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ABROGATION OF ADRIAMYCIN TOXICITY *IN VIVO* BY CYCLOHEXIMIDE

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Abstract—The processes involved in cell killing by Adriamycin® (ADR) and other agents that interact with topoisomerase II are unclear. To investigate the mode of ADR cytotoxicity *in vivo*, we have investigated the effects of the protein synthesis inhibitor, cycloheximide (CH), on cell killing by ADR in the murine intestinal tract. We have used morphological criteria to assay the cell death. ADR rapidly induces cell death in this tissue that has the morphology of apoptosis or programmed cell death. CH, when administered immediately after ADR, reduced the incidence of cell death by ~ 81% at 3 hr and ~ 51% at 6 hr after treatment. The inhibitor was only effective when administered within 0.5 hr of ADR suggesting that critical events leading to cell death may occur during this period. The inhibitor did not interfere with the ADR uptake or retention. Significant positive correlation was observed between protein and DNA synthesis inhibition (as measured by precursor uptake) and apoptosis inhibition. CH delayed progression of cells through all phases of the cell cycle except mitosis. However, ADR also had a similar effect, suggesting that progression through the cell cycle is not necessary for the expression of apoptosis. The effectiveness of CH in apoptosis inhibition, even when administered 0.5 hr after the ADR, coupled with the rapid uptake of ADR by the intestinal epithelium suggests that the mode of inhibition is unlikely to be modulation of cellular targets of ADR such as topoisomerase II or inhibition of formation of ADR–topoisomerase II complex. These data indicate that topoisomerase II-interacting agents such as ADR may induce apoptosis; the processes leading to cell death in this situation are thought to be gene dependent and require protein synthesis for their expression. Thus, the cytoprotective effect of CH may be due directly to the inhibition of protein synthesis.

Anticancer agents such as intercalators, e.g. Adriamycin® (ADR‡), and epipodophyllotoxins are thought to act via interaction with topoisomerase II (reviewed in Ref. 1). These drugs affect the breakage–reunion process of the DNA topoisomerase II by stabilizing the topoisomerase II–DNA cleavable complex. The intracellular content of topoisomerase II varies with the cell cycle phase [2–4] and may be correlated with the cell cycle-dependent cytotoxicity of these agents [5]. However, the exact mechanism whereby interaction of the drugs with the topoisomerase II–DNA cleavable complex leads to cell death is unclear.

Protein synthesis inhibitors such as cycloheximide (CH) can confer cytoprotection against such agents *in vitro* [6–9]. The cytoprotective action of CH may be via disruption of the cell cycle and alteration of the cellular topoisomerase II content. It has been shown that CH treatment can reduce cellular topoisomerase II [9] in A31 mouse fibroblast and human lymphoblast CCRF-CEM cells. However, topoisomerase content is not proliferation dependent in every cell line and there does not appear to be a correlation in any case between intracellular topoisomerase content and sensitivity to the drugs [10]. Furthermore, CH may have a cytoprotective action without a significant change in the enzyme content or DNA cleavage [9]. Thus, the mechanism of cytoprotection by agents such as CH against topoisomerase II-targeting drugs is unclear.

We have investigated the effect of CH on the cytotoxic effects of ADR on the murine intestinal tract *in vivo*. We have used morphological criteria to assay the cell death. The effect of CH on (a) ADR uptake and retention, (b) cell cycle and (c) macromolecular synthesis has also been investigated.

MATERIALS AND METHODS

Animals. Male BDF1 (B6D2F1 cross: C57BL6 × DBA2) mice (10–12 weeks old) were used for all experiments. Animals were kept under a 12-hr dark (18.00 to 06.00 hr)/12-hr light regimen. They were given food and water *ad lib.* The animals were killed by cervical dislocation.

Drugs. ADR (Pharmacia, Italy) and CH (Sigma Chemical Co., St Louis, U.S.A.) were dissolved, immediately before use, in sterile isotonic saline to give an appropriate concentration in 0.2 mL. All drugs were administered i.p. The inhibitor was generally administered immediately after the ADR and the doses of drug used were 20 (ADR) and 16 (CH) mg/kg unless stated otherwise. In these

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‡ Abbreviations: ADR, Adriamycin; CH, cycloheximide.

experiments 20 mg/kg of ADR were used because maximum cell death is observed at this dose.

Reagents. The radiolabelled compounds were purchased from NEN, Du Pont, U.K. The reagents for flow cytometry were purchased from Sigma. Other reagents except where stated, were purchased from BDH (Poole, U.K.).

Sample preparation and scoring of apoptosis/mitosis. The middle third of the small intestine was removed and fixed in Carnoy's solution for at least 30 min. Bundles of 10 pieces of small intestine were prepared as described previously [11] and histological transverse 5-μm-thick sections cut. The apoptotic cells were counted in good longitudinal sections of crypts. These are defined as those showing (a) at least 17 cells along the crypt length, (b) Paneth's cells at the base of the crypt and (c) at least two thirds of the lumen in the same plane. The crypt cells were numbered from the base of the crypt upwards [12, 13] and the position of apoptotic cells recorded at each cell position. Apoptotic cells in the crypt, as elsewhere, tend to fragment; to maintain objectivity in counting, a cluster of one or more apoptotic bodies at a particular cell position along the side of the crypt was scored as a single event. Twenty-five crypt sections were scored for each mouse.

Adriamycin uptake. Following removal, the small intestine was washed and the luminal contents flushed out with ice-cold isotonic saline. The intestine was then laid on filter paper and cut open to expose the luminal surface. Using a microscope slide, the epithelium was scraped off. ADR was extracted from the epithelium using the 50% ethanol–0.3 N HCl procedure described by Bachur *et al.* [14]. The fluorescence of the samples and ADR standards prepared in 50% ethanol–0.3 N HCl was measured at an excitation wavelength of 475 nm and emission wavelength of 585 nm using a Shimadzu RF540 fluorospectrophotometer (Shimadzu Corp., Kyoto, Japan). The ADR content was determined from standard curves. The total protein in the tissues was determined as described above. The data were expressed as micrograms ADR per microgram protein.

Precursor incorporation. DNA, RNA and protein synthesis was assayed by incorporation of the following radiolabelled precursors: [^{3}H]thymidine ([^{3}H]Tdr), sp. act. 247.9 GBq/mM, [^{3}H]uridine ([^{3}H]Urd), sp. act. 1.01 TBq/mM, [^{3}H]leucine ([^{3}H]Leu), sp. act. 2.22 TBq/mM, respectively. The animals were injected i.p. with 370 kBq of either [^{3}H]Tdr, [^{3}H]Urd or [^{3}H]Leu in 0.2 mL, 20 min prior to killing. Following removal, the tissues were washed in ice-cold isotonic saline, frozen in liquid nitrogen and pulverized. The proteins were precipitated in 5% TCA and solubilized in 1 M NaOH. The total protein was determined using the bisinchoninic acid (BCA) protein assay kit BCA-1 (Sigma) based on the Lowry method [15]. Aliquots of the solubilized

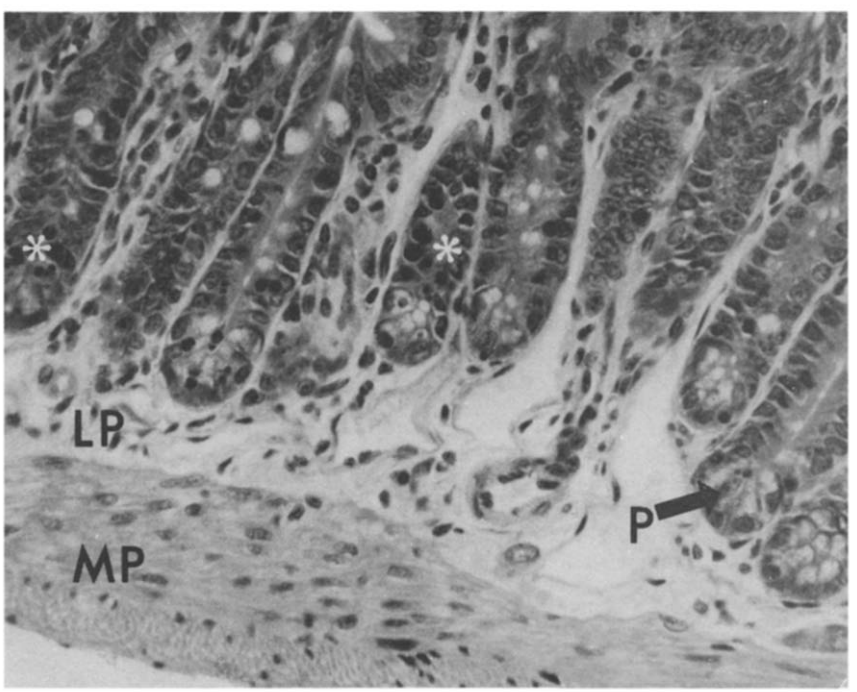

Fig. 1. (a) Photomicrograph of transverse section through murine small intestine showing ADR-induced apoptosis (*) in crypts. Clusters of apoptotic cells are observable predominantly in the base of the crypt with individual cells often lying within the cytoplasm of the neighbouring cells or within the crypt lumen (magnification × 160). P, Paneth cells; LP, lamina propria; MP, muscularis propria. (b) Photoelectronmicrographs of apoptotic cells and fragments (*) in mouse small intestinal epithelium at 6 hr after ACR (20 mg/kg) treatment (magnification × 1500). (c) Higher magnification of one of the apoptotic cells shown in (b). Characteristic nuclear and cytoplasmic condensation and fragmentation are seen. Nucleolar desegregation is also observed (magnification × 5500).

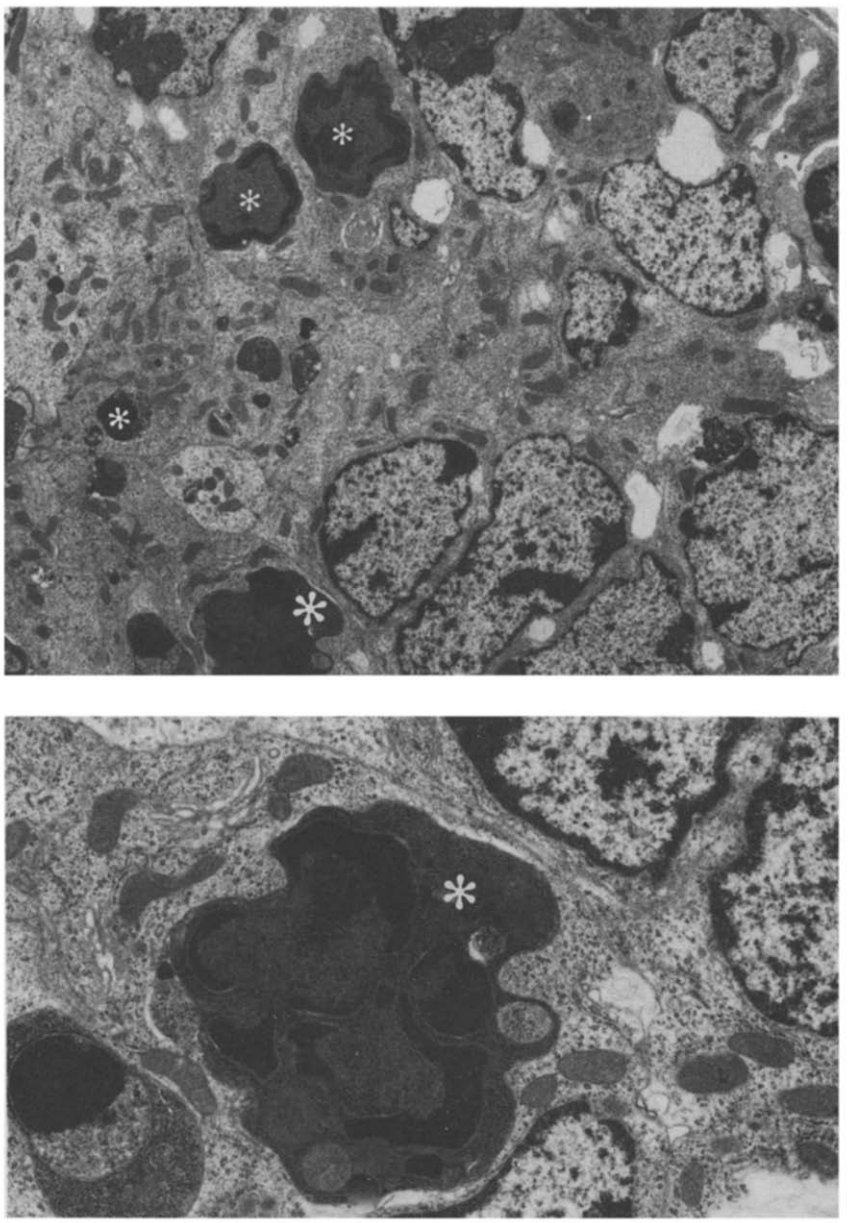

Fig. 1. Continued.

proteins and TCA supernatants were used for scintillation counting. The data were expressed as specific activity (dpm acid insoluble fraction per μg protein) and corrected for the effects of the drugs on precursor uptake (specific activity: dpm acid soluble fraction per unit volume).

Flow cytometry. A modification of the method of Flint *et al.* [16] was used to isolate the crypts. Briefly, everted 1.5-cm pieces of the small intestine were spun gently for 40 min in the isolation buffer (5.6 mM disodium hydrogen orthophosphate, 8.0 mM potassium dihydrogen orthophosphate, 96.0 mM sodium chloride, 1.5 mM potassium chloride, 27.0 mM trisodium citrate, 0.5 mM dithiothreitol, 55 mM sorbitol, 44 mM sucrose, 5 mM EDTA, 5 mM ethyleneglycolbis(aminoethylether)tetraacetate, in distilled water, pH 7.3)at 37°. The tissues were then subjected to serial vortexing to remove the crypts. The samples were allowed to sediment for 5 min on ice and the sediment was examined in an inverted microscope. Only samples showing in excess of 90% crypts (subjectively) were processed further. The crypts were collected by centrifugation at 800 rpm (4°) for 5 min. The supernatant was discarded and the crypts resuspended in 0.5 mL citrate buffer (250 mM sucrose, 40 mM tri-sodium citrate, 5% dimethyl sulphoxide in distilled water). The preparation of nuclei and staining with propidium iodide for flow cytometry was as described by Vindeløw *et al.* [17]. A Coulter Epics V (Coulter, Luton, U.K.) flow cytometer with an argon laser at excitation wavelength 488 nm and emission wavelength > 610 nm was used. A total of 30,000 nuclei/animal was examined and the cells were

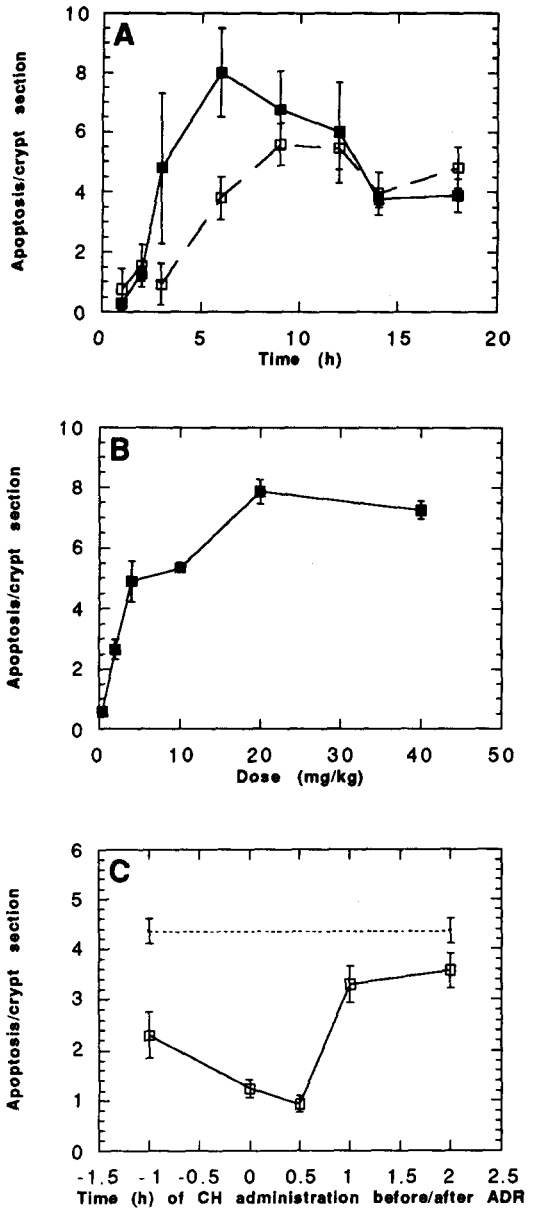

Fig. 2. The yield of apoptosis per crypt section (A) with time following treatment with 20 mg/kg ADR alone (■) or together with 16 mg/kg CH (□); (B) at 3 hr following treatment with ADR (0–40 mg/kg); (C) at 3 hr after treatment with varying the time of administration of CH (16 mg/kg). ADR (20 mg/kg) was administered at time 0 (arrow) and CH was administered either immediately after or at times before or after the ADR. The level of apoptosis in ADR-treated animals is also shown by the broken line. In each graph the points represent the means ± 1 SE of data from at least four animals.

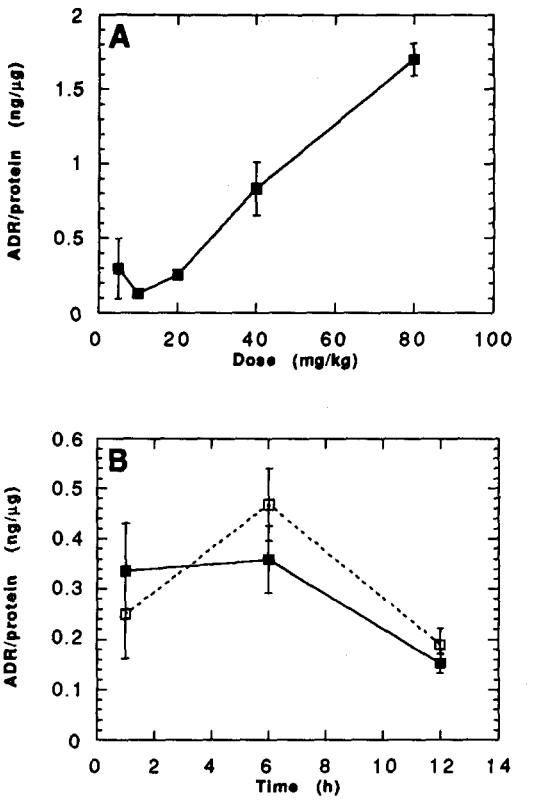

Fig. 3. (A) Uptake of ADR (0–80 mg/kg) 1 hr post administration and (B) the effect of CH (16 mg/kg) on the uptake and retention of ADR (20 mg/kg) in mouse intestinal epithelium. The data are expressed as amount of ADR per acid insoluble protein. Each point represents the mean ± 1 SE of data from at least four animals. ADR (■), ADR+CH (□).

gated by size and fluorescence. The coefficient of variation of the G_1 peak in the flow cytometry studies was less than 6% (range: 3.68–5.98) and the noise levels were low.

Statistical analysis. Where appropriate the statistical significance was tested by Student's *t*-test with the accepted significance level of $P < 0.01$. Pearson correlation coefficient was used to test the correlation between apoptosis inhibition and inhibition of protein, DNA and RNA synthesis.

RESULTS

Cell deletion by ADR

ADR-induced cell death was characterized by nuclear and cytoplasmic condensation, and preservation of the organelles in the early stages (Fig. 1). These changes are typical of apoptosis or programmed cell deletion (reviewed in Ref. 18). Secondary degenerative changes were only observed at the later stages (12–24 hr after treatment). Apoptosis was observed within 2 to 3 hr of treatment (Fig. 2A). Peak incidence occurred after 6 hr. Although there was a decrease in the incidence beyond this period, apoptosis was still observed in the crypts 18–20 hr after treatment. A biphasic dose–response was observed with increasing doses of ADR (Fig. 2B), with no further cell killing observed with doses greater than 20 mg/kg. This was not due to saturation of ADR uptake (Fig. 3A). The cell killing occurred predominantly though not exclusively in the base of the crypt (Fig. 4). The median cell

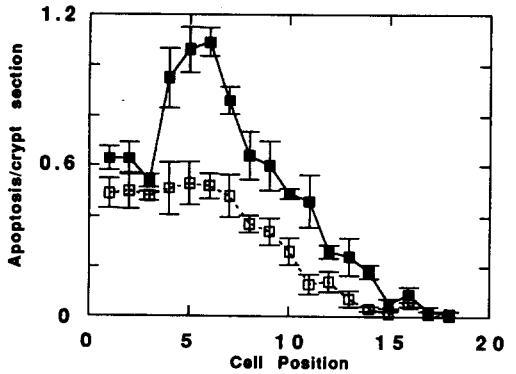

Fig. 4. The spatial distribution of ADR-induced apoptosis in the crypt. The data are presented as yield of apoptosis at each cell position in the crypt 6 hr following injection with 20 mg/kg ADR (■) alone or together with 16 mg/kg CH (□). Each point represents the mean ± 1 SE of data from at least four animals.

position of spatial distribution of apoptosis was between cell positions 5 and 7. Since the intestinal epithelium is a highly organized structure with its topographical organization related to the kinetic hierarchy [19], inferences about the identity of the target population can be made from the topographical distribution of apoptosis in the crypt. The occurrence of ADR-induced cell death in the base of the crypt suggests that the target population for this agent consists of stem cells and early transit cells.

Effects of CH on ADR-induced cell killing

A single dose of CH administered immediately after ADR inhibited cell death by ~ 81% at 3 hr and ~ 51% at 6 hr after treatment (Fig. 2A). The differences between the means of apoptosis in the cytotoxic only-treated group (4.79 and 7.99 at 3 and 6 hr, respectively) and the cytotoxic plus inhibitor-treated group (0.93 and 3.79 at 3 and 6 hr, respectively) were statistically significant ($P < 0.01$). Significant inhibition was not observed at any other time. The incidence of apoptosis in the inhibitor-treated animals did not increase at any time to the peak levels observed in the cytotoxic only-treated group (Fig. 2A). CH was only effective in apoptosis inhibition if administered within 45 to 60 min of ADR treatment (Fig. 2C). The inhibition was observed at most cell positions (Fig. 4) but was greatest over cell positions 4–7.

Effects of CH on ADR uptake/retention

CH, when administered immediately after the ADR, did not affect the uptake and retention of ADR (Fig. 3B).

Effects of CH on macromolecular synthesis.

CH showed significant ($P < 0.01$) dose-dependent inhibition of protein and DNA synthesis but not RNA synthesis, 30 min after treatment (Fig. 5B, C,

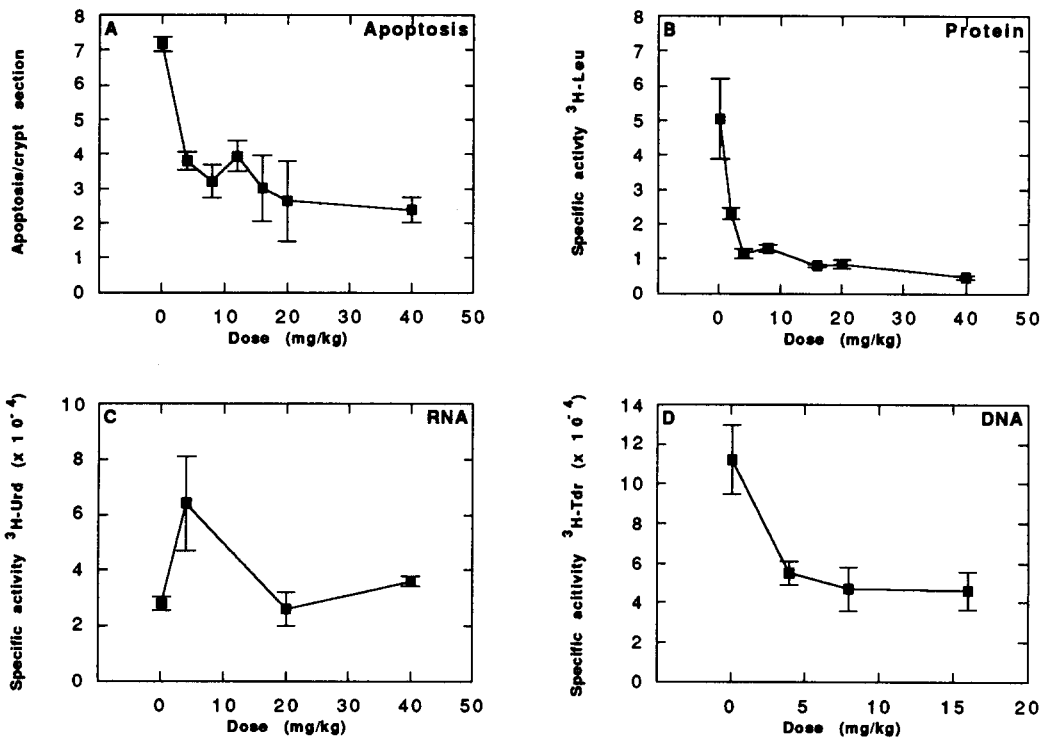

Fig. 5. The effect of CH (0–40 mg/kg) on (A) the incidence of ADR-induced apoptosis 3 hr after treatment: (B) the incorporation of [^{3}H]Leu; (C) the incorporation of [^{3}H]Udr and (D) the incorporation of [^{3}H]Tdr into acid insoluble material. Each point represents the mean ± 1 SE of data from at least four animals.

Table 1. Pearson correlation coefficients of mean percentage inhibition of apoptosis and macromolecular synthesis by CH

	ACR-induced apoptosis	
	Correlation coefficient (r)	Significance (P)
Protein synthesis	0.98	<0.01
DNA synthesis	0.99	<0.01
RNA synthesis	−0.15	NS

The significance of correlation (i.e. deviation of r from 0) was tested by the Student's t-test.

D). The corresponding dose-dependent inhibition of apoptosis is shown in Fig. 5A. The Pearson correlation coefficients between the percentage inhibition by CH of macromolecular synthesis and ADR-induced apoptosis are given in Table 1. Significant positive correlation was observed between inhibition of protein and DNA synthesis and inhibition of ADR-induced apoptosis by CH. With respect to the time course of inhibition, CH showed inhibition of both DNA (84%) and protein synthesis (93%) within 15 to 30 min (data not shown). Near normal levels of synthesis were observed 6 hr after treatment.

Effects of CH and ADR on the crypt cytokinetics.

The cell cycle distribution in the G_1, S and G_2+M phases with time following treatment with CH alone is shown in Fig. 6A. There was no change in cell cycle compartment distribution in CH-treated animals compared to saline-treated controls. The effect of CH on the mitotic activity in the crypt is shown in Fig. 7. There was a transient decrease in the number of mitoses 1 hr post treatment suggesting

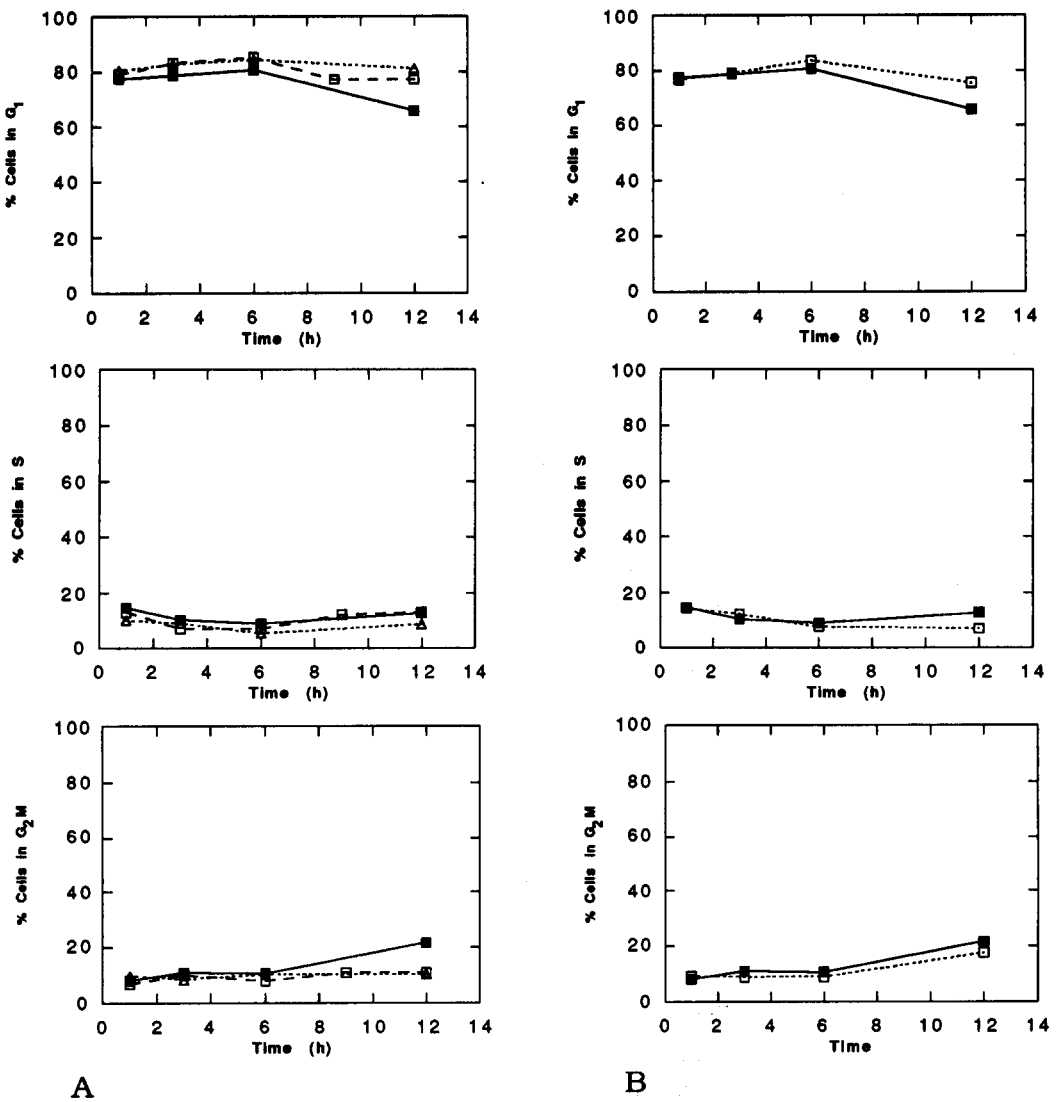

Fig. 6. The % of crypt cells in G_1, S and G_2+M phases at times following treatment with (A) isotonic saline (△), 20 mg/kg ADR (■) and 16 mg/kg CH (□), and (B) 20 mg/kg ADR alone (■) and together with 16 mg/kg CH (□).

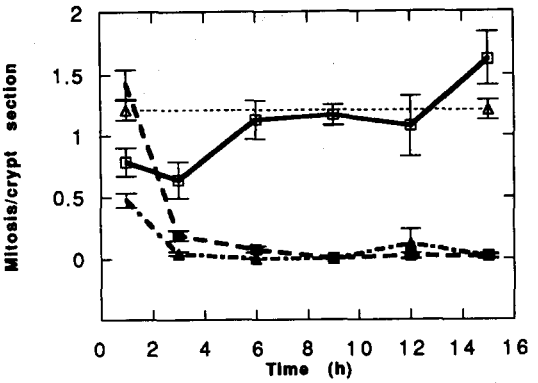

Fig. 7. Mitoses per crypt section with time following treatment with 20 mg/kg ADR (■), 16 mg/kg CH (□) and both agents together (▲). The level of mitosis in saline-treated animals is also shown (△). Each point represents the mean ± 1 SE of data from at least four animals.

a transient G_2 delay; however, an overshoot above the normal levels was not observed until 15 hr after treatment. This suggests that CH delays progression of cells through all phases except for mitosis. This is compatible with the depression of incorporation of [^{3}H]Tdr (Fig. 5C).

The effect of ADR alone and together with CH on the crypt cytokinetics is shown in Figs 6 and 7. With ADR, an accumulation of cells in the G_2+M phase largely at the expense of the cells in the G_1 phase was observed at 12 hr (Fig. 6A,B). Since there are virtually no mitotic cells observed (histologically) beyond 2 hr after drug administration (Fig. 7), it is apparent that the G_2 block is effective by this time. Since (a) the G_2 population does not show an increase until 12 hr after treatment and (b) there is no emptying of the G_1 population by 6 hr (assuming a T_{G1} of ~4 hr) and the S population by 12 hr (assuming T_S of ~6 hr), ADR must also induce a transient cell cycle delay (of between 6 and 12 hr) in the other cell cycle phases as well. Compared to the ADR only-treated group, the CH-treated group showed an earlier decrease in the mitotic activity in the crypt similar to that observed in animals treated with CH only (Fig. 7) and a relative emptying of the S phase fraction (Fig. 6B). The latter is probably due to a longer G_1 delay induced by CH.

DISCUSSION

Previous studies have shown inconsistent correlation between the cytoprotective effects of CH against agents that act via topoisomerase II and intracellular topoisomerase content. The mechanism of the cytoprotective action of this agent remains to be elucidated. In the present study, we have demonstrated that ADR induces cell death in the murine small intestinal tract that has the morphology associated with apoptosis (or programmed cell death) and that the mechanism of cytoprotection by CH may be related to the inhibition of protein synthesis. Apoptosis is normally observed in physiological situations such as development, normal tissue turnover and involution, and in immune-mediated cell killing. However, it is also observed in pathological atrophy of tissues and following cytotoxic treatment (reviewed in Ref. 18). It is thought to be a gene-dependent process requiring protein synthesis for its expression (reviewed in Ref. 18).

Since ADR appears to kill cells early in the cell kinetic hierarchy, it is possible that the programmed cell death in this situation represents altruistic cell suicide to prevent immortalization of damaged DNA [20].

In the present investigation, CH inhibited apoptosis induced by ADR. CH has been shown previously to inhibit apoptosis occurring in physiological situations. CH has also been shown to inhibit cell death induced by other cytotoxic agents in this tissue [21, 22].

The critical events leading to cell death in this tissue appear to be occurring within a short period after exposure to the cytotoxic agent since CH is ineffective if administered after this period. These observations are not only similar to those observed previously in the rat small intestine [21, 22] but also to those of Ben-Ishay and Farber [23] on the cytoprotective effects of CH on bone marrow against cytosine arabinoside and nitrogen mustard. Galili *et al.* [24] have also reported similar findings concerning inhibition of glucocorticoid-induced apoptosis of human chronic lymphocytic leukaemia cells *in vitro* by cycloheximide, puromycin and actinomycin D.

Investigation of the mechanism by which CH inhibits the ADR-induced apoptosis *in vivo* is difficult since about 2% of crypt cells undergo cell death and, at present, isolation of these cells for biochemical analysis is not possible. In the present study, whole intestinal tissue or isolated epithelium have been used for biochemical assays and the findings may not necessarily reflect the events occurring in the cell undergoing apoptosis. However, with respect to the effects of CH on macromolecular synthesis, it is unlikely that there is a difference in effects on different cell types since the agent has been shown to have protein and DNA synthesis inhibitory activity in a wide variety of tissues. With respect to ADR uptake and retention in the intestinal epithelium, it has been demonstrated previously [25] that there does not appear to be a preferential uptake or retention by any cells in the murine intestinal epithelium.

The mechanism by which CH inhibits apoptosis is thought to be inhibition of synthesis of proteins required for expression of cell death. This was supported in the present study by the strong correlation between protein synthesis inhibition and inhibition of cell death. However, a strong correlation is also observed with DNA synthesis inhibition. Although this cannot be excluded as a possible mode of inhibition of apoptosis by CH, it is unlikely since ADR itself has been shown to inhibit DNA synthesis both *in vitro* [26] and *in vivo* including the murine intestinal epithelium [27, 28]. Protein synthesis inhibitors may also have other actions that may confer cytoprotection. CH clearly did not affect the uptake and retention of the ADR. *In vitro,* protein synthesis inhibitors confer cytoprotection against ADR [6, 8] and the mechanism of cytoprotection in these cases appears to be inhibition of cell cycle progression. The effect of the inhibitors on the cell

cycle kinetics could change the incidence of cell death in two possible ways: (i) if the cytotoxic agent is cell cycle active or cell cycle specific, then the size of its target population could be reduced by alteration of the kinetic state of these cells by a metabolic inhibitor; and (ii) if the expression of cell death occurs in a specific phase, then the inhibition of cell cycle progression would affect its incidence.

The first of these is possible because the intracellular content of topoisomerase II, the cellular target of ADR, varies with the cell cycle phase [2–4] and may be correlated with the cell cycle-dependent cytotoxicity of these agents [5]. Obviously, the alteration in the cellular kinetics by CH could significantly affect the cellular topoisomerase II levels. However, this would require administration of the inhibitor before the cytotoxic agent so that the kinetic state of the cells is altered by the time of exposure to the cytotoxic agent. In the present investigation, CH was effective not only when administered immediately before or after the cytotoxic agent, but even when administered up to 45–60 min later. Furthermore, administration of the CH 1 hr prior to the cytotoxic treatment appears to decrease the inhibition. These considerations coupled with the observation of rapid uptake of ADR by the intestinal epithelium also suggest that the mechanism of inhibition by CH is not through inhibition of formation of the ADR–topoisomerase II complex.

It has been shown previously that (a) the intracellular topoisomerase II content is not cell cycle dependent in every cell type [10], (b) there does not appear to be a consistent correlation between intracellular topoisomerase content and sensitivity to the drugs [10], and (c) CH may have a cytoprotective action without causing a significant change in the enzyme content or DNA cleavage [9].

The inhibition of cell cycle progression by CH could alter the incidence of cell death if expression of apoptosis only occurred in a specific phase. However, ADR also induces a transient delay in progression of cells through all cell cycle phases. These findings are consistent with previous observations (reviewed in Ref. 29). Since apoptosis continues to occur during this period, its expression is unlikely to be related to cell cycle phase.

CH may also confer cytoprotection by induction of stress-like proteins. Such proteins are induced by CH within 1 hr *in vitro* [30] and have been implicated in cytoprotection conferred against ADR by hypoxic insult and the glycolytic pathway inhibitor, 2 deoxy-D-glucose. This has not been excluded in the present study.

In conclusion, ADR induces cell death that has the morphology of apoptosis. The cytoprotective effect of CH appears to be related to the inhibition of this process by the inhibition of protein synthesis. Factors such as effects of CH on the pharmacokinetics of ADR and cell cycle kinetics do not appear to be important. Since cytotoxic-induced apoptosis is also observed *in vitro*, cytoprotection in this situation by agents such as CH may be related to the inhibition of apoptosis.

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REFERENCES

1. Kohn KW, Pommier Y, Kerrigan D, Markovits J and Covey JM, Topoisomerase II as a target of anticancer drug action in mammalian cells. *NCI Monog* **4**: 61–71, 1987.
2. Duguet M, Lavenot C, Harper F, Mirambeau G and DeRecondo AM, DNA topoisomerases from rat liver: physiological variations. *Nucleic Acids Res* **11**: 1059–1075, 1983.
3. Taduo G, Mirambeau G, Lavenot C, der Garabedian A, Vermeesch J and Duguet M, DNA topoisomerase activities in concanavalin A-stimulated lymphocytes. *FEBS Lett* **176**: 431–435, 1984.
4. Heck MMS, Hittelman WN and Earnshaw WC, Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* **85**: 1086–1090, 1988.
5. Sullivan DM, Glisson BS, Hodges PK, Smallwood-Kentro S and Ross WE, Proliferation dependence of topoisomerase II mediated drug action. *Biochemistry* **25**: 2248–2256, 1986.
6. Hromas R, Barlogie B, Swartzendruber D and Drewinko B, Potentiation of DNA-reactive antineoplastic agents and protection against S-phase-specific agents by anguidine in Chinese hamster ovary cells. *Cancer Res* **43**: 3070–3073, 1983.
7. Charcosset J-Y, Bendridjian J-P, Lantieri M-F and Jacquemin-Sablon A, Effects of 9-OH-Ellipticine on cell survival, macromolecular synthesis and cell cycle progression in sensitive and resistant Chinese hamster lung cells. *Cancer Res* **45**: 4229–4236, 1985.
8. Teodori L, Barlogie B, Drewinko B, Swartzendruber D and Mauro F, Reduction of 1-β-D-arabinofuranosylcytosine and adriamycin cytotoxicity following cell cycle arrest by anguidine. *Cancer Res* **41**: 1263–1270, 1981.
9. Chow K-C, King CK and Ross WE, Abrogation of etoposide-mediated cytotoxicity by cycloheximide. *Biochem Pharmacol* **37**: 1117–1122, 1988.
10. Sullivan DM, Latham MD and Ross WE, Proliferation-dependent topoisomerase II content as a determinant of antineoplastic drug action in human, mouse and Chinese hamster ovary cells. *Cancer Res* **47**: 3973–3979, 1987.
11. Potten CS and Hendry JH, The microcolony assay in small intestine. In: *Cell Clones, Manual of Mammalian Cell Techniques* (Ed. Potten CS), pp. 50–60. Churchill Livingstone, Edinburgh, 1985.
12. Potten CS, Chwalinski S, Swindell R and Palmer M, The spatial organization of the hierarchical proliferative cells of the crypts of the small intestine into clusters of synchronized cells. *Cell Tissue Kinet* **15**: 351–370, 1982.
13. Potten CS, Kellet M, Roberts SA, Rew DA and Wilson GD, The measurement of *in vivo* proliferation in human colorectal mucosa using bromodeoxyuridine. *Gut* **33**: 71–78, 1992.
14. Bachur NR, Leone Moore A, Bernstein JG and Liu A, Tissue distribution and deposition of Daunomycin in mice: fluorometric and isotopic methods. *Cancer Chemother Rep* **54**: 89–94, 1970.
15. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Flint N, Cove FL and Evans G, A low temperature method for the isolation of small intestinal epithelium

along the crypt–villus axis. *J Biochem* **280**: 331–334, 1991.
17. Vindeløw LL, Christensen IJ and Nissen NI, A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* **3**: 323–327, 1983.
18. Wyllie AH, Cell death. *Int Rev Cytol* **517**: 755–785, 1987.
19. Potten CS and Loeffler M, Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110**: 1001–1020, 1990.
20. Potten CS, Extreme sensitivity of some intestinal crypt cells to X and γ-irradiation. *Nature* **269**: 518–521, 1977.
21. Lieberman MW, Verbin RS, Landay M, Liang H, Farber E, Lee T-N and Starr R, A probable role for protein synthesis in the intestinal epithelial cell damage induced *in vivo* by cytosine arabinoside, nitrogen mustard or X-irradiation. *Cancer Res* **30**: 942–951, 1971.
22. Verbin RS, Diluiso G and Farber E, Protective effects of cycloheximide against 1-β-D arabinosylcytosine-induced intestinal lesions. *Cancer Res* **33**: 2086–2092, 1973.
23. Ben-Ishay Z and Farber E, Protective effects of an inhibitor of protein synthesis, cycloheximide, on bone marrow damage induced by cytosine arabinoside or nitrogen mustard. *Lab Invest* **33**: 478–490, 1975.
24. Galili U, Leizerowitz R, Moreb J, Gamliel H, Gurfel D and Polliack A, Metabolic and ultrastructural aspects of the *in vitro* lysis of chronic lymphocyte leukaemia cells by glucocorticoids. *Cancer Res* **42**: 1433–1440, 1982.
25. Dethlefsen LA, Riley RM and Lehman CM, Delayed enhanced effects of adriamycin on the X-irradiation-induced gastrointestinal toxicity in mice. *Radiat Res* **100**: 157–170, 1984.
26. Israel M, Idriss JM, Koseki Y and Khetarpal VK, Comparative effects of adriamycin and DNA-non-binding analogues on DNA, RNA and protein synthesis *in vitro*. *Cancer Chemother Pharmacol* **20**: 277–284, 1987.
27. Dethlefsen LA and Riley RM, The effects of adriamycin on murine duodenal crypt cell proliferation. *Int J Radiat Oncol Biol Phys* **5**: 501–506, 1979.
28. Burholt DR, Hagemann RF, Schenken LL and Lesher S, Influence of adriamycin and adriamycin-radiation combination on jejunal proliferation in the mouse. *Cancer Res* **31**: 22–27, 1977.
29. Barranco SC, Cellular and molecular effects of Adriamycin on dividing and non-dividing cells. In: *Cell Cycle Effects of Drugs* (Ed. Dethlefsen LA), pp. 251–268. Pergamon Press, Oxford, 1986.
30. Sorrentino V, Battistni A, Curatola AM, DiFrancesco P and Rossi GB, Induction and/or selective retention of proteins in mammalian cells exposed to cycloheximide. *J Cell Physiol* **125**: 313–318, 1985.