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EFFECTS OF ADRIAMYCIN AND ETOPOSIDE ON THE REPLICATION OF ADENOVIRUS 5 IN SENSITIVE AND RESISTANT HUMAN TUMOUR CELLS

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Abstract—Adenovirus is a potential probe for identifying and understanding drug sensitivity in primary, nonproliferating cultures of human normal and tumour cells but the scope and limitations of such an approach first need to be evaluated in established cell lines. For this purpose we have identified an ovarian tumour cell line (CI-80-13S) with natural resistance to adriamycin, etoposide and crosslinking agents compared with other human tumour lines. Resistance to adriamycin correlated poorly with resistance to etoposide in these cell lines ($r = 0.05$). Adenovirus replication in drug-treated cells (viral capacity) was found to be differentially inhibited in sensitive cells when the drug was administered to cells simultaneously with infection (adriamycin) or 20 hr after infection (etoposide). Viral capacity could not be inhibited by more than 90% in sensitive cells. In contrast, no such plateau was exhibited in the dose–responses of cell survival or inhibition of cellular DNA synthesis, both of which distinguished sensitive from resistant cells. Adenovirus was not inactivated by preincubation with high doses of adriamycin or etoposide, thus confirming that no functionally-relevant damage is directly induced by these agents in DNA. Uptake of adriamycin and etoposide was similar in sensitive and resistant cells and both agents blocked cells in the G2 phase of the cell cycle. Protein-linked DNA was induced in sensitive cells. The results indicate that (a) these drugs have two dose-dependent effects in cells, one of which does not inhibit replication of adenovirus; and (b) inhibition of adenovirus replication could in principle be used to predict sensitivity to adriamycin and etoposide.

Chemotherapy of ovarian cancer achieves response rates of up to 50% with crosslinking agents but resistance often develops [1], even with multidrug protocols such as PAC‡ therapy [2]. To minimise side-effects and the induction of resistant tumour cells, it would be desirable to use only those agents to which the individual tumour is sensitive. There have therefore been many attempts to devise *in vitro* assays for predicting the chemosensitivity of human tumours, including ovarian cancer [3, 4]. This approach, together with attempts to overcome natural or induced resistance to present agents, remains an important aspect of improving current therapy.

Adriamycin and etoposide, used for first or second line therapy in ovarian cancer, have dissimilar structures but both appear to interact with topoisomerase II leading to the formation of protein-associated DNA strand breaks [5–16]. It is not clear how these breaks, which disappear after removal of the drug, are related to subsequent events such as inhibition of DNA synthesis, blocking in G2 and chromosome aberrations [17–20]. Resistance to this class of agent has been associated in various cell lines with lowered intracellular levels of drug due to facilitated efflux, inhibited transport in a multidrug resistant phenotype [21–25], or altered or decreased activity of topoisomerase II [5–7, 11]; there have been few studies of resistance in human cells [12].

Most of the present techniques can only be applied to permanent cell lines, which unfortunately suffer from the disadvantage of being available from few human tumours and which are selected for growth *in vitro*. There is a need for *in vitro* functional methods of accessing the response of a much higher proportion of tumor and normal tissues. Adenovirus may have potential value in this respect, based on studying replication of viral DNA in the target cells as a substitute for studying replication of cellular DNA. The virus has a double-stranded DNA genome which depends on a number of cellular proteins for its replication [26] and could in principle be inhibited by a wide range of antitumor agents due to effects on cell functions. Two complementary approaches are possible, depending on the nature of the agent to be tested. In the HCR assay the virus is treated with drug prior to infection. It is assumed that agents will either have no effect or will damage the viral DNA, thus inhibiting virus replication especially in cells which lack appropriate DNA repair mechanisms. In the VC assay, the cells are treated before or during infection with untreated virus. Virus replication will therefore be inhibited by agents which affect any cellular function needed by the virus (DNA, RNA or protein synthesis). For example, cell lines sensitive to the DNA methylating agent MTIC replicated drug-treated virus poorly (inhibited

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‡ Abbreviations used: PAC, cyclophosphamide, adriamycin and cisplatin; HCR, host cell reactivation; VC, viral capacity; ID, infectious dose; PBS, phosphate-buffered saline, pH 7.2; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide.

HCR) because of a deficiency in cellular DNA repair; and cell lines sensitive to inhibitors of DNA synthesis (hydroxyurea and deoxyadenosine) replicated virus poorly (inhibited VC) when the cells were treated with drug during infection with untreated virus because viral DNA synthesis relies on cellular nucleotide metabolism [27].

An advantage of this approach is that the epigenetic action of an agent can be distinguished from the consequences of its direct damage to the genome. This is possible because in the HCR assay, excess drug can be removed by dilution and/or dialysis before treated virus is given to the cells. Intracellular virus is necessarily exposed to drug in the VC assay, but inhibition of cell metabolism and virus replication is achieved at drug levels 100–1000-fold less than those required to directly inactivate viral DNA [27]. Since viral DNA damage is negligible, inhibition of virus replication in the VC assay is considered to result from epigenetic effects of the drug.

While limitations in this approach are expected, the relative simplicity and detailed knowledge of adenovirus biochemistry have made the system a useful model for elucidating basic processes in eukaryotic cells [28]. Of critical importance to the proposed application is the fact that adenovirus replicates well in human normal and tumour cells, whether proliferating or not, and can be readily quantitated two days after infection on an individual cell basis [27]. Thus for the purpose of determining the chemosensitivity of a primary culture, virus assays have the potential for accessing the response of all of the cells present and, by morphological or immunological markers, of allowing tumor cells to be distinguished from normal cells.

The aim of the present work was to identify a panel of sensitive and resistant human cell lines, for use in evaluating adenovirus as a probe for predicting sensitivity to adriamycin and etoposide in primary cultures, and for investigating the mechanism of action of these drugs.

MATERIALS AND METHODS

The origin of Hela-S_3 and the human melanoma cell lines MM96L, MM127, MM138, MM253cl and MM418 have been described [27, 29]. MM138L is a late passage subline of MM138. MM474F is a strain of normal fibroblasts established from a human melanoma biopsy. HET is a strain of human embryonic tongue fibroblasts (Commonwealth Serum Laboratories, Melbourne, Australia) provided by Dr K. A. O. Ellem. The ovarian tumour cell line CI-80-13S [30] was provided by Dr R. Bradley, Cancer Institute, Melbourne.

Cells were cultured in 5% CO_2/air at 37° in Roswell Park Memorial Institute medium 1640 (Flow Laboratories, Sydney, Australia) supplemented with 1 mM pyruvate, 200 μM nicotinamide, 100 IU/ml penicillin, 100 μg/ml streptomycin, 3 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid and 10% fetal calf serum. Assays for *Mycoplasma* by culture on agar were negative.

Due to failure of the CI-80-13S and MM127 lines to form discrete colonies cell survival was determined by a modified colony assay. This method, which gives similar results to visual counting of colonies for a variety of agents [31, 32], involved addition of drug to cells (2×10^3/16-mm well) plated on the previous day, and, after 5–7 days of continuous exposure, labeling the cultures with [^{3}H]-thymidine for 2–4 hr. Cells were detached with trypsin, lysed with water and harvested onto glass-fibre disks for liquid scintillation counting. The D_{37} (dose required to give 37% survival) was calculated from dose–response curves obtained using 5 doses.

For the virus assays, duplicate cultures (5×10^3/6-mm well) were infected with 10-fold dilutions of adenovirus 5 for 1 hr and then washed once with medium. Viral replication was determined after 2 days by counting the number of virus-infected, immunoperoxidase-labeled cells, a method which detects inhibition of viral replication as reliably as plaque assay [27]. One ID was defined as the amount of virus required to produce one infected cell.

Cell DNA synthesis was determined by prelabeling cultures (5×10^4 cells/16-mm well) with [2-C^{14}]thymidine (0.005 μCi/ml; 20 Ci/mol) for 24 hr, followed by drug treatment for the periods described in the text and then pulse labeling for 45 min with [methyl-^{3}H]thymidine (5 μCi/ml; 40 Ci/mmol). The cells were detached with trypsin, lysed and harvested onto glass-fibre discs with H_2O and solubilised in Soluene 350 (Packard Instruments, Zurich, Switzerland) prior to liquid scintillation counting. The $^3H/^{14}C$ ratio was expressed as a percentage of untreated controls harvested at the same time.

Flow cytometry of DNA and adriamycin fluorescence was performed with a FACS IV instrument (Becton–Dickinson FACS Systems, Sunnyvale, CA) operated at 488 nm. DNA histograms were obtained by staining with propidium iodide [33]. Adriamycin fluorescence was determined [22, 34] in cells treated as monolayers and then washed in ice-cold PBS, detached with 0.02% trypsin in PBS and kept at 0° for analysis.

The uptake and efflux of etoposide were determined by treating cell monolayers (5×10^5 per 60-mm dish seeded 24 hr previously) with 1 ml of medium containing 10 μM [^{3}H–G]-etoposide (300 Ci/mole; Moravek Biochemicals, Brea, CA) at 37°. At various times, the cells were washed rapidly by aspiration with ice-cold PBS (3×5 ml) and detached with 0.5 ml 0.02% trypsin in PBS. The cell number was determined (hemacytometer) and the suspension transferred to a scintillation vial. The plate was washed with 250 μl of H_2O and the combined solutions lysed with Sarkosyl (0.2% final concentration). Instagel (4 ml; Packard Instruments, Zurich, Switzerland) was added and the dpm of the mixture determined by liquid scintillation counting. Blanks (plates without cells) typically gave values of 400–600 dpm.

For determination of protein-linked DNA, cells (10^5 per 16-mm well) were labeled overnight with [^{3}H-methyl]thymidine (0.8 μCi/ml, 40 Ci/mmole) and treated with drug for 2 hr. The cell monolayer was lysed in 1 ml of lysis buffer (1.25% SDS, 0.4 mg/ml DNA and 5 mM EDTA, pH 8) at 65°, transferred to a microfuge tube containing 250 μl of 325 mM KCl and vortexed for 10 min. After cooling in ice for 10 min and centrifugation (microfuge) the pellet was

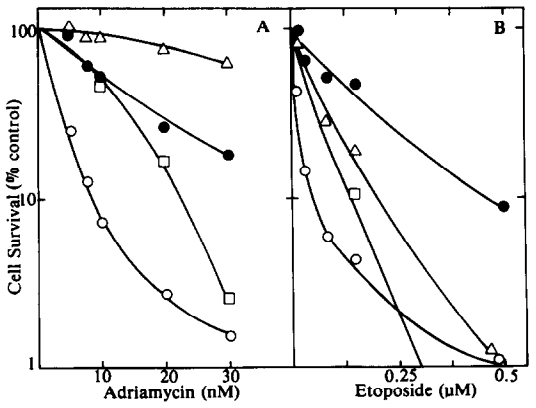

Fig. 1. Dose–response for cell survival in human tumour cell lines. (A) Adriamycin. ●, CI-80-13S; □, HeLa; ○, MM127; △, MM138L. (B) Etoposide. Symbols as in (A). Points are means of duplicates.

resuspended in wash buffer (100 mM KCl, 0.1 mg/ml DNA and 1 mM EDTA, pH 8) incubated at 65° for 10 min, cooled and centrifuged as above. After a second identical washing procedure, the pellet was dissolved in 200 μl H_2O and mixed with 4 ml Instagel (Packard Instruments, Zurich, Switzerland) for scintillation counting.

Table 1. Toxicity of adriamycin and etoposide in human tumour cell lines

Cell	D_{37}* Adriamycin (nM)	D_{37}* Etoposide (μM)
CI-80-13S	13 ± 3†	0.26 ± 0.03
HeLa	14 ± 3	0.083 ± 0.01
HET	29	0.13
MM474F	NT‡	0.037
Melanoma		
MM96L	16 ± 4	0.26 ± 0.07
MM127	3.4 ± 0.5	0.028 ± 0.007
MM138L	77	0.11 ± 0.02
MM253cl	19	0.143
MM418	28 ± 4	0.24 ± 0.04

* Dose required to reduce survival to 37%.
† Mean and SE for separate experiments (N = 2–5).
‡ NT, not tested.

RESULTS

Cell survival

Preliminary experiments showed that assessment of cell survival by [^{3}H]thymidine labeling after prolonged drug treatment correlated with the visual observation that sensitive cells rounded and detached from the plastic surface at doses which did not affect resistant cells. Dose–response curves for cell survival showed that 3 human tumour cell lines (CI-81-13S, MM96L and MM418) were resistant to both adriamycin and etoposide when compared with 4 other cell lines and human fibroblasts (Fig. 1 and Table 1). One cell line (MM138L) was very resistant to adriamycin but not to etoposide, and the MM127 line was highly sensitive to both agents. Overall, there was no correlation between the D_{37} values for adriamycin and etoposide ($r = 0.05$).

Comparison of other agents (Table 2) showed that CI-80-13S was significantly more resistant to nitrogen mustard derivatives (chlorambucil and melphalan) than other cell lines, but was not resistant to MTIC, hydroxyurea or vincristine. HeLa cells were exceptionally sensitive to MTIC and hydroxyurea, as reported previously [35]. Further studies were carried out with CI-80-13S and MM127, cell lines resistant or sensitive respectively to the topoisomerase II inhibitors.

Virus replication

Two types of assay were performed. In the HCR assay, virus was treated with high levels of adriamycin (0–100 μM) or etoposide (0–1 mM) in culture medium for 24 hr, dialysed to remove excess drug and then used to infect cells. No significant decrease in viral replication was found in MM127 or CI-80-13S cells compared with controls (results not shown), indicating that no functional damage was directly induced in viral DNA.

The VC assay tested the ability of drug-treated cells to replicate untreated adenovirus. Preliminary experiments determined the maximum drug levels which allowed cells to remain attached for subsequent analysis. The time period was chosen after consideration of the temporal response of cellular DNA synthesis to the drugs (see below), and of the fact that viral DNA synthesis occurs 12–24 hr after infection, at which time cellular proliferation and DNA synthesis has ceased [26]. A combined dose/treatment time response was carried out, comparing resistant CI-80-13S with sensitive MM127 cells (Fig. 2). The overall results showed that drug treatment

Table 2. Sensitivity of human tumour cell lines to various agents

Cell line	D_{37} (μM) Chlorambucil	Melphalan	MTIC	Vincristine	Hydroxyurea
CI-80-13S	6.9	4.9	430	4.0	320
HeLa	2.3	1.2	50	NT*	60
MM127	1.8	1.7	450	7.5	700
MM96L	1.4	1.7	550	6.6	300
MM418	NT	2.6	NT	NT	NT

* NT, not tested.

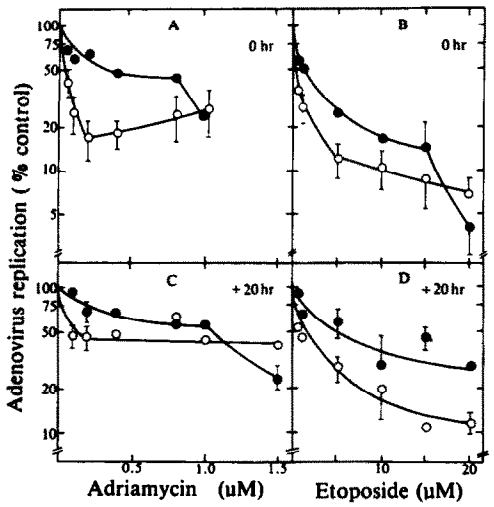

Fig. 2. Dose–response for inhibition of viral capacity in CI-80-13S (●) and MM127 cells (○). (A) Adriamycin added to cultures immediately after infection. (C) Adriamycin added 20 hr after infection. (B) Etoposide added immediately after infection. (D) Etoposide added 20 hr after infection. Points are means of 3 separate experiments. Bars, SE.

of cells before or after infection inhibited VC by up to 90% in both cell lines. The possibility of further inhibition could not be tested in CI-80-13S because the cells detached at higher drug doses and were lost for analysis. The MM127 line, however, demonstrated a distinct dose plateau at this level of inhibition. At low doses, MM127 cells showed differential inhibition of viral capacity compared with CI-80-13S when treated with adriamycin immediately after infection (Fig. 2A) or when treated with etoposide 20 hr after infection (Fig. 2D). No significant difference between the 2 cell lines was found with the other combinations (Figs. 2B and 2C) or when the drugs were added 24 hr before infection (results not shown).

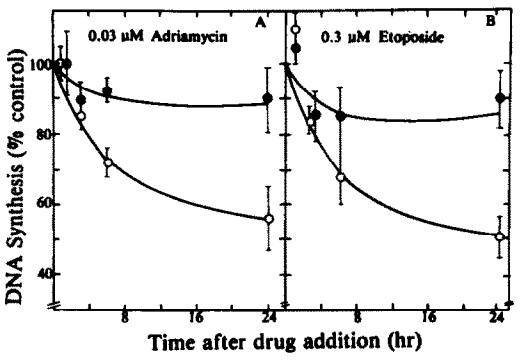

Fig. 3. Temporal response of inhibition of DNA synthesis in CI-80-13S (●) and MM127 (○) cells: (A) Adriamycin (10 μM); (B) etoposide (1 μM). Points are means of duplicates.

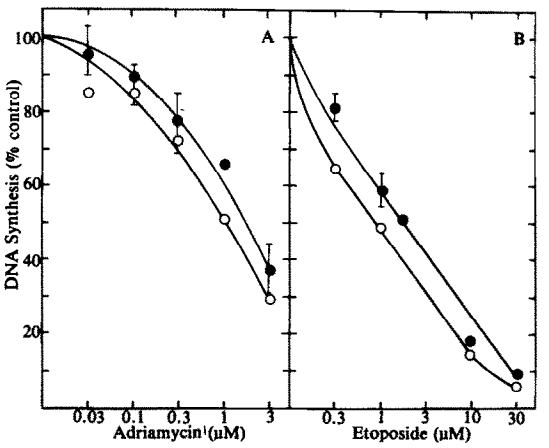

Fig. 4. Dose–response for inhibition of DNA synthesis in CI-80-13S (●) and MM127 cells (○), after a 3 hr treatment with drug: (A) Adriamycin; (B) etoposide. Points are means of duplicates.

Cellular DNA synthesis and the cell cycle

Cellular DNA synthesis, as judged by incorporation of [^{3}H]thymidine, was gradually inhibited during exposure to adriamycin and etoposide (Fig. 3). In treatments longer than 3 hr the sensitive MM127 cells were inhibited more than CI-80-13S. A dose–response study carried out 3 hr after treatment showed that DNA synthesis was inhibited to a similar extent in both cell lines over the whole of the dose range (Fig. 4), as found also in sensitive and resistant Ehrlich ascites tumor cells [36].

The effects of adriamycin and etoposide on cell cycle progression was determined by flow cytometry. As found previously in other cell lines [17–20], both drugs arrested cells in the G2 phase during a 48-hr treatment period (Fig. 5). Stathmokinetic experiments involving simultaneous addition of colcemid (1–5 μg/ml) to arrest cells in G2/M did not detect any blocking by adriamycin or etoposide in G1 (results not shown); and with colcemid alone confirmed that Hela cells (>95% arrested in G2/M after

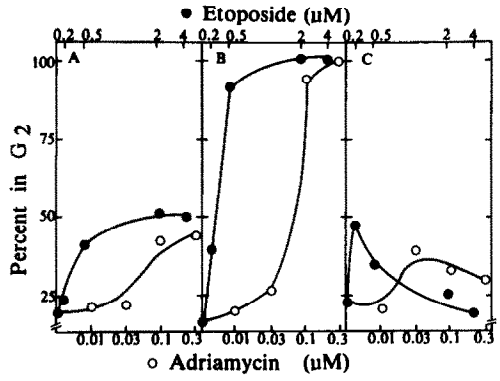

Fig. 5. Cell cycle arrest in G2 after 24 hr treatment with adriamycin (○) or etoposide (●): (A) CI-80-13S; (B) HeLa, (C) MM127.

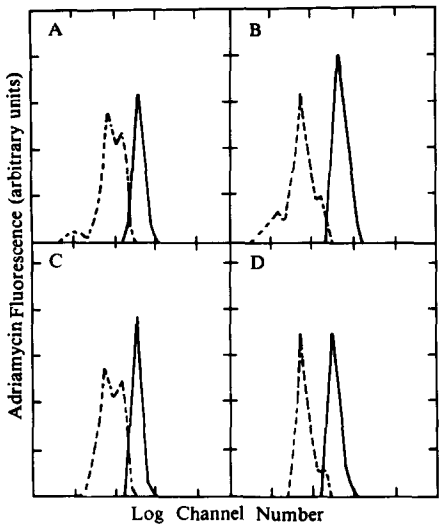

Fig. 6. Accumulation of adriamycin by cells as detected by flow cytometry: (A) CI-80-13S; (B) MM127; (C) HeLa; (D) MM138L. Broken line, no drug. Solid line, cell monolayers treated with 10 μM adriamycin for 1 hr.

24 hr) cycled more rapidly than CI-80-13S (40%) or MM127 (30%). This accounts for HeLa showing the highest proportion of cells blocked in G2. MM127 cells exhibited a G2 block which could only be detected at low doses; the results at higher doses are not representative because > 80% of cells were not recovered, presumably due to detachment or lysis.

Accumulation of adriamycin and etoposide

The fluorescent properties of adriamycin enable uptake to be compared on an individual cell basis by flow cytometry [22, 34]. The cells were treated with drug as monolayers and harvested by scraping to minimise any artifactual effects of trypsin detachment and treatment in suspension. Preliminary experiments with the latter method yielded qualitatively similar results but the total uptakes by most cell lines were much higher (results not shown). As judged by the increase in peak channel number for fluorescence, accumulation of adriamycin during a 1-hr exposure was similar in all 4 lines tested: MM127, CI-80-13S, MM138L and HeLa (Fig. 6).

Etoposide accumulation and efflux was determined in cell monolayers using the ^{3}H-labeled compound. There was no correlation between accumulation and cell sensitivity, whether cell lines were compared on the basis of the amount of drug per cell or, more appropriately, on the concentration ratio (Table 3). When the drug was removed from the culture medium, the intracellular level fell to background values during the subsequent 2 hr in all 4 cell lines.

Table 4. Induction of protein-linked DNA in drug-treated cells

	^{3}H precipitated (% control dpm)*	
Cell line	Adriamycin (1 μM)	Etoposide (10 μM)
CI-80-13S	109	68
MM127	187	217

* Controls 10,000 dpm.

Induction of protein-linked DNA

Cells were prelabeled with ^{3}H-thymidine, treated with drug and the SDS–KCl precipitable radioactivity was determined [37] as a measure of the protein-linked DNA breaks [9, 38]. The level of such material in untreated control cells was higher than in previous studies [9, 38], possibly because the high DNA content of the present cell lines (nearly tetraploid) prevented sufficient shearing prior to the precipitation step. The precipitate was increased by adriamycin and etoposide in the sensitive MM127 line but not in CI-80-13S (Table 4).

DISCUSSION

The drug-resistant phenotype comprising natural resistance to topoisomerase II inhibitors and cross-linking agents and found in the ovarian tumour cell line CI-80-13S appears to be similar in some respects to that reported previously in some ovarian [39–40]

Table 3. Accumulation and retention of 10 μM ^{3}H-etoposide by human tumour cells

		Accumulation			
		pmol/cell × 10^3 after 1 hr exposure	Concentration ratio*		Retention† (%)
Cell line	Cell volume (μm^3)		1 hr	3 hr	
CI-80-13S	2730	6.55	4.78 ± 0.76‡	3.89 ± 0.24	<8
MM127	2930	3.21	2.23 ± 0.17	4.32 ± 0.09	<8
MM138L	1570	4.05	5.25 ± 0.29	2.71 ± 0.11	<8
HeLa	1930	2.64	2.79 ± 1.59	0.87 ± 0.06	<8

* Concentration inside the cell divided by the concentration oustide the cell, determined after various exposure times.

† ^{3}H dpm retained by cells treated for 1 hr and incubated in fresh medium for a further 2 hr, expressed as % of 1 hr level.

‡ Mean and SD of duplicates.

and glioma [41] cell lines. It was distinguished from the transport-dependent alkaloid/adriamycin-resistant phenotype [24] by sensitivity to vincristine and lack of altered drug transport. Clinical resistance to the former two classes of drug is frequently observed in ovarian cancer [1, 2], yet the biochemical basis for resistance, including the functional relationship, if any, between two apparently diverse mechanisms of action, remains unknown.

This study focussed on comparing the action of topoisomerase II inhibitors in cells of different natural sensitivities with emphasis on using adenovirus as a probe for investigation. However, due to the parallel comparison of the toxicity of adriamycin and etoposide in a range of human cells in this study, several observations were made which are of more general interest. First, the variable degree of cross-resistance between adriamycin and etoposide, found also in other systems [12, 36, 39–41], indicates that each drug in this class of agent may require individual evaluation. It is possible that resistance to the DNA intercalating topoisomerase inhibitor adriamycin in a cell line such as MM138L arises from a difference in DNA binding, whereas for etoposide, which does not intercalate, resistance may arise from an altered enzyme activity [12]. Secondly, a tumour cell line was identified (MM127) which was highly susceptible to both agents. Sensitive also to deoxyadenosine and its 2-halogeno analogues [43], these cells may be unable to deal with topological alterations resulting from DNA–protein crosslinks and strand breaks. Thirdly, the finding that fibroblasts were as sensitive as some of the tumor cell lines diminishes the possibility that transformed cells are intrinsically more susceptible to topoisomerase II inhibitors than normal cells [6]. This question should now be addressed by comparing tumour with clinically-relevant normal cells.

SV-40 virus previously served as a model genome for identifying topoisomerase II as a target of etoposide in monkey cells [8]. New evidence concerning the mechanism of action of adriamycin and etoposide was obtained in the present study of adenovirus replication in sensitive and resistant human cells. The failure of the drugs to inactivate virus by direct treatment in the HCR assay showed that DNA was not functionally damaged. The epigenetic effects of the drugs on viral replication in treated cells (VC assay) were then examined. At low doses, inhibition of VC closely paralleled the difference in cellular sensitivity, indicating a mechanism associated with inhibition of cellular DNA synthesis. Experiments with temperature-sensitive mutants have failed to detect any requirement by adenovirus for topoisomerase II [44, 45]. Adenovirus replication requires cellular topoisomerase I [46, 47]; and etoposide-resistant Chinese hamster ovary cells were found to have a DNA linking activity which co-purified with topoisomerase I, with no significant alteration in topoisomerase II [16]. Furthermore, topoisomerase II is a chromatin scaffold protein [48], a function which may not be required for replication of adenovirus. The low-dose sensitivity mechanism in MM127 identified by the VC assay may therefore be associated with a saturable target which is not topoisomerase II.

The fact that VC but not cell survival reaches a plateau in sensitive cells indicates that these drugs might have at least two dose-dependent modes of cytotoxic action on cells, one of which does not affect adenovirus replication. The latter, high-dose mechanism may well involve topoisomerase II because protein-linked DNA was induced in sensitive cells. It should now be possible to clarify such aspects by comparing the dose–responses of protein-associated DNA breaks in cellular and viral DNA.

With appropriate drug doses and treatment periods, the results of the VC assay correlated with cellular sensitivity to adriamycin and etoposide in the cell lines studied. The difference between sensitive and resistant cells occurred over a narrower dose range than that obtained by measuring inhibition of cell DNA synthesis 24 hr after treatment but should be sufficient to detect clinically-sensitive tumours. Primary cultures of ovarian tumours incorporate ^{3}H-thymidine poorly but support the replication of adenovirus 5 (unpublished results). It should therefore be feasible to determine the predictive value of the VC assay in a suitable series of patients.

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