MECHANISM OF ACTION OF MACROMOMYCIN: DNA STRAND SCISSION, INHIBITION OF DNA SYNTHESIS AND MITOSIS

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The effects of macromomycin (MCR), a high molecular weight peptide antibiotic, on cell division, DNA synthesis and DNA fragmentation were examined in cultured mammalian tumor cells. When MCR was added to HeLa cell culture simultaneously with [^{3}H]thymidine, inhibition of DNA synthesis was observed depending on the amount of the drug present, although the inhibition was partial even at a high concentration of the drug. Preincubation of cells with MCR for 2 hours before assay was required for the complete inhibition of DNA synthesis. Cell division of synchronized L5178Y cells, arrested at metaphase, was strongly inhibited by MCR, indicating that the inhibition of cell mitosis by the drug was not dependent on the inhibition of DNA synthesis.

Strand scission of DNA in MCR-treated cells was observed by alkaline sucrose gradient centrifugation. The fragmentation of cellular DNA occurred at low concentration of the drug and within a very short incubation time (37°C, 5 minutes). At high concentrations of the drug, however, the size of the fragmented DNA remained constant. DNA polymerase activity in isolated nuclei from HeLa and L5178Y cells was stimulated by MCR. These data suggest that MCR works directly on cell nuclei and strand scission of DNA is one of the more important actions of the drug.

An antitumor antibiotic, macromomycin (MCR) is an acidic polypeptide isolated from the culture filtrate of *Streptomyces macromomyceticus*.[1] Its molecular weight is about 12,000 and it does not contain arginine or methionine.[2] It inhibits the growth of some experimental tumors[1,3] and Gram-positive bacteria.[1,4]

It has been demonstrated that MCR binds to the membrane of tumor cells and inhibits DNA synthesis preferentially.[5,6] The cytotoxicity can be abolished by the treatment of cells with trypsin after exposure of the cells to MCR.[6,7,8] The mechanism of action of MCR on mammalian cells is still unknown.

We found that the inhibition of cell mitosis by MCR was not a consequence of the inhibition of DNA synthesis and low concentrations of the drug caused DNA strand scission in intact cultured cells during a short incubation period. This paper describes the action of MCR on growing HeLa and L5178Y cells with special reference to DNA synthesis, cell mitosis and DNA strand breaks.

Materials and Methods

[^{3}H]-Thymidine (55.2 Ci/mmole) and [^{3}H]-dGTP (12.2 Ci/mmole) were purchased from New England Nuclear, Boston, Mass., deoxyribonucleoside triphosphates from Boehringer Mannheim, Germany, colcemid from Wako Pure Chemical Industries, Osaka, Japan. MCR (lot 11-2) was provided by Kanegafuchi Chemical Industry Co., Hyogo, Japan and kept at −80°C. MCR was dissolved in phosphate-buffered saline (PBS) each time just prior to use.

Cultivation of Cells:

HeLa S3 cells were cultured in monolayers in EAGLE's minimum essential medium supplemented

with 10% calf serum (doubling time about 24 hours). Mouse lymphoblastoma L5178Y cells were cultured in FISHER's medium with 10% horse serum in suspension (doubling time about 10 hours). Cell numbers of HeLa cells were determined in a hemocytometer by counting cell nuclei stained with crystal violet (0.05%) in 0.1 M citric acid solution. L5178Y cells were counted by Coulter counter.

Synchronous Culture of Cells:

(1) Randomly growing HeLa cells in short test tubes were treated with 2 mM thymidine for 24 hours, washed with PBS and cultured in fresh medium for 10 hours, followed by incubation in a medium containing 1 mM hydroxyurea for 16 hours. Finally, the cells were washed with PBS and cultured in fresh medium. DNA synthesis started immediately and continued for about 10 hours.

(2) Logarithmically growing L5178Y cells in a 100-ml medium bottle were treated with 0.025 μg/ml of colcemid for 7 hours. The cells were washed with warm PBS twice and suspended in warm fresh medium. The cultures in short test tubes with rubber-stopper were incubated immediately in a water-bath at 37°C. The cell number remained constant for 30 minutes and then began to increase rapidly for 2 hours.

Measurement of DNA Synthesis in HeLa Cells:

HeLa S3 cells grown in short test tubes in 2 ml medium synchronously or asynchronously were incubated with 1 μCi/ml of [^{3}H]-thymidine at 37°C for 60 minutes with or without MCR. After incubation, cells were washed with cold PBS twice. The cells were further incubated with 2 ml of 0.02% EDTA solution in PBS. An equal volume of cold 10% trichloroacetic acid (TCA) was added to the cell suspension and the TCA-insoluble materials were collected on glass fiber filters (Whatman GF83). The radioactivity was measured in a liquid scintillation counter.

DNA Polymerase Activity in Isolated Nuclei from HeLa and L5178Y Cells:

Nuclei which should have high DNA polymerase activity were collected from synchronized HeLa cells in the S phase. HeLa cells incubated with a 2 mM thymidine-containing medium overnight were cultured in a thymidine-free medium for 3 hours, at which time DNA synthesis was almost maximum, and harvested for isolation of nuclei. The nuclei were prepared by the procedure of MURAMATSU *et al.*[9] The cells (total 7×10^7) were kept in an ice-bath for 10 minutes in 5 ml of reticulocyte standard buffer (RSB) which contained 10 mM Tris-HCl, pH 7.4, 10 mM NaCl and 1.5 mM $MgCl_2$, centrifuged at 2,000 rpm for 5 minutes and suspended in 5 ml of RSB containing 0.3% of Nonidet P40 (Shell Chemical Co.). The mixture was homogenized in a tightly fitted glass homogenizer with a Teflon pestle giving 10 strokes. The homogenate was centrifuged at 2,500 rpm for 5 minutes. The pellet was suspended in 2 ml of 0.25 M sucrose - 3.3 mM $CaCl_2$, layered over an equal volume of 0.88 M sucrose, and centrifuged at 2,500 rpm for 10 minutes. The pellet was suspended as purified nuclei in 1.0 ml of glycerol-Tris buffer (50 mM Tris-HCl, pH 7.4, 7.5 mM $Mg(OAc)_2$, 5 mM DTT, 0.1 mM EDTA and 25% glycerol) and used immediately for the DNA polymerase reaction.

In the case of nuclei from L5178Y cells, nuclei were collected from the randomly growing culture because enough cells for determining DNA polymerase activity were easily obtained.

The assay was carried out at 37°C in a total volume of 100 μl which contained 50 mM Tris-HCl, pH 7.5, 9.5 mM $MgCl_2$, 5 mM ATP, 0.15 mM dATP, dCTP, TTP, 0.5 μCi of [^{3}H]-dGTP and 50 μl of nuclei suspension. The reaction was stopped by the addition of 10% cold TCA-1% sodium pyrophosphate and the cold TCA-insoluble radioactivity was measured as described above.

Alkaline Sucrose Gradient Analysis of Strand Scission of DNA in HeLa Cells:

Randomly growing HeLa cells were labeled overnight with [^{3}H]-thymidine (0.2 μCi/ml), washed with PBS and suspended in a fresh medium at the concentration of 25,000 cells/ml. MCR was added to 2 ml cell suspension and incubated for indicated periods at 37°C except otherwise indicated. The cells were washed with PBS, concentrated in *ca.* 0.2 ml cell suspension by centrifugation and placed on the top of 5~20% alkaline sucrose gradient (4.6 ml) on which 0.2 ml of the lysis solution (0.5 M NaOH, 0.02 M EDTA and 0.1% Triton X100) had been layered. The gradient contained 0.3 M NaOH, 0.7 M NaCl and 0.01 M EDTA. After kept at room temperature for 30 minutes for lysis, centrifugation was performed in a Beckman SW50L rotor at 30,000 rpm for 90 minutes at 20°C. Samples were fractionated

in 0.2 ml from the top of the gradient with an ISCO fractionator, Model 640. Under these centrifuge conditions, DNA of the control cells sedimented at the bottom of the tube and was suspended in water as the pellet fraction. Each fraction was precipitated, after the addition of 100 μg of bovine serum albumin as carrier, by cold 5% TCA and the radioactivity was measured as described above.

Results

Effect of MCR on the Growth of Cultured Mammalian Cells

MCR inhibited the growth of HeLa S3 cells completely at concentration higher than 0.74 μg/ml and the concentration for 50% growth inhibition was 0.11 μg/ml (Fig. 1). MCR also inhibited the growth of L5178Y cells completely at 0.11 μg/ml (data not shown). When L5178Y cells were incubated with MCR (0.11 μg/ml) for 5 minutes at 37°C, the growth of the cells did not recover by simple washing or even trypsin treatment (Fig. 2).

Fig. 1. Effect of MCR on the growth of HeLa S3 cells.

MCR was added at the indicated final concentrations to each culture tube (2 ml/tube)at 0 day (24 hours after the inoculation). The cell numbers were determined at every other day by counting stained nuclei and average number of three tubes was shown in the figure.

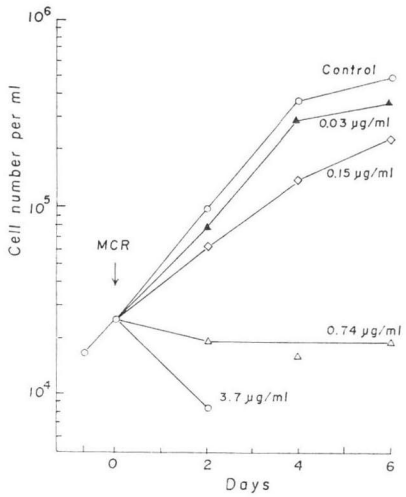
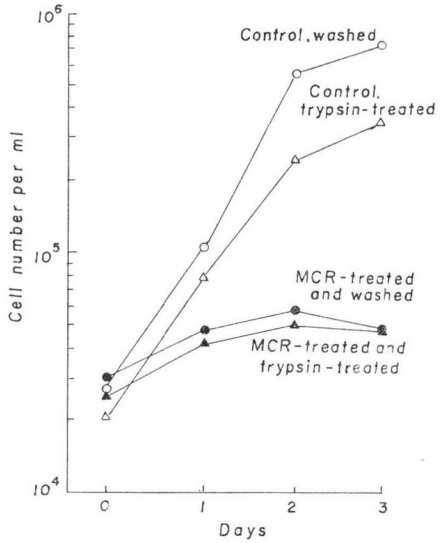


Fig. 2. Effect of washing or trypsin treatment on the growth of MCR-treated L5178Y cells.

L5178Y cell suspension in 40 ml centrifuge tubes (*ca.* 20,000 cells/ml) were incubated with MCR (0.11 μg/ml) or PBS (control) at 37°C for 5 minutes and immediately chilled in ice-water. After 3 washings with cold PBS, the cells were suspended in fresh medium (washing effect). Another MCR-treated and control cells were suspended in PBS containing 0.08% trypsin (GIBCO) and incubated at 37°C for 5 minutes. The cells were removed from trypsin-containing PBS by centrifugation and suspended in fresh medium (trypsin effect). Cell suspension (2 ml) was inoculated in short test tubes and incubated at 37°C. Inoculum cell number was not identical to each group because of the loss of cells by repeated centrifugations.



Inhibition of DNA Synthesis in HeLa Cells

MCR specifically inhibited DNA synthesis in cultured mammalian cells but the inhibition was not complete even at high concentrations.[6] So, the effect of a preincubation period with MCR on DNA synthesis was examined in HeLa cells. Without preincubation, the inhibition of DNA synthesis was 23% at 0.74 μg/ml and only 59% at 92.6 μg/ml. After a one-hour preincubation, however, the

Fig. 3. Effect of preincubation on inhibition of DNA synthesis in HeLa cells by MCR.

HeLa S3 cells in short test tubes (46,000 cells/ml) were preincubated with MCR at various concentrations at 37°C for indicated periods, then incorporation of [^{3}H]-thymidine into acid-insoluble fraction was determined. Percent of inhibition was shown in the figure. Incorporation of control cells was 1445, 1422, 1483 cpm/tube at 0, 1, 2 hour preincubation, respectively.

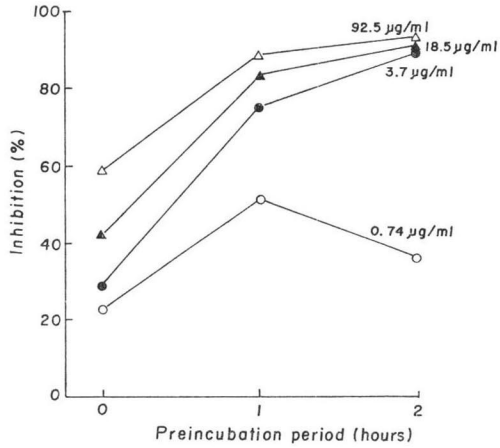
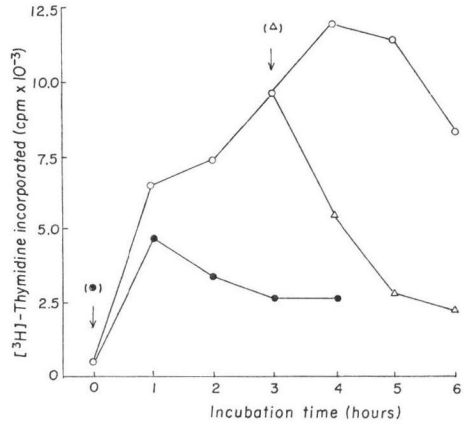


Fig. 4. Effect of MCR on DNA synthesis in synchronized HeLa S3 cells.

MCR (3.7 μg/ml) was added at 0 or 3 hours after the start of DNA synthesis in synchronized HeLa cells (20,000 cells/ml) and incorporation of [^{3}H]-thymidine into DNA was determined at every one hour as described in the text.



inhibition was greatly increased at each concentration (50% inhibition, 0.69 μg/ml) and after 2-hour preincubation, the degree of inhibition reached about 90% at concentrations 3.7 μg/ml or higher (Fig. 3).

Inhibition of DNA synthesis by MCR was examined in synchronous culture of HeLa cells. Those cells treated with excess thymidine and hydroxyurea started DNA synthesis immediately after changing the medium and continued it for about 10 hours. The cell number remained constant for this period and then increased (data not shown). The activity of DNA synthesis at each period was measured by incorporation of [^{3}H]-thymidine into the cold TCA-insoluble fraction after 60-minute incubation. When MCR at 3.7 μg/ml was added at 0 hour, inhibition of DNA synthesis was 27% in the first hour and 52% in the next hour. When MCR was added 3 hours after the onset of DNA synthesis, the inhibition was 53% and 75% in the first and second hour after the addition of MCR, respectively (Fig. 4). These results suggest that MCR may inhibit chain elongation but not initiation of DNA synthesis.

Effect of MCR on Cell Division of Synchronized L5178Y Cells

To examine whether the inhibition of cell division by MCR resulted from the inhibition of DNA synthesis, another synchronous culture was employed. L5178Y cells arrested at metaphase by colcemid treatment began cell division after a 0.5-hour lag in a fresh medium and the cell number increased rapidly for 2 hours. DNA synthesis began 3 hours after the removal of colcemid (data not shown). When MCR (1 μg/ml) was added at 0 hour in a fresh medium, cell division was strongly inhibited, suggesting that the inhibition of cell division by MCR was not a consequence of the inhibition of DNA synthesis. However, when MCR was added after 30 minutes or 1 hour, the inhibition of cell division was not as strong as when added at 0 hour. (Fig. 5). These results suggested that a certain period might be necessary for MCR to cause inhibition of cell division or that cells at metaphase are sensitive

Fig. 5. Effect of MCR on the division of synchronized L5178Y cells.

MCR (1 μg/ml) was added to the culture at 0, 0.5 and 1 hour after the removal of colcemid and the cell number at each period was determined by Coulter counter.

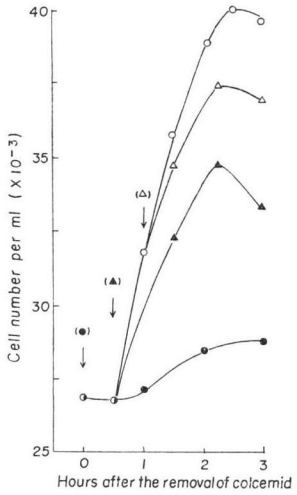
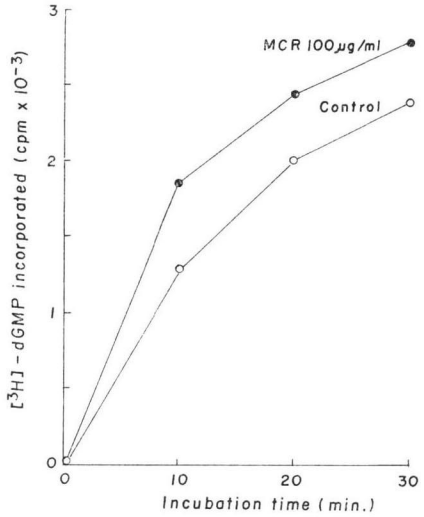


Fig. 6. DNA polymerase assay in isolated nuclei from synchronized HeLa S3 cells.

Procedure was described in Materials and Methods.



to MCR, but the cells in the later stage of metaphase may not be sensitive.

DNA Polymerase Activity in Isolated Nuclei

MCR showed a stimulative effect on DNA polymerase activity in isolated nuclei of synchronized HeLa cells at each time examined, when nuclei (3.6×10^6 nuclei/ml) were incubated with 100 μg/ml of MCR at 37°C (141% at 10 minutes, 121% at 20 minutes, 117% at 30 minutes) as shown in Fig. 6. In another experiment using isolated nuclei of L5178Y cells, MCR also showed a stimulative effect on DNA polymerase activity with 20-minute incubation, whereas adriamycin inhibited it strongly (Table 1).

Table 1. Effect of MCR on DNA polymerase assay in isolated nuclei of L5178Y cells.

	[³H]-dGMP incorporated
Control	1,035 (100)
MCR 100 μg/ml	1,399 (135)
MCR 50 μg/ml	1,225 (118)
ADM 20 μg/ml	393 (38)

The DNA polymerase assay was carried out at 37°C for 20 minutes incubation with or without antibiotics. Nuclei were prepared from randomly growing L5178Y cells and 2×10^7 nuclei/ml were used. The number represents cpm/tube, and that in the parentheses relative incorporation.

ADM: adriamycin

DNA Profiles of MCR-Treated HeLa Cells in Alkaline Sucrose Gradients

The single strand scission of cellular DNA of MCR-treated cells was clearly observed by alkaline sucrose density gradient centrifugation. In the centrifuge conditions used here, the DNA of control cells was pelleted to the bottom of the tube because of its very large size and amounts of fragmented DNA of drug-treated cells appeared in the gradients depending on the drug concentrations. Even at a low concentration of 0.03 μg/ml, which was lower than the concentration for 50% growth inhibition (0.11 μg/ml), scission of DNA was observed with incubation at 37°C for 30 minutes and at 0.15 μg/ml the increased amounts of fragmented DNA appeared around the same position (fractions 18~23). At a concentration of 0.75 μg/ml, the peak of fragmented DNA shifted to fraction 18 and at 3.7 μg/ml,

Fig. 7. DNA strand scission in HeLa cells treated with MCR as a function of drug concentrations.

The cells (25,000 cells/ml) prelabeled with [^{3}H]-thymidine were incubated with MCR at the concentrations indicated for 30 minutes at 37°C and DNA profiles were analyzed on the alkaline sucrose gradient as described in the text. Sedimentation was carried out from the left to the right.

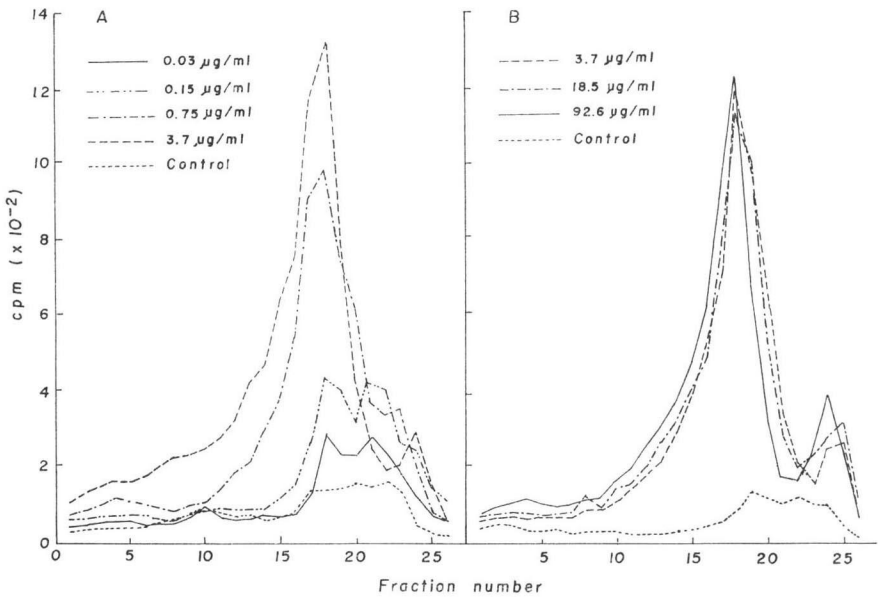


Fig. 8. Sedimentation profiles of DNA of MCR-treated HeLa cells at different periods of incubation.

The cells were incubated at 37°C for indicated period with MCR (3.7 μg/ml).

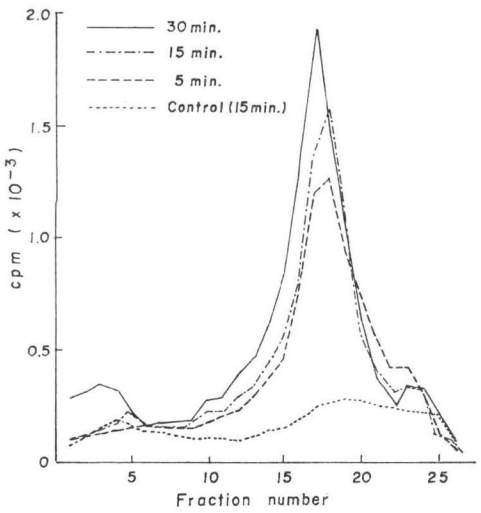
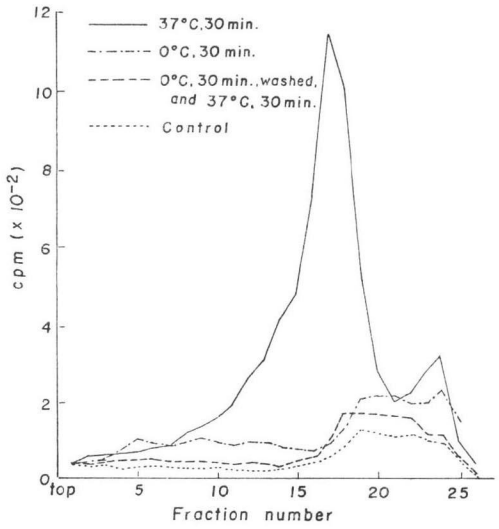


Fig. 9. Effect of temperature on DNA strand scission by MCR in HeLa cells.

The cells were incubated with MCR (18.5 μg/ml) for 30 minutes at 37°C or 0°C (in ice-water). One half of the cells which were incubated at 0°C with MCR were washed twice with cold PBS and incubated for another 30 minutes at 37°C in fresh medium. Control cells were incubated for 30 minutes at 37°C.



the peak at fraction 18 was much higher with a concomitant increase in smaller fractions (Fig. 7A). However, at drug concentrations of 3.7~92.6 μg/ml, there was no change in DNA profiles (Fig. 7B). Next, the cells were incubated with the drug for a much shorter incubation time and the sedimentation profile of DNA was examined. After a 5-minute incubation of the cells with MCR at 3.7 μg/ml at 37°C, strand scission was clearly observed and the amounts of fragmented DNA increased up to 30 minutes (Fig. 8). Longer incubation of 45 minutes gave a similar profile to that of 30 minutes (data not shown). These results suggest that the strand scission of DNA occurred rapidly after MCR treatment, probably within 5 minutes.

The effect of incubation temperature on strand scission was examined (Fig. 9). At 0°C for 30 minutes MCR (18.5 μg/ml) gave very slight fragmentation of DNA, whereas at 37°C it showed great degradation, indicating that strand scission of DNA by MCR was highly dependent on incubation temperature. When the cells were treated with MCR at 0°C for 30 minutes and incubated in fresh medium at 37°C for more 30 minutes, no more strand scission was observed. These results suggest that MCR does not bind effectively to cells at 0°C. Strand scission of DNA by MCR was also seen in intact L5178Y cells and the size of fragmented DNA was again limited (data not presented).

Discussion

KUNIMOTO *et al.* demonstrated that [^{131}I]-labeled MCR bound to the mammalian cell surface.[5,6] Moreover, the inhibition of the cell growth of cultured YOSHIDA sarcoma was recovered by a brief treatment with trypsin.[6] Similar results were also reported using cultured mouse mammary adenocarcinoma TA3Ha cells[7] and B16 melanoma cells.[8] MCR inhibited DNA synthesis in cultured cells but did not affect the DNA polymerase activity in an isolated nuclei system.[6] All these results suggest that the interaction of MCR to the cell surface causes inhibition of DNA synthesis and cell mitosis.

In our studies, DNA synthesis inhibition was partial at high concentrations of MCR as KUNIMOTO *et al.* reported,[6] and the degree of inhibition increased with increase of incubation time (Fig. 3). MCR was also found to inhibit cell division in the absence of DNA synthesis inhibition using a synchronized culture of L5178Y cells (Fig. 5). This suggests that the inhibition of cell mitosis is not a consequence of inhibition of DNA synthesis and it results from another mechanism.

We found that MCR caused strand scission in cellular DNA of intact cultured cells. It appeared at a lower concentration than that required for 50% growth inhibition (Fig. 7A) and occurred very rapidly after the exposure of the cells to MCR (Fig. 8). Thus, strand scission of DNA might be the most important action of MCR and might result in inhibition of cell mitosis and/or DNA synthesis. The reason why the size of the fragmented DNA remained constant at higher concentrations of MCR is unclear. Its size was more than 100 S as determined by T7 phage DNA as a sedimentation marker.

MCR stimulated DNA polymerase activity in an isolated nuclei system. In this case, a high concentration of MCR (50 and 100 μg/ml) was used. It should be noted, however, that the concentration of nuclei was about one hundred times higher than the cell number in the case of assays of DNA synthesis, the cell growth or DNA strand-breaks and that the ratios of the concentrations of the drug and cells or nuclei were comparable. We reported that the antitumor antibiotic bleomycin bound to DNA molecules directly and caused DNA strand scission *in vitro* and *in vivo*,[10,11] and that it stimulated DNA polymerase activity in an early stage in an isolated *E. coli* enzyme system, probably due to the increase of primer sites in DNA or repair activity.[12] We attempted to see whether the stimulation of DNA polymerase activity in isolated nuclei by MCR was due to strand scission which occurred in the DNA of nuclei. However, DNA in nuclei used as the control for the DNA polymerase assay fragmented to very small fractions and additional strand scission was not observed in MCR-treated nuclei.

Neocarzinostatin is also a tumor-inhibitory, high molecular peptide antibiotic. MCR is differentiat-

ed from neocarzinostatin by the composition of amino acids, isoelectric point, antigenecity, *etc.*[2,13] EBINA *et al.* reported that neocarzinostatin blocked S phase, especially initiation of DNA synthesis, and G2 phase[14] and it also inhibited microtubular paracrystal formation induced by vinblastin in HeLa cells.[15] They speculated that neocarzinostatin affects microfilaments by binding to receptors on the cell surface resulting in the prevention of the assembly of microtubules, cell mitosis and initiation of DNA synthesis. We examined the effect of MCR on paracrystal formation in the same system as they used. MCR at a concentration as high as 50 μg/ml inhibited paracrystal formation by 80% when cells were preincubated with the drug for 16 hours. When preincubation was 4 hours, however, no inhibition was observed (data not presented). We concluded that the inhibition by MCR of paracrystal formation was not the primary action but rather a secondary one.

BEERMAN and GOLDBERG reported that neocarzinostatin caused strand-breaks in intact HeLa cells, in isolated nuclei[16] and in isolated DNA from SV40[17] or PM2 phage.[18] They have proposed that DNA itself is the target of neocarzinostatin. The results of strand scission caused by MCR in intact HeLa cells were quite similar to those reported by BEERMAN and GOLDBERG. We are now studying the interaction of MCR to isolated DNA.

Contrary to papers by KUNIMOTO *et al.*,[6] LIPPMAN[7] and WINKELHAKE and BUCKMIRE,[8] we did not obtain evidence that MCR would bind to the cell surface. The reason for the discrepancy is not clear. However, this might be due to the difference of MCR samples used and/or the difference of experimental conditions including the difference of cells. In our study, it was quite difficult to make cells recover their growth by treatment with trypsin after exposure of MCR because MCR caused strand scission in cellular DNA rapidly. IM *et al.* reported that the cytotoxicity of MCR to P388 leukemia cells could not be reversed by treating the cells with trypsin.[19]

Recently, it has been revealed that the MCR-producing strain also produces different but similar high-molecular peptide antibiotics named auromomycin and neomacromycin (personal communication by Dr. K. WATANABE). These are similar in biological activity to MCR but different in several aspects. We will describe the modes of action of these antibiotics elsewhere.

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References

1) CHIMURA, H.; M. ISHIZUKA, S. HORI, K. KIMURA, J. IWANAGA, T. TAKEUCHI & H. UMEZAWA: A new antibiotic, macromomycin, exhibiting antitumor activity and antimicrobial activity. J. Antibiotics 21: 44~49, 1968
2) YAMASHITA, T.; N. NAOI, K. WATANABE, T. TAKEUCHI & H. UMEZAWA: Further purification and characterization of macromomycin. J. Antibiotics 29: 415~423, 1976
3) LIPPMAN, M. M.; W. R. LASTER, B. J. ABBOTT, J. BENDITTI & M. BARATTA: Antitumor activity of macromomycin B (NSC-170105) against murine leukemias, melanoma and lung carcinoma. Cancer Res. 35: 939~945, 1975
4) BUCKMIRE, F. L. A. & J. L. WINKELHAKE: Bacterial activity of macromomycin, an antitumor antibiotic. Antimicr. Agents & Chemoth. 11: 1010~1016, 1977
5) KUNIMOTO, T.; M. HORI & H. UMEZAWA: Reversible binding of macromomycin, a macromolecular peptide antibiotic, to cell membranes. J. Antibiotics 24: 203~205, 1971
6) KUNIMOTO, T.; M. HORI & H. UMEZAWA: Macromomycin, an inhibitor of membrane function of tumor cells. Cancer Res. 32: 1251~1256, 1972
7) LIPPMAN, M. M.: *In vitro* studies on macromomycin (NSC-170105), effects on cultured TA3Ha and L1210 cells. Cancer Chemoth. Rept. 58: 181~187, 1974
8) WINKELHAKE, J. L. & F. L. A. BUCKMIRE: Radioimmune assay and characteristics of antibodies to

macromomycin (NSC 170105). Cancer Res. 37: 1197~1204, 1977

9) MURAMATSU, M.; Y. HAYASHI, T. ONISHI, M. SAKAI, K. TAKAI & T. KASHIYAMA: Rapid isolation of nucleoli from detergent purified nuclei of various tumor and tissue culture cells. Exptl. Cell Res. 88: 345~351, 1974
10) SUZUKI, H.; K. NAGAI, H. YAMAKI, N. TANAKA & H. UMEZAWA: On the mechanism of action of bleomycin. Scission of DNA strands *in vitro* and *in vivo*. J. Antibiotics 22: 446~448, 1969
11) SUZUKI, H.; K. NAGAI, E. AKUTSU, H. YAMAKI, N. TANAKA & H. UMEZAWA: On the mechanism of action of bleomycin: Strand scission of DNA caused by bleomycin and its binding to DNA *in vitro*. J. Antibiotics 23: 473~480, 1970
12) YAMAKI, H.; H. SUZUKI, K. NAGAI, N. TANAKA & H. UMEZAWA: Effects of bleomycin A2 on deoxyribonuclease, DNA polymerase and ligase reactions. J. Antibiotics 24: 178~184, 1971
13) SAMY, T. S. A. & V. RASO: Radioimmunoassay of neocarzinostatin, an antitumor protein. Cancer Res. 36: 4378~4381, 1976
14) EBINA, T.; K. OHTSUKI, M. SETO & N. ISHIDA: Specific G2 block in HeLa S3 cells by neocarzinostatin. Europ. J. Cancer 11: 155~158, 1975
15) EBINA, T. & N. ISHIDA: Inhibition of formation of microtubular paracrystals in HeLa-S3 cells by neocarzinostatin. Cancer Res. 35: 3705~3709, 1975
16) BEERMAN, T. A. & I. H. GOLDBERG: The relationship between DNA strand-scission and DNA synthesis inhibition in HeLa cells treated with neocarzinostatin. Biochim. Biophys. Acta 475: 281~293, 1977
17) BEERMAN, T. A. & I. H. GOLDBERG: DNA strand scission by antitumor protein neocarzinostatin. Biochem. Biophys. Res. Commun. 59: 1254~1261, 1974
18) BEERMAN, T. A.; R. POON & I. H. GOLDBERG: Single-strand nicking of DNA *in vitro* by neocarzinostatin and its possible relationship to the mechanism of drug action. Biochim. Biophys. Acta 475: 294~306, 1977
19) IM, W. B.; C. CHIANG & R. MONTGOMERY: Studies on macromomycin, an antitumor protein. J. Biol. Chem. 253: 3259~3264, 1978