells were maintained in suspension culture in Joklik's minimal essential medium with 5% fetal calf serum, as described previously (15). For all experiments with MCR-I, the cells were suspended in medium at an initial concentration of 1×10^6 cells/ml. G_1/S synchronization of the cells was accomplished by a double thymidine block technique (15): Exponentially growing cells were blocked for 15 h at 37°C with 0.25 M thymidine. Then the cells were centrifuged and resuspended in fresh medium at 37°C for 12 h. At that time, a second 0.25 mM thymidine block was imposed. Twelve hours later, the cells were released from the block by centrifugation and resuspended in fresh medium. Synchrony was monitored based on cell growth kinetics. The cell number started to increase 7–8 h after release from the second block and was almost doubled by 13 h after release.

Incorporation of [^{3}H]thymidine into macromolecules in HeLa S_3 cells. The cells were incubated with MCR-I and [^{3}H]thymidine (2 μCi/ml) as specified in the figure legends. At the indicated times, triplicate samples (0.5–1.0×10^6 cells) from each suspension were given 10 ml of cold 0.5 M PCA (perchloric acid) containing 5 mM sodium pyrophosphate. After at least 2 h of standing in ice, the PCA-insoluble material was collected by filtration onto GF/C filters (Whatman, England). The precipitates were washed three times with 10 ml of cold 0.5 M PCA, followed by two washes with ethanol. The filters were then dried and their radioactivity was counted in standard toluene scintillation fluid in a Beckman scintillation counter.

Radioactivity incorporated into HeLa cells incubated with [^{3}H]thymidine in the presence of 10 mM hydroxyurea was determined in a similar way except that PCA-precipitated macromolecules were kept in ice for 4 h, then spun down (1500*g*, 15 min), resuspended in fresh cold PCA, and after several hours of standing in ice, collected onto GF/C filters. The filters were washed six times with 8 ml of cold PCA, followed by washing with ethanol, and processed further as described above.

Incorporation of radioactive dTTP into lysates and purified nuclei from HeLa S_3 cells. The cells were synchronized at G_1/S as described above and treated with MCR-I as specified in the figure legends. Four hours after release from the block, the cells were harvested. Cellular lysates, purified nuclei, and cytoplasmic fractions were prepared from these cells according to Fraser and Huberman (16, 17). The lysates or nuclei suspensions (50×10^6 nuclei/ml unless otherwise stated) were combined at a ratio of 9:1 with an assay mix similar to that of Fraser and Huberman (16, 17). The assay mix in the final reaction mixture contained the following: 36 mM KCl, 9 mM Na_2HPO_4, 45 mM Hepes, 8 mM glucose, 2 mM DTT, 1 mM EDTA, 0.45 mM EGTA, 10 mM $MgCl_2$, 30 μM spermine, 2.5 mM ATP, 0.5 mM each GTP, UTP, and CTP, 0.1 mM each dCTP, dGTP, and dATP, and 0.03 mM [^{3}H]dTTP (6.5–15 μCi/ml), pH 7.8. In some experiments, instead of [^{3}H]dTTP, ^{32}P-labeled dTTP was used at 2.4 μCi/ml. The aliquots (75–100 μl) of the final reaction mixtures were incubated at 37°C for the indicated time. The reaction was terminated by cooling the samples in an ice bath and the addition of 0.3 ml of cold buffer H [2 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, 10 mM Na_2HPO_4, pH 8.0 (16)], followed by the addition of 5 ml of 0.5 M

PCA containing 5 mM sodium pyrophosphate. After at least 4 h of standing in ice, the samples were centrifuged (1500*g*, 15 min), and the pellets were resuspended in cold 0.5 M PCA containing 5 mM pyrophosphate and spun down again. This operation was repeated three more times, after which the final pellets were resuspended in 0.3 ml of 0.4 M PCA and heated for 45 min at 90°C. Then the samples were transferred quantitatively to scintillation vials with 10.5 ml of 3a70 scintillation fluid (Research Products Inc.). In some cases, cpm values were converted to dpm using a quench correction curve based on H numbers (according to the Beckman Instrument Manual).

RESULTS

Inhibition of [^{3}H]thymidine incorporation into DNA in nonsynchronous and G_1/S-synchronized HeLa S_3 cells treated with macromomycin-I. The effect of MCR-I on the incorporation of radioactive thymidine into DNA was measured in nonsynchronous HeLa S_3 cells treated with drug for 4 h. Under these conditions, MCR-I at concentrations of 1.0 μg/ml and higher appeared to block thymidine incorporation almost completely, with significant inhibition observed at an antibiotic level as low as 20 ng/ml (Fig. 1). Shorter incubation of cells with MCR-I resulted in a less pronounced effect, e.g., incubation of cells with MCR-I at 0.5 μg/ml for 1 h caused 62% inhibition of thymidine incorporation as compared with 86% inhibition after 4 h of incubation.

To determine whether the inhibition of thymidine incorporation by MCR-I is independent of the S cell cycle position, drug was given for 1 h to G_1/S-synchronized HeLa S_3 cells before, at, or after release of cells from the block. In all cases, the subsequent incorporation of [^{3}H]thymidine was inhibited to a similar extent (Fig. 2). The degree of this inhibitory effect was comparable with that found in nonsynchronized cells (data not shown). Figure 2 also indicates that thymidine incorporation remained suppressed even at 6 h after the end of drug treatment.

Inhibition of DNA synthesis in lysates derived from macromomycin-I-treated HeLa S_3 cells. Lysates from HeLa S_3 cells capable of incorporating deoxynucleoside triphosphates into DNA (16) were used to establish if the inhibition of thymidine incorporation by MCR-I reflects the drug's effect on DNA replication. HeLa S_3 cells synchronized at G_1/S were incubated for 4 h with various levels of MCR-I, then lysates were obtained and the rate of DNA synthesis was measured. The data shown in Fig. 3A clearly demonstrate that the treatment of cells with drug results in an impaired capability of lysates to incorporate [^{3}H]dTTP into DNA. The dependence of this effect on the drug concentration (Fig. 3B) closely follows the inhibition of thymidine incorporation in intact cells (Fig. 1). Another experiment showed that the inhibition of dTTP incorporation into DNA in lysates from MCR-I-treated cells depends on the time of drug treatment (Figs. 4A and B), similar to the inhibition of thymidine incorporation in MCR-I-treated nonsynchronous cells.

There was a possibility that the drug's action on cells synchronized by thymidine block may disturb the endogeneous dTTP pool and, therefore, the incorporation of labeled dTTP in lysates may not be a proper measure of DNA synthesis. This appeared to be unlikely, as we found that incorporation of either [^{3}H]dTTP or [^{3}H]-dGTP in lysates from drug-treated cells (0.5 μg/ml MCR-I for 4 h) is inhibited to a similar degree (85 and 79% of inhibition, respectively).

Inhibition of DNA synthesis in purified nuclei and reconstituted lysates from Macromomycin-I-treated HeLa S_3 cells. Cell lysates can be fractionated into nuclei and cytoplasmic fraction, the latter containing most of

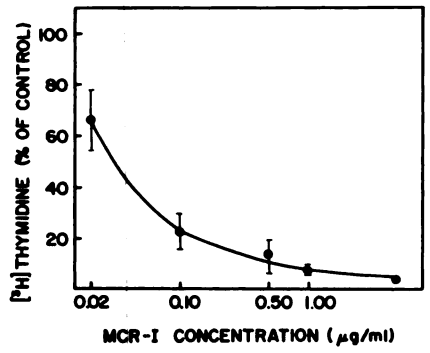

FIG. 1. *Effect of MCR-I on the incorporation of [^{3}H]thymidine into macromolecules in HeLa S_3 cells*

The cell suspensions were incubated for 4 h with the drug, and during the last hour, [^{3}H]thymidine (2 μCi/ml) was added. Triplicate samples (0.5 ml) were withdrawn and the PCA-insoluble radioactivity was determined as described in Materials and Methods. Incorporation of [^{3}H]thymidine into control samples was about 1.7×10^4 cpm. Points represent average values from two or three experiments. Bars indicate range.

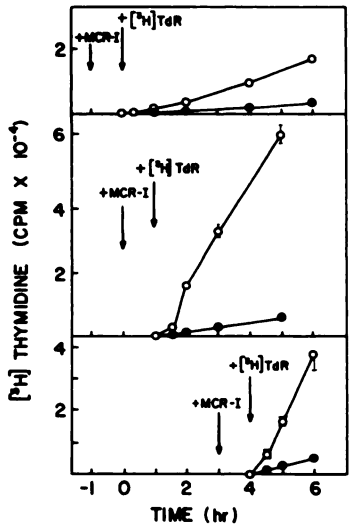

FIG. 2. *Effect of MCR-I on the incorporation of [^{3}H]thymidine into macromolecules in G_1/S-synchronized cells*

MCR-I (1 μg/ml) was given to the cells 1 h before release from the G_1/S block (upper panel), at release (middle panel), and 3 h after release from the block (lower panel). In each case, the cells were incubated with drug for 1 h, then spun down and resuspended in fresh medium with [^{3}H]thymidine. Control cultures underwent the same operations as drug-treated cells. At the indicated times, triplicate samples (0.8 ml) were withdrawn and cold PCA-insoluble radioactivity was determined as described in Materials and Methods. Control cells (○); MCR-I-treated cells (●). The standard deviation is indicated by bars except for the cases where it was smaller than the size of the symbols.

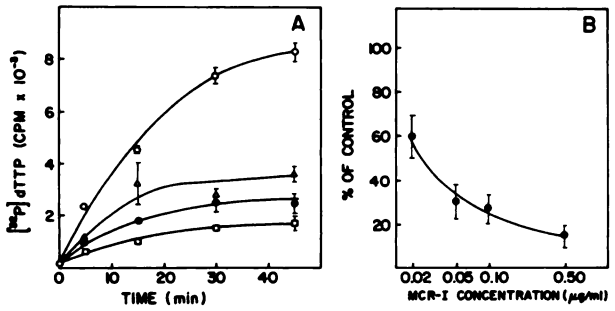

FIG. 3A. *Effect of MCR-I treatment of HeLa S_3 cells on the incorporation of dTTP into DNA in cell lysates*

HeLa S_3 cells synchronized at the G_1/S transition were given MCR-I at the release from the block. Four hours after release, cellular lysates were prepared and the incorporation of [^{32}P]dTTP into DNA was measured at the indicated times as described in Materials and Methods. Control (○); MCR-I at 0.05 μg/ml (△), 0.1 μg/ml (●), and 0.5 μg/ml (□). The reaction mixture volume was 75 μl. Points represent average values for triplicate samples. The standard deviation is indicated by bars except for the cases where it was smaller than the size of the symbols.

FIG. 3B. *Incorporation of radioactive dTTP into DNA in lysates from HeLa S_3 cells incubated with various concentrations of MCR-I, under conditions as in Fig. 3A*

The points are average values for duplicate samples from two or three experiments (± range) and represent the incorporation of ^{3}H- or ^{32}P-labeled dTTP, expressed as percentage of control, after 30-min incubation of lysates with radioactive precursor.

the DNA polymerase activity as well as other factors involved in replication (17). Purified nuclei are able to incorporate triphosphates into DNA, though at a lower rate than original lysates or lysates reconstituted from nuclei and cytoplasmic fraction (17). To determine if MCR-I inhibits DNA replication by interfering with the DNA template, as opposed to an effect on DNA polymerase(s) or other cytoplasmic factors, we assayed the incorporation of [^{3}H]dTTP into purified nuclei and reconstituted lysates, including "hybrid" lysates obtained by mixing components from control and drug-treated cells. Under conditions which resulted in DNA synthesis in a lysate from drug-treated cells suppressed to 18% of control (Table 1, 1A versus 1B), nuclei from the same lysate showed a rate of DNA synthesis that was 15% of control (Table 1, 2A versus 2B). The addition of a cytoplasmic fraction from control cells to nuclei produced an expected (five- to sixfold) stimulation of DNA synthesis, but it did not overcome the inhibition observed in nuclei from MCR-I-treated cells (Table 1, 3A, 3B versus 2B). On the other hand, a cytoplasmic fraction from drug-treated cells was still able to stimulate efficiently DNA synthesis in nuclei from control cells (3C). The extent of stimulation was somewhat lower when compared with that for the cytoplasmic fraction from control cells (3A). This difference may result from possible accumulation of unused triphosphates in drug-treated cells. This would lead to a small decrease in the specific radioactivity of [^{3}H]dTTP in the assays with the cytoplasmic fraction from these cells.

If MCR-I interfered with the DNA template, while not affecting the replication enzymes, the DNA polymerase(s) associated with nuclei from drug-treated cells would be expected to be able to utilize exogeneous DNA as a template. We found that the addition of salmon sperm DNA to nuclei from both control and drug-treated

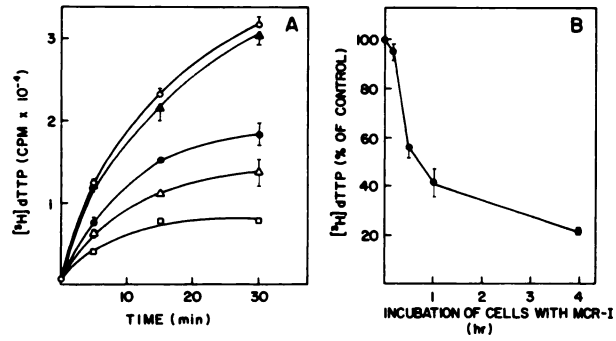

FIG. 4A. *Time dependence of MCR-I effects on the incorporation of dTTP into DNA in lysates from drug-treated HeLa S_3 cells*

The cells synchronized at the G_1/S transition were given MCR-I (0.5 μg/ml) at various times after release from the block: (□) 0 h; (△) 3 h; (●) 3 h, 30 min; (▲) 3 h, 50 min; (○) control. Four hours after release, cellular lysates were prepared and the incorporation of [^{3}H]dTTP into DNA was measured at the indicated times as described in Materials and Methods. The reaction mixture volume was 100 μl, and the [^{3}H]dTTP concentration was 15 μCi/ml. Points represent average values for triplicate samples. The standard deviation is indicated by bars except for the cases where it was smaller than the size of the symbols.

FIG. 4B. *Incorporation of [^{3}H]dTTP into DNA in lysates vs time of incubation of cells with MCR-I*

These data were derived from Fig. 4A for 30-min incubation of lysates with labeled dTTP.

TABLE 1

Incorporation of [^{3}H]dTTP into DNA in original lysates, purified nuclei, and reconstituted lysates from control and MCR-I-treated HeLa S_3 cells

The cells synchronized at G_1/S were incubated without and with 0.1 μg/ml MCR-I for 4 h after release from the block. Lysates, purified nuclei, and cytoplasmic fractions from these cells were obtained and assayed for [^{3}H]dTTP incorporation as described in Materials and Methods. Reaction mixture volumes were 100 μl and the [^{3}H]dTTP concentration was 6.5 μCi/ml in the case of original lysates (1A, 1B) and 13.8 μCi/ml in the remaining assays. Numbers represent average values for triplicate samples ± SD.

No.	System	pmol of [^{3}H]-dTMP incorporated/4.5 × 10^6 nuclei	Percentage of control
1A	Lysate from control cells	130.4 ± 0.9	100
1B	Lysate from MCR-I-treated cells	22.8 ± 1.5	18
2A	Nuclei from control cells	20.1 ± 1.1	100
2B	Nuclei from MCR-I-treated cells	2.9 ± 0.6	15
3A	Nuclei from control cells plus cytoplasmic fraction from control cells[a]	109.3 ± 3.0	100
3B	Nuclei from MCR-I-treated cells plus cytoplasmic fraction from control cells	17.5 ± 1.9	16
3C	Nuclei from control cells plus cytoplasmic fraction from MCR-I-treated cells	76.6 ± 4.1	73

[a] Incorporation of radioactive label into the cytoplasmic fraction alone (without nuclei) did not exceed background level (<0.25 pmol of [^{3}H]dTTP).

TABLE 2

Effects of exogeneous DNA on incorporation of [^{3}H]dTTP in nuclei from control and MCR-I-treated HeLa S_3 cells

The cells were incubated with the drug and nuclei prepared as stated in Table 1, except that a less active MCR-I preparation was used at 1 μg/ml. Nuclei were suspended at 20 × 10^6/ml in buffer H without or with the indicated amount of salmon sperm DNA (Sigma Type III). Incubation of nuclei with [^{3}H]dTTP (0.94 μCi/nmol) was carried out for 20 min as described in Materials and Methods. The results are expressed as pmol of [^{3}H]dTTP incorporated per 5 × 10^6 nuclei and as % of control. The numbers represent average values for triplicate samples ± SD.

DNA concentration	Nuclei from control cells		Nuclei from drug-treated cells	
	pmol	% of control	pmol	% of control
μg/ml				
0	12.4 ± 0.9	100	3.1 ± 0.8	25
5	16.7 ± 1.5	100	9.4 ± 1.0	56
100	32.4 ± 3.3	100	26.3 ± 2.8	81
800	155.5 ± 6.4	100	139.7 ± 12.5	90
1200	172.8 ± 16.6	100	190.4 ± 12.3	110

cells resulted in a large stimulation of [^{3}H]dTTP incorporation (Table 2). The inhibition of triphosphate incorporation observed in nuclei from drug-treated cells in the absence of exogeneous template decreased with increasing concentrations of salmon sperm DNA and was completely reversed at 1200 μg/ml DNA (Table 2).

As in the case of the complete lysates, incorporations of [^{3}H]dTTP and [^{3}H]dGTP into purified nuclei were inhibited to a similar degree (data not shown).

The effect of macromomycin-I on repair synthesis in HeLa S_3 cells. The ability of MCR to damage cellular DNA suggested that, besides inhibition of DNA replication, the drug may stimulate DNA repair synthesis as is the case with neocarzinostatin (18). To determine if MCR-I has any effect on repair synthesis, we studied the incorporation of [^{3}H]thymidine into cells incubated with drug in the presence of 10 mM hydroxyurea to reduce replication synthesis. As shown in Fig. 5, at drug concentrations which inhibit DNA replication, repair synthesis is substantially enhanced, with the maximal effect (four-fold stimulation) observed at a drug concentration of 1.0 μg/ml.

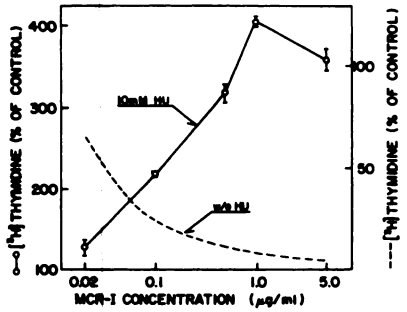

FIG. 5. *Effect of MCR-I on incorporation of [^{3}H]thymidine into HeLa S_3 cells in the presence of 10 mM hydroxyurea*

The cells were incubated for 4 h at 37°C with and without MCR-I in medium containing 10 mM hydroxyurea. One hour before the end of incubation, [^{3}H]thymidine was added (10 μCi/ml). Triplicate samples (0.8 ml) were withdrawn and cold PCA-insoluble radioactivity was determined as described in Materials and Methods. The incorporation at 0 min after the addition of [^{3}H]thymidine was 51 ± 17 cpm and did not depend on the presence or absence of MCR-I in cell suspensions. The radioactivity of control samples after 60 min of incubation with [^{3}H]thymidine was 312 ± 7 cpm. Bars indicate standard deviations. The broken line is shown for comparison of the effect of MCR-I on the incorporation of [^{3}H]thymidine in HeLa cells in the absence of hydroxyurea (data from Fig. 1).

DISCUSSION

Though the results reported in this paper were found with a drug preparation containing predominately MCR-I, we have obtained qualitatively similar data in lysate and nuclei systems using MCR isolated according to Yamashita *et al.* (1). The latter preparation, however, was less active. We found also that selected fractions of different MCR-I contents (>90% and ~50%) obtained by our isolation procedure inhibited thymidine incorporation to a similar degree when the drug concentration was expressed with regard to MCR-I rather than total protein (data not shown). Thus, the chromophore-containing protein is distinctly the more active form of the drug. It seems likely that varying amounts of MCR-I were a major factor responsible for the biological and biochemical activities of MCR purified by previously published procedures. (It should be noted that we obtained MCR-I from the same crude material that was used by others to isolate MCR.)

MCR-I is a potent inhibitor of thymidine incorporation into DNA in HeLa cells and can inhibit this process at concentrations as low as 20 ng/ml. A similar degree of inhibition of thymidine incorporation has been observed previously in the case of auromomycin (11) and macromomycin isolated by Sawyer *et al.* (6). Other preparations of MCR were considerably less active (5, 8, 10).

Our experiments with G_1/S-synchronized cells indicated that the inhibition of thymidine incorporation by MCR-I is independent of the cell cycle position (within the G_1 through S phases) during drug treatment. The extent of inhibition was similar to that of the inhibition observed in nonsynchronous cells. In addition, the drug effect seems to be irreversible, as there is no recovery of thymidine incorporation at 6 h following drug treatment.

Our results are also consistent with the finding that MCR-I inhibits [^{3}H]dTTP incorporation in lysates from drug-treated synchronized (G_1/S) cells. The effect of MCR-I on DNA synthesis in lysates from drug-treated cells closely resembles the inhibition of thymidine incorporation in intact (nonsynchronous) cells with respect to dose and time dependence. Hence, these data indicate that the decreased incorporation of thymidine in intact cells reflects the inhibition of cellular DNA synthesis by MCR-I and is not an artifact arising from altered transport or phosphorylation of thymidine. Moreover, the drug's effect on DNA synthesis is due to an impaired incorporation of triphosphates into DNA rather than an inhibition of the synthesis of DNA precursors.

The question of whether MCR-I inhibits DNA replication by acting on the DNA template, as expected from its ability to damage cellular DNA, was examined by experiments with purified nuclei and reconstituted lysates from control and drug-treated cells. If the drug interfered with the DNA template while not affecting

DNA polymerases and other factors involved in replication, one would expect the following: (a) DNA synthesis in purified nuclei from drug-treated cells would be inhibited to a similar degree as observed in whole lysates; (b) the addition of the cytoplasmic fraction from control cells to nuclei from drug-treated cells would not overcome this inhibition; (c) the cytoplasmic fraction from drug-treated cells would stimulate DNA synthesis in nuclei from control cells to a degree comparable with the effect of the cytoplasmic fraction from control cells; and (d) the inhibition of DNA synthesis in nuclei from drug-treated cells would be reversed by the addition of an exogenous template. All of these conditions were met. Thus, the experiments clearly demonstrate that the DNA template is a target in the action of MCR-I on DNA replication.

Suzuki *et al.* (8) reported that treatment of isolated nuclei by MCR results in a stimulation of DNA synthesis. These data suggest differences between isolated nuclei treated with drug and nuclei from drug-treated cells. Direct comparison of both systems is difficult, however, as these procedures require considerably different conditions of incubation of cells or nuclei with drug. We found that the effect of MCR-I on DNA synthesis in nuclei from drug-treated cells is strongly decreased by such factors as increased cell concentrations or the presence of sulfhydryl compounds during incubation with drug (Woynarowski and Beerman, unpublished data). On the other hand, increased incorporation of triphosphates in MCR-treated nuclei may reflect repair-type DNA synthesis that arises from excessive cutting of DNA, thereby generating additional initiation sites. Our experiments indicated that MCR-I stimulates DNA repair synthesis in intact HeLa S_3 cells, as measured by the incorporation of radioactive thymidine into DNA in the presence of hydroxyurea. The stimulation is first seen at a drug level (20 ng/ml) which begins to inhibit replication synthesis and also has been shown to cleave cellular DNA (7).

The hitherto discussed effects of MCR-I on DNA replication and repair seem to arise from the drug's damage to DNA and are comparable to data obtained for neocarzinostatin. The latter antibiotic was shown to inhibit DNA synthesis in both cellular and cell-free systems (15, 19) as a result of breaks caused in the DNA template (15). Neocarzinostatin was also reported to stimulate repair synthesis in intact cells (18).

Currently, studies are being done to establish the effects of MCR-I on DNA template activity with respect to replication and repair synthesis using an *in vitro* system with purified DNA polymerases α, β, and γ. Further studies are planned with MCR-I to explore relationships between DNA damage and DNA synthesis and repair in cellular systems.

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