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INHIBITION OF PROTEIN SYNTHESIS REDUCES THE CYTOTOXICITY OF 4′-(9-ACRIDINYLAMINO)METHANESULFON-*m*-ANISIDIDE WITHOUT AFFECTING DNA BREAKAGE AND DNA TOPOISOMERASE II IN A MURINE MASTOCYTOMA CELL LINE

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Abstract—Stimulation of cleavable complex formation by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (mAMSA) and related anticancer drugs is an important initial event in drug action which correlates with cytotoxicity. However, it was recently suggested that factors in addition to cleavable complex formation are needed to express lethality. Therefore we investigated the effects of inhibitors of DNA replication and RNA and protein synthesis on mAMSA-induced cell killing in the K21 subline of the P815 murine mastocytoma cell line. This showed that RNA and protein synthesis, but not DNA replication, was necessary for maximal mAMSA cytotoxicity. Moreover, inhibition of RNA synthesis with cordycepin or protein synthesis with cycloheximide protected cells from the cytotoxic action of mAMSA without reducing DNA breakage or cleavable complex formation and there was no decrease in DNA topoisomerase II activity in nuclear extracts from cells treated with cordycepin or cycloheximide. We conclude that cleavable complex formation is independent of RNA and/or protein synthesis and we propose that the subsequent conversion into a lethal event requires an additional labile protein factor.

Several anticancer drugs including amsacrine (4′-9-acridinylamino)methanesulfon-*m*-anisidide, mAMSA), adriamycin, the ellipticines and the epipodophyllotoxins have been shown to induce the formation of a cleavable complex between DNA topoisomerase II (EC 5.99.1.3.) and DNA [1–4]. The formation of this complex has been related to drug mediated cell killing [5–7], although the precise mechanisms involved are still unknown. Recently it has been suggested that secondary events beyond formation of the cleavable complex may be important for the expression of lethality [8–11] and that additional factors are involved in converting the initial cleavable complex into a lethal lesion [11–16], although no experimental evidence has so far been presented. In addition there have been reports of a lack of correlation between cleavable complex formation and cytotoxicity [17, 18], suggesting that a more complex relationship exists between DNA damage and cell death than previously thought. For example, when Estey *et al.* [17] and Chow and Ross [18] studied amsacrine- or etoposide-mediated cell killing and DNA breakage in relation to the cell cycle stage, maximal cytotoxicity was observed in S phase concomitant with DNA synthesis and maximal DNA topoisomerase II activity, whereas maximal DNA breakage occurred in G2/M phase. However, Balb/c 3T3 cells were protected from the cytotoxic effects of etoposide by prior incubation with cycloheximide [18]. The effect of cycloheximide was attributed to the inhibition of topoisomerase II synthesis, which reduced the enzyme content of cells, although other explanations such as chromatin changes were not excluded. In conclusion it appears that the formation of the cleavable complex and DNA breakage are important steps in drug action, which do not, however, necessarily lead to drug-induced cell killing, and that additional events, possibly mediated by other factors are necessary for maximal cytotoxicity.

To test the assumption that the disruption of the cleavable complex by cellular processes such as moving DNA replication forks, RNA transcription or other, yet undefined factors could lead to cell death we investigated the possible influence of DNA replication and RNA or protein synthesis on amsacrine cytoxocity and DNA damage by inhibiting DNA replication with aphidicolin, RNA synthesis with cordycepin and protein synthesis with cycloheximide. Our results show that RNA and protein synthesis, but not DNA replication are necessary for maximal cytotoxicity of amsacrinc. In contrast, neither DNA damage nor topoisomerase II appeared to be affected after inhibiting RNA or protein synthesis. We propose that a labile cleavable complex disrupting factor converts the initial drug-induced DNA breaks into lethal lesions.

MATERIALS AND METHODS

Materials. [Methyl-^{3}H]thymidine (70–90 Ci/mmol) was from Amersham and α[^{35}S]dATP (500 Ci/mmol) was from NEN (Boston, MA). Phage P4 was a generous gift of Dr. R. Calendar, University of Cali-

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fornia, Berkeley. The restriction enzyme EcoRI was from Promega Biotec and DNA Pol I Klenow fragment and proteinase K were from Boehringer Mannheim (F.R.G.). Amsacrine (4′-(9-acridinylamino)methanesulfon-*m*-anisidide; mAMSA) provided as the isethionate salt by Dr. B. C. Baguley, University of Auckland Medical School, was stored as 1 mM stock solution in water at −20° and working dilutions were made immediately prior to use. Dextran grade B 150–200 was from BDH (Poole, U.K.), Hoechst 33258 was from Calbiochem Biochemicals (Los Angeles, CA) and p-iodonitrotetrazolium violet was from Serva (Heidelberg, F.R.G.). The protease inhibitors aprotinin, leupeptin, α_2-macroglobulin, phenylmethylsulfonylfluoride and diisopropylfluorophosphate were from Sigma Chemical Co. as were aphidicolin, cordycepin and cycloheximide. Aphidicolin was added to cells to a final concentration of 1 μg/ml, cordycepin to 10 μM and cycloheximide to 10 μg/ml. These concentrations were found to inhibit [^{3}H]thymidine incorporation into acid-precipitable material by at least 95%, [^{3}H]uridine incorporation by at least 80%, and [^{35}S]methionine incorporation by at least 95%, respectively.

Cell culture. The clonal wild-type subline K21 of the P815-X2 mouse mastocytoma cell line [19] was kindly provided by Dr. R. Schindler, University of Berne, Switzerland. The cells were grown in RPMI 1640 medium supplemented with 10% horse serum in a 5% CO_2/95% air atmosphere. Aphidicolin (1 μg/ml), cordycepin (10 μM) or cycloheximide (10 μg/ml) were added for two or six hours as indicated.

Drug toxicity assays. mAMSA sensitivity of K21 cells treated with or without 1 μg/ml aphidicolin, 10 μM cordycepin or 10 μg/ml cycloheximide for 2 or 6 hr was determined by colony formation in soft agarose. 0–10 μM mAMSA was added to cultures during the last hour of either aphidicolin, cordycepin or cycloheximide treatment. The cells were washed twice with medium, resuspended and diluted with fresh medium to between 250 cells/ml and 10^6 cells/ml and aliquots (0.25 ml) mixed with 5 ml 0.2% agarose in medium were poured onto a layer of 0.3% agarose in medium in 60 mm plastic petri dishes. The agarose was supplemented with 10% horse serum, 0.1 mg/ml folic acid, 20 μM sodium pyruvate, 20 μM each L-alanine, L-aspartic acid, L-glutamic acid and L-proline and 0.2 mM cysteine. The plates were incubated at 37° for 10–12 days. Colonies were stained overnight with *p*-iodonitrotetrazolium violet (1 mg/ml; 1 ml/plate) and the relative survival of drug treated cultures compared to untreated controls was determined.

Extraction of DNA topoisomerase II activity. The following protease inhibitors were added to all extraction buffers at the indicated final concentrations immediately prior to use: aprotinin, 1% (v/v); leupeptin, 0.1 mg/ml; α_2-macroglobulin, 0.01 mg/ml; phenylmethylsulfonylfluoride, 1 mM; diisopropylfluorophosphate, 0.1 mg/ml. This did not adversely affect topoisomerase II activity *per se*. Aliquots of 3×10^7 cells were collected by centrifugation, washed once in ice-cold Tris-buffered saline (25 mM Tris–HCl, pH 7.5/135 mM NaCl/5 mM KCl) and resuspended in 0.4 ml buffer A (20 mM Tris–HCl, pH 7.2/150 mM KCl/2 mM $MgCl_2$/2 mM $CaCl_2$/0.1 mM dithiothreitol/10 mM $Na_2S_2O_5$/2% (w/v) dextran grade B) to a cell density of 4–5 × 10^7 cells/ml. Triton X-100 was added to a final concentration of 0.1% and the cells were lysed for 10 min at 0° with occasional gentle mixing, followed by centrifugation at 1200 *g* for 10 min at 4°. The nuclei pellet was resuspended in 0.4 ml buffer A, layered on 3 ml 30% (w/v) sucrose in buffer A and sedimented at 1200 *g* for 10 min at 0°. The nuclei were resuspended in 0.4 ml buffer B (buffer A with 5 mM $MgCl_2$ but without $CaCl_2$), centrifuged at 1200 *g* for 10 min at 4° and resuspended in 0.4 ml buffer C (20 mM Tris–HCl, pH 8.0/150 mM KCl/5 mM $MgCl_2$/0.1 mM dithiothreitol/10 mM $Na_2S_2O_5$). Topoisomerase II extraction from isolated nuclei was as previously described [16]. Nuclear extracts were diluted to equal protein concentrations of approx. 0.25 mg/ml and stored at −70°.

The phage P4 DNA unknotting assay. The phage P4 DNA unknotting assay was used to quantitate topoisomerase II activity in nuclear extracts as described elsewhere [16]. One unit of topoisomerase II activity was defined as the amount of extract (in μg protein) that completely unknotted 0.24 μg phage P4 DNA.

Quantitation of mAMSA-stimulated covalent protein–DNA complex formation. The SDS/K^+ precipitation assay described by Liu *et al.* [20] and Rowe *et al.* [21] was used to measure the drug-induced formation of protein–DNA complexes in whole cells and nuclear extracts essentially as described elsewhere [16]. For studies with cells the DNA of K21 cells was labelled with 3 μCi/ml [methyl-^{3}H]thymidine (70–90 Ci mmol) in AHTG-medium (medium supplemented with 0.01 mM amethopterin/0.03 mM hypoxanthine/0.01 mM non-radioactive thymidine/0.1 mM glycine) overnight. The cells were collected by centrifugation and washed once with Tris-buffered saline. The cells were resuspended in fresh growth medium to a final density of 10^5 cells/ml and incubated with or without 10 μM cordycepin or 10 μg/ml cycloheximide for 1 to 5 hr at 37°. The cells were then distributed in 1 ml aliquots into 24-well microtiter plates and treated with 0–10 μM mAMSA for 1 hr at 37°. Following drug treatment the cells were collected by centrifugation, lysed, the protein–DNA complexes precipitated by SDS/K^+ and collected on GF/C filters as previously described [16]. The total acid-precipitable radioactivity per assay (i.e. per 10^5 cells) was routinely found to be approx. 5 × 10^4 cpm.

Determination of protein–DNA complex formation in 10 μl of nuclear extracts containing approx. 0.25 μg protein was essentially as previously described [16] but α[^{35}S]dATP was used to 3′-end label EcoRI digested pBR322 DNA instead of α[^{32}P]dATP.

DNA breakage. K21 cells were treated with or without 10 μM cordycepin or 10 μg/ml cycloheximide for 2 or 6 hr and 0–1 μM mAMSA was added during the last hour. DNA breakage induced by mAMSA was then determined by the fluorescence enhancement assay for DNA unwinding (FADU) described by Kanter and Schwartz [22]. In this technique DNA breaks are detected through the enhancement of the rate of alkaline denaturation of DNA, using the

bisbenzamide fluorophore H33258 as a probe for residual double-stranded DNA after a fixed denaturation time. Following drug treatment the fraction of residual double-stranded DNA, F, in group B samples after the fixed unwinding period was calculated using the relationship $F = (B - C)/(A - C)$ where A, B, and C are the mean relative fluorescence intensities in group A (no unwinding), B (sample) and C (total unwinding) respectively.

Protein determination. Protein concentrations were determined according to Bradford [23].

RESULTS

mAMSA cytotoxicity in cordycepin, cycloheximide or aphidicolin treated K21 cells

To determine whether inhibition of RNA or protein synthesis or DNA replication in K21 cells had an effect on mAMSA cytotoxicity, cells were treated for 2 or 6 hr with 10 μM cordycepin (RNA synthesis inhibitor) or 10 μg/ml cycloheximide (protein synthesis inhibitor) or for 2 hr with 1 μg/ml aphidicolin (to inhibit DNA replication) and exposed to 0–10 μM mAMSA during the last hour. Cell survival was assessed by colony formation in soft agarose. As shown in Fig. 1, inhibition of RNA synthesis or protein synthesis had a marked effect on mAMSA-induced cell killing, increasing C_{10} (the drug concentration needed to produce 90% cell killing) more than 4-fold from 0.9 μM in control cells to between 3.8 μM and 4.4 μM (average 4.1 μM) in 2 and 6 hr cordycepin-treated and 2 hr cycloheximide-treated cells. After 6 hr cycloheximide treatment C_{10} increased a further 50% to 6.7 μM, corresponding to an almost 8-fold increase in drug resistance. These results suggested that continuous RNA and/or protein synthesis was necessary for maximum mAMSA cytotoxicity. In contrast DNA replication was not necessary for mAMSA cytotoxicity since no changes in cell survival rates were detected after a 2 hr treatment with 1 μg/ml aphidicolin.

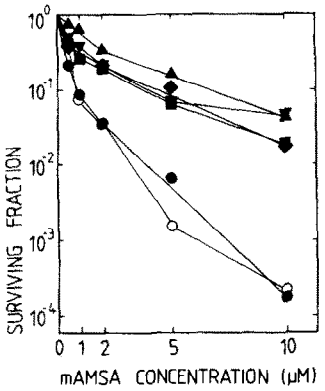

Fig. 1. Effect of mAMSA on survival of K21 cells after inhibiting DNA replication and RNA or protein synthesis. Aliquots of K21 cells were incubated with or without 10 μM, cordycepin or 10 μg/ml cycloheximide for 2 hr or 6 hr or 1 μg/ml aphidicolin for 2 hr and 0–10 μM mAMSA was added during the last hour. Cells were washed, then plated in soft agarose and incubated for 10–12 days at 37°. Surviving fractions were determined relative to non-drug treated controls. All values are means of at least three separate experiments with six different cell densities per mAMSA dose. The mean cloning efficiencies of cordycepin or cycloheximide treated cells without mAMSA relative to cells without inhibitor were: ●——●, no inhibitor, 87% = 1.0; ◆——◆, 2 hr cordycepin 0.87; ■——■, 6 hr cordycepin 0.42; ▼——▼, 2 hr cycloheximide 1.25; ▲——▲, 6 hr cycloheximide 0.95; ○——○, 2 hr aphidicolin 0.5.

DNA topoisomerase II activities

While this work was in progress other reports suggested that DNA topoisomerase II was a rapid turnover protein [18, 24]. Therefore we compared topoisomerase II strand passing activities of cells that had been incubated with or without 10 μM cordycepin or 10 μg/ml cycloheximide for 2 or 6 hr. Nuclear extracts were prepared, serially 2-fold diluted and a P4 DNA unknotting assay was performed with each dilution. The results of a typical experiment are presented in Fig. 2. No significant changes in topoisomerase II activity were observed and each extract contained approx. 1 unit of topoisomerase II activity per 50 ng protein (dilution 1/16). To further confirm that the inhibition of RNA or protein synthesis had no effect on topoisomerase II in our cells we also measured mAMSA-mediated stimulation of protein–DNA complex formation with nuclear extracts from cells treated with or without cordycepin or cycloheximide using [^{35}S]3′-end labelled DNA as a substrate (Fig. 3). Approximately 15-fold stimulation of protein–DNA complex formation by 10 μM mAMSA was observed with only minor differences between extracts from differently treated cells. In view of these results it was unlikely that a change in topoisomerase II activity was responsible for the significant decrease in mAMSA sensitivity of K21 cells treated with RNA or protein synthesis inhibitors. In separate experiments no direct effect of cordycepin, cycloheximide or aphidicolin on topoisomerase activity *per se* was detected (data not shown).

mAMSA-induced DNA breakage

To measure drug-induced DNA breakage in K21 cells after inhibiting RNA or protein synthesis we used a fluorescence enhancement assay for DNA unwinding (FADU) and compared the results with those from uninhibited control cells. Figure 4 shows that there was little change in mAMSA-induced DNA breakage after inhibiting RNA or protein synthesis for 6 hr with the appropriate inhibitor. The same results were also obtained after only 2 hr inhibitor treatment (data not shown) and neither of the inhibitors alone appeared to cause DNA breakage as indicated by the very similar *F*-values without mAMSA.

Stimulation of protein–DNA complex formation

Possible effects of RNA or protein synthesis inhibition on mAMSA-induced stimulation of protein–DNA complex formation in whole cells were measured by the SDS/K^+ precipitation assay (Fig. 5). A marked mAMSA dose-dependent stimulation of protein–DNA complex formation up to approximately 16-fold was observed in uninhibited cells, comparable to the stimulation obtained with the

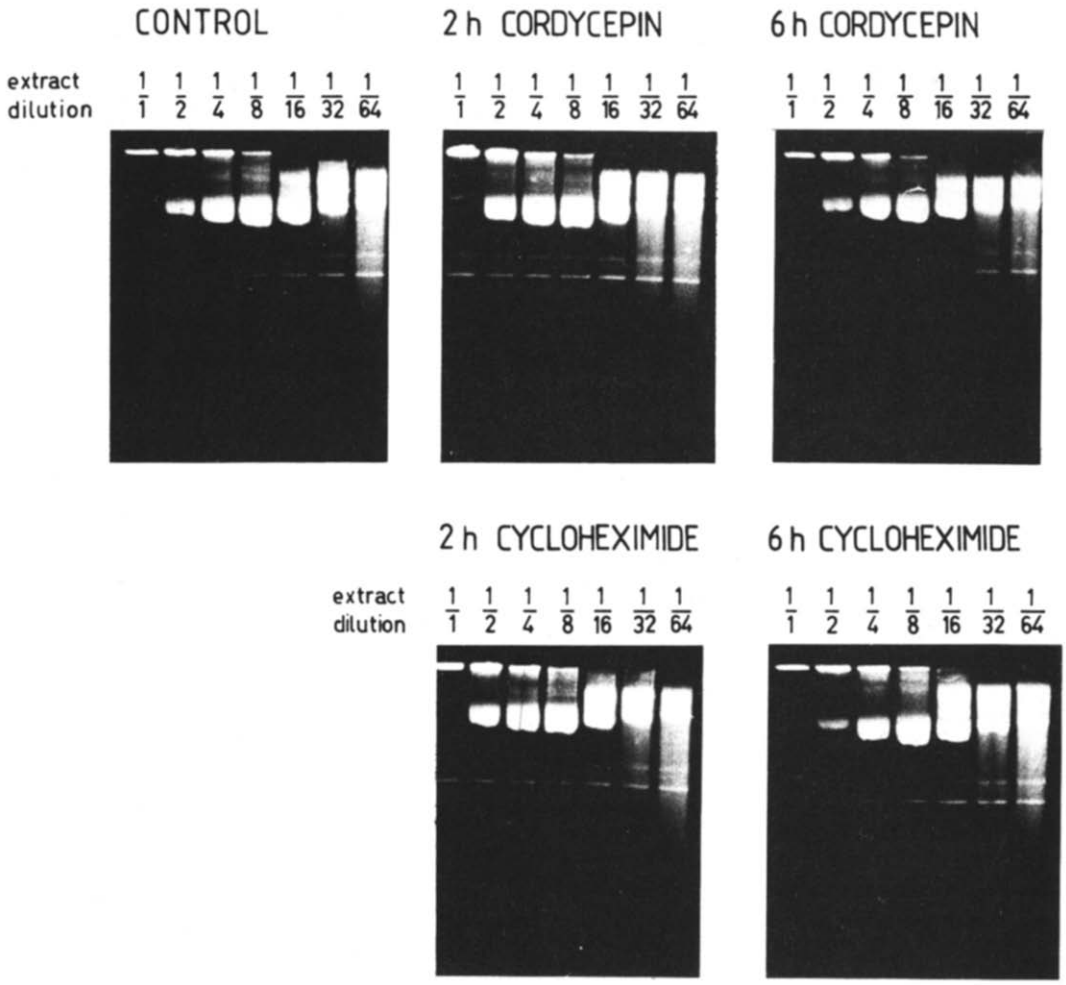

Fig. 2. Quantitation of nuclear DNA topoisomerase II activities by P4 DNA unknotting assays. Nuclear extracts from K21 cells grown for 2 hr or 6 hr with or without 10 μM cordycepin or 10 μg/ml cycloheximide were serially 2-fold diluted and with each dilution a P4 DNA unknotting assay was performed. Reaction products were separated on 0.7% agarose gels, stained with ethidium bromide and photographed under UV light.

corresponding nuclear extract. Treatment with cordycepin for 2 hr did not significantly affect protein–DNA complex formation. The apparent stimulation of complex formation observed after 2 hr cycloheximide treatment may not be significant in view of the considerable variation between individual experiments indicated by the standard error bars. In contrast, 6 hr cordycepin or cycloheximide treatment consistently reduced the mAMSA-induced stimulation of protein–DNA complex formation by approximately 50%.

DISCUSSION

Several studies have described correlations between cleavable complex formation, DNA breakage and cytotoxicity of anticancer drugs [13, 21, 25–27], although complex formation is not always related to cytotoxicity [17, 18, 28]. Rowe *et al.* [21] proposed a model whereby the cleavable complex is disrupted by additional factors such as moving replication forks or other functions involving DNA, thus generating open double strand breaks which may be lethal and/or recombinogenic. Indeed, it has recently been shown that topoisomerase II is involved in illegitimate recombination [29, 30].

To investigate the hypothesis that additional events or factors are needed to convert the cleavable complex into a lethal lesion, we studied the cytotoxicity of mAMSA in K21 cells after inhibiting DNA replication and RNA or protein synthesis. In agreement with others [13, 18] we found no evidence that DNA replication was necessary for mAMSA cytotoxicity whereas maximal cell killing appeared to require RNA and/or protein synthesis. We are not aware of any previous study that investigated the effect of inhibiting RNA synthesis on drug cytotoxicity although cycloheximide has previously been shown to protect Balb/c 3T3 cells from etoposide-mediated cytotoxicity [18]. These results suggested

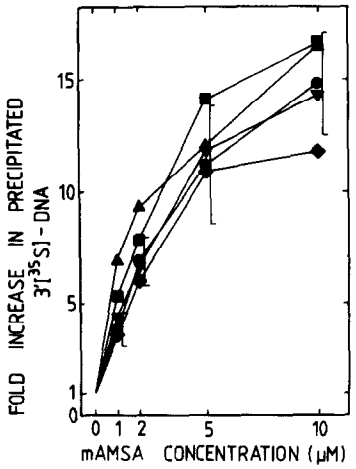

Fig. 3. Stimulation of topoisomerase II–DNA complex formation in nuclear extracts from K21 grown for 2 hr or 6 hr with or without 10 μM cordycepin or 10 μg/ml cycloheximide. Stimulation by mAMSA of the topoisomerase II–DNA complex was determined using 50 ng 3'-end labelled pBR322 DNA as substrate. ●——●, nuclear extract from cells grown without inhibitor (control); ▲——▲, nuclear extract from cells grown for 2 hr with 10 μM cordycepin; ■——■, nuclear extract from cells grown for 6 hr with 10 μM cordycepin; ▼——▼, nuclear extract from cells grown for 2 hr with 10 μg/ml cycloheximide; ◆——◆, nuclear extracts from cells grown for 6 hr with 10 μg/ml cycloheximide. Values given are means from four experiments. For clarity, only standard errors of control extracts are given.

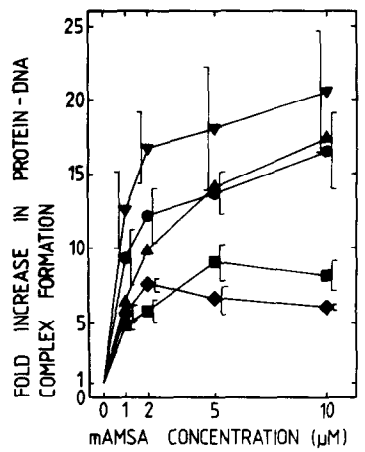

Fig. 5. Stimulation of protein–DNA complex formation in K21 cells after inhibiting RNA or protein synthesis. mAMSA-stimulated formation of protein–DNA complexes was measured in K21 cells from cultures grown for 2 hr or 6 hr with or without 10 μM cordycepin or 10 μg/ml cycloheximide. Cellular DNA was prelabelled with [^{3}H]thymidine and protein–DNA complexes formed were precipitated by SDS/K$^+$. ●——●, Cells grown without inhibitor (control); ▲—▲, cells grown for 2 hr with 10 μM cordycepin; ■——■, cells grown for 6 hr with 10 μM cordycepin; ▼——▼, cells grown for 2 hr with 10 μg/ml cycloheximide; ◆——◆, cells grown for 6 hr with 10 μg/ml cycloheximide. Values given are means plus standard errors from four experiments.

that the continuous synthesis of a short-lived protein might be necessary for maximal cytotoxicity of mAMSA. The obvious candidate for such a protein was DNA topoisomerase II, since this enzyme has been shown to vary during the cell cycle [24], to have a short half life [24] and to be in a continuing dynamic equilibrium between synthesis and degradation in proliferating cells [18].

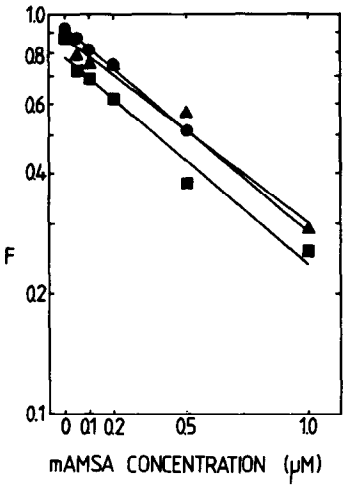

Fig. 4. Effect of mAMSA on DNA breakage after inhibiting RNA or protein synthesis. Aliquots of K21 cells were grown for 6 hr with or without 10 μM cordycepin or 10 μg/ml cycloheximide and 0–1 μM mAMSA was added during the last hour. Drug-induced DNA breakage was measured by the fluorescence enhancement assay for DNA unwinding (FADU). F was calculated as described in Materials and Methods. ●——●, Cells grown in the absence of inhibitor (control); ▲——▲, cells grown with 10 μM cordycepin; ■——■, cells grown with 10 μg/ml cycloheximide. Values given are means from three independent experiments.

To test whether the effect of RNA or protein synthesis inhibition resulted from topoisomerase II depletion in K21 cells, we measured strand passing activity (by P4 DNA unknotting) and mAMSA-stimulated protein–DNA complex forming activity (using 3'-end labelled DNA as substrate) in nuclear extracts from RNA and protein synthesis inhibited cells. However, there was no significant decrease in enzyme activity and the fact that protein–DNA complex formation was very similar in all extracts suggested that there was also no qualitative or quantitative modification of topoisomerase II. These results also indicated that topoisomerase II was relatively stable, possibly as a consequence of cell transformation as reported for transformed chicken cells [24].

To determine whether the reduced killing of K21 cells by mAMSA after inhibiting RNA or protein synthesis was accompanied by a reduction in DNA breaks, we measured mAMSA-induced DNA damage in cells by the fluorescence enhancement assay for DNA unwinding and mAMSA-stimulation of protein–DNA comlex formation by SDS/K$^+$ precipitation. There was little effect of RNA or protein synthesis inhibition on mAMSA-induced DNA breakage and a substantial reduction in protein–DNA complex formation only after 6 hr cordycepin or cycloheximide treatment. This suggested that the formation of the cleavable complex was independent of RNA and/or protein synthesis.

Our data suggest that the concealed DNA breaks in cleavable complexes induced by mAMSA, are formed independently of RNA and/or protein synthesis, but that their subsequent conversion into a lethal event is dependent upon RNA and/or protein synthesis or more likely on a product of protein synthesis, a protein factor. The possibility can not be excluded that the protective effect of inhibiting RNA or protein synthesis on mAMSA-induced cell killing was through a modification of intracellular drug concentration. However, we consider this to be unlikely because the protective effect is only on cell survival and not on DNA breakage in whole cells, which would be expected to be equally affected if it was concentration related. We propose the following model. After formation and stabilisation of the cleavable protein(topoisomerase II)–DNA complex by mAMSA or other drugs an additional protein factor acts to separate the topoisomerase II monomers bound to each 5′-end of the broken DNA, producing overt DNA breaks. Whether anticancer drugs directly activate the cleavable complex disrupting factor or enhance its action by increasing the persistence time of the cleavable complex remains to be resolved. The cleavable complex disrupting factor appears to be a labile protein that is presumably involved with topoisomerase II during normal DNA replication and possibly in sister chromatid exchanges and recombination, both of which involve topoisomerase II [8, 10, 29, 30]. It is interesting that Alexander *et al.* [31, 32] recently reported a synergistic enhancement of anticancer drug cytotoxicity by tumour necrosis factor. This effect was only seen when tumour necrosis factor was added with or after anticancer drugs and it occurred only with drugs that stabilise the cleavable complex. The authors suggested that "the enhancement by tumour necrosis factor of topoisomerase-targeted drug cytotoxicity involves an increase in lethality to the cell from the unique form of DNA damage mediated by these drugs" [32].

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