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Overproduction of topoisomerase II in an ataxia telangiectasia fibroblast cell line: comparison with a topoisomerase II-overproducing hamster cell mutant

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ABSTRACT

Ataxia telangiectasia (AT) cell lines are characterised by their hypersensitivity to ionizing radiation and bleomycin, and their failure to inhibit DNA synthesis after DNA damage. A recent report [Singh et al. (1988) Nucl. Acids Res. **16**, 3919-3929] indicated that a reduction in topoisomerase II (topo II) activity was a feature of AT lymphoblast cell lines. We have studied the possible role of DNA topoisomerases in determining the phenotype of an AT fibroblast cell line. AT5BIVA cells are sensitive to the topo II inhibitors etoposide (VP16) and amsacrine (m-AMSA), compared to normal human fibroblasts (MRC5-V1 and VA13). AT5BIVA cells express a 3-fold higher level of topo II protein than MRC5-V1 cells, and 6-fold higher than VA13. This is reflected in elevated topo II activity in AT5BIVA cells. Untransformed AT5BI cells also show elevated topo II activity compared to untransformed normal cells. The extent of overproduction of topo II in AT5BIVA cells is comparable with that seen in a mutant Chinese hamster cell line, ADR-1, which is similarly hypersensitive to both bleomycin and topo II inhibitors. However, ADR-1 cells show neither hypersensitivity to ionizing radiation nor abnormal inhibition of DNA synthesis following DNA damage. Topo II overproduction per se does not appear sufficient to generate an "AT-like" phenotype.

AT5BIVA cells express a reduced level of topoisomerase I (topo I) and are hypersensitive to the topo I inhibitor, camptothecin. ADR-1 cells express a normal level of topo I, indicating that a reduction in the level of topo I is not the inevitable consequence of an elevation in topo II.

INTRODUCTION

AT is a rare, autosomal recessive disease displaying a wide variety of phenotypic characteristics. AT patients suffer neurological degeneration, immunological deficiencies and a high incidence of cancers, particularly lymphoid malignancies (1). Hypersensitivity to ionizing radiation is also a major feature (2,3). Cell lines derived from AT patients retain their hypersensitivity to ionizing radiation and the radiomimetic agent bleomycin (4,5), and are further characterised by their failure to inhibit DNA synthesis after radiation or chemical damage (6,7,8). AT cell lines have also been shown to exhibit hypersensitivity to certain cytotoxic drugs whose

action is mediated principally via an interaction with the nuclear enzyme topo II (8).

DNA topoisomerases appear to be involved in several aspects of DNA metabolism, in particular genetic recombination, DNA transcription, and chromosome organisation and segregation (9). For this reason, it is possible that both the hypersensitivity to DNA damaging agents and the radioresistant DNA synthesis seen in AT cells are a consequence of an abnormality in topo I or topo II.

Type II topoisomerases, including the mammalian topo II, catalyse the concerted breakage and rejoining of double-stranded DNA. An intermediate in the reaction, termed the 'cleavable complex', consists of an enzyme subunit bound via a covalent link to the 5' phosphoryl end of each DNA strand (10). Hence, to reveal these strand breaks requires deproteinization. The drugs which depend upon topo II for at least part of their cytotoxic action include the intercalating agents, such as Adriamycin and m-AMSA, and the epipodophyllotoxins, VP16 and VM26 (also termed etoposide and teniposide, respectively). Their mode of action appears to be via stabilization of the 'cleavable complex' resulting in the persistence of potentially cytotoxic, but protein-concealed, strand breaks (11,12). It has been suggested that the alkaloid, camptothecin, acts in an analogous way to these drugs, but via an interaction with topo I (13).

We have investigated the possibility that an abnormality in topo I or topo II is consistent with the major phenotypic differences between AT and normal cell lines. In contrast to a previous report (23) of a reduction in topo II activity in AT lymphoblast cell lines, we find that the AT fibroblast cell line AT5BIVA expresses a high level of topo II protein compared to control fibroblasts. We have compared the phenotype of AT5BIVA cells with that of a hamster cell mutant which similarly overproduces topo II protein.

MATERIALS AND METHODS

Cell Culture and Media.

AT5BIVA and untransformed AT cells were kindly provided by Dr A. Lehmann, MRC Cell Mutation Unit, Sussex. AT5BIVA cells are SV40 transformed fibroblasts from a patient suffering from AT. Normal and AT Cells were routinely maintained in EMEM with 15% FCS. CHO cells were grown in Ham's F10

supplemented with 10% FCS. Cells were routinely tested for mycoplasma contamination and found to be negative.

Survival Curves.

Exponentially growing cells were trypsinised and seeded in 100-mm petri dishes. The cells were allowed to adhere for four hours prior to drug treatment. Following this, cells were allowed to grow for 14 days with medium changes every five days. Colonies were fixed with methanol:acetic acid (3:1), stained with crystal violet and counted. Colonies containing 50 cells or more were counted as survivors.

Filter Binding Assay.

Measurement of the efficiency of covalent binding of topo II to DNA was carried out essentially as described by Minford et al. (14). Nuclear extracts were prepared from cells by the method of Glisson et al. (15), and their protein content determined by the method of Bradford (16). The extracts were incubated with linearized plasmid DNA labeled at the 3' end, together with various concentrations of VP16, at 37^{o}C for 20 minutes. The reactions were stopped by the addition of 20mM EDTA, pH10, and the mixture applied to a polyvinyl chloride filter (Millipore, 2 um pore). The filters were processed as described by Minford et al. (14).

Immunoblotting.

Nuclear extracts, equalised for protein as before, were electrophoresed on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane by electrophoretic blotting. The nitrocellulose was then washed in TTBS (50mM Tris-HCl, pH 7.9, 150mM NaCl, 0.05% Tween-20) containing 3% BSA for 16 hours. The membrane was incubated at room temperature for 24 hours with rabbit anti-serum against either chicken topo I or calf thymus topo II (kindly provided by Dr L.F. Liu) before extensive washing with TTBS. Immunocomplexes were reacted with (^{125}I)-labeled protein-A in TTBS before washing and autoradiography. To confirm that gel loadings and transfers were comparable, a control set of identically loaded gel lanes was stained with Coomassie Blue, as was the gel after electrophoretic transfer. Densitometry of autoradiograms was performed using an LKB laser densitometer.

Inhibition of DNA Synthesis.

Measurement of inhibition of DNA synthesis after treatment with DNA damaging agents was performed essentially as described by Jeggo (17), using a double

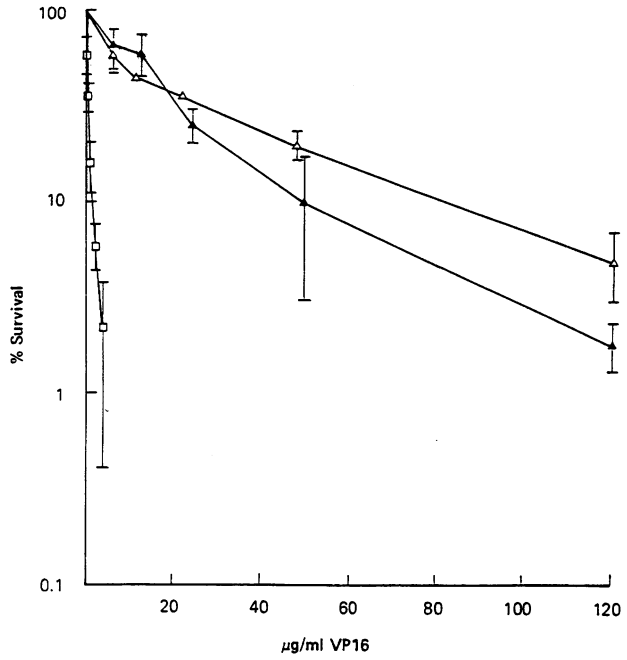

Figure 1
Survival of AT5BIVA (□), VA13 (▲) and MRC5-V1 (△) cells following exposure to VP16 for 1 hour. Points represent the mean of 4 independent experiments. Bars, SE.

label technique. Briefly cells were seeded into 35mm tissue culture dishes and grown in (^{14}C)-thymidine, (0.008 uCi/ml), for 24 hours prior to irradiation or drug treatment. Medium was replaced with warm PBS during irradiation (or sham irradiation of controls). After irradiation, cells were

TABLE 1

SUMMARY OF CELL SENSITIVITIES AND TOPOISOMERASE LEVELS

Cell Line (transformed)	D_{37} values VP16 (ng/ml)	m-AMSA (ng/ml)	campto (nM)	Topo II activity	Relative protein level Topo I	Topo II
VA13	18.5	1.50	160	+	5	1
MRC5-V1	19.5	0.41	2100	+	4	2
AT5BIVA	0.5	0.15	10	++	1	6

campto = camptothecin

incubated with unlabelled medium for 90 minutes, (for 20 minutes after drug treatment), then pulse-labelled with (^{3}H)-thymidine (1 - 2 uCi/ml; 42 Ci/mmole) for 60 minutes. Cells were washed, removed by cell scraping, and the DNA precipitated and collected on filters, as described by Jeggo (17). TCA precipitable radioactivity was determined by scintillation counting.

RESULTS

Sensitivity to topo II and topo I inhibitors

The SV40-transformed AT fibroblast cell line, AT5BIVA, was compared with 2 transformed normal human fibroblast lines, MRC5-V1 and VA13, for sensitivity to topo II inhibitors. The normal fibroblast lines show a similar level of sensitivity to the epipodophyllotoxin, VP16 (Fig. 1). AT5BIVA cells are significantly more sensitive than the normal lines, greater than 20-fold based on D_{37} values (Table 1).

A different pattern of relative sensitivities to the intercalating agent m-

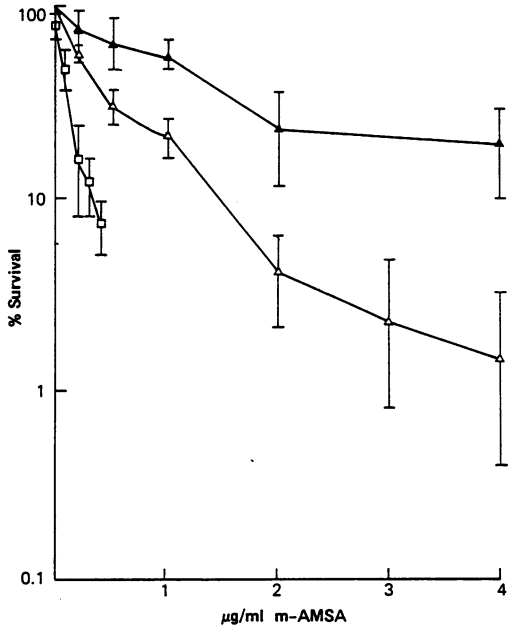

Figure 2
Survival of AT5BIVA (□), VA13 (▲) and MRC5-V1 (△) cells following exposure to m-AMSA for 1 hour. Points represent the mean of 4 independent experiments. Bars, SE.

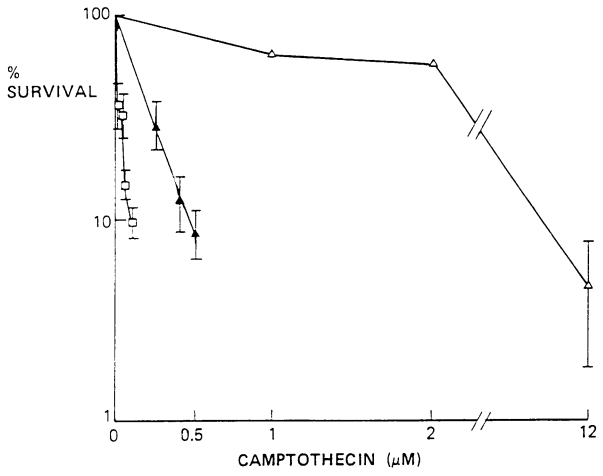

Figure 3
Survival of AT5BIVA (□), VA13 (▲) and MRC5-V1 (△) cells following exposure to camptothecin for 1 hour. Points represent the mean of 4 independent experiments. Bars, SE.

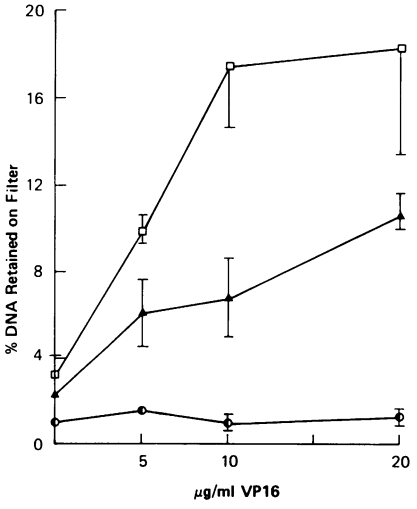

Figure 4
Topo II activity in nuclear extracts from AT5BIVA (□), VA13 (▲) and the untransformed counterpart of VA13, WI38 (◐) cells. Results are expressed as the % DNA retained on the filter as a function of VP16 concentration. Points represent the mean of 3 independent experiments. Bars, SE.

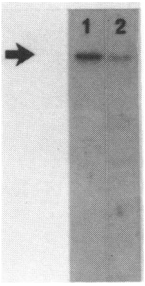

Figure 5
Western blot of nuclear extracts of AT5BIVA (Lane 1) and MRC5-V1 cells (Lane 2) using specific topo II antibodies.

AMSA is seen (Fig. 2). MRC5-V1 cells are around 3-fold more sensitive to this agent than are VA13 cells. AT5BIVA cells are 3- to 4-fold more sensitive than MRC5-V1 cells (Table 1).

The relative sensitivities of normal and AT cells to the topo I inhibitor, camptothecin, are shown in Figure 3. There is a marked difference in sensitivity between MRC5-V1 and VA13 cells, the latter being 13-fold more sensitive than MRC5-V1, based on D_{37} values. AT5BIVA cells are approximately 16-fold more sensitive than VA13 cells (Table 1).

Measurements of topo II activity and protein content

The relative level of topo II activity in normal and AT cells was determined by a specific filter binding assay (14). This assay measures the covalent binding of topo II to the 5' termini of radiolabeled duplex DNA as a function of the concentration of a topo II inhibitor. The amount of DNA retained on PVC filters is proportional to topo II activity (14). Figure 4 shows that nuclear extracts from AT5BIVA cells contain a higher level of topo II activity than those from the normal fibrobast line, VA13. This figure also shows that an extract from the untransformed counterpart of VA13, designated WI38, gives essentially no detectable topo II activity (see section below). The level of topo II activity in MRC5-V1 cells was comparable with that in VA13 cells (data not shown).

To determine whether this elevation in activity in AT5BIVA cells was due to an increase in topo II protein, Western analysis was performed on nuclear extracts from AT5BIVA and normal cells. The result (Fig. 5) shows that the elevated topo II activity in AT5BIVA cells is reflected in an approximately 3-fold increase in topo II protein in this line relative to MRC5-V1 cells.

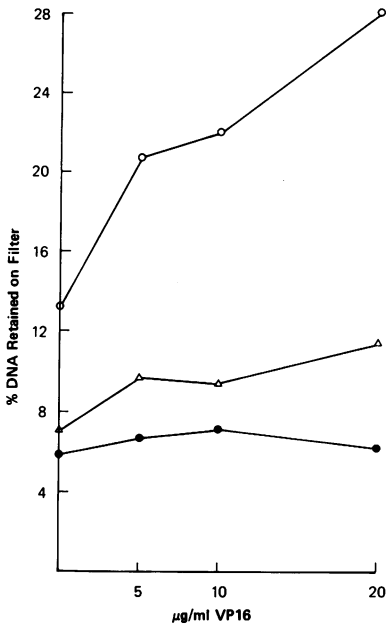

Figure 6
Topo II activity in nuclear extracts from untransformed AT5BI cells (○), untransformed AT4BI cells (●) and an untransformed human fibroblast cell (△). Points represent the mean of 2 experiments.

Figure 7 (see section below) shows that AT5BIVA cells contain an approximately 6-fold higher level of topo II protein than VA13 cells. These data are summarized in Table 1.

Effects of transformation on topo II expression

It has previously been reported that SV40 transformation of cells increases their topo II content (18). This raised the possibility that any change in topo II expression in AT5BIVA cells was due to an artefact of transformation. Hence, we measured topo II activity and topo II protein levels in untransformed normal and AT fibroblast cells. Figure 6 shows that AT5BI, the untransformed parent of AT5BIVA cells, maintains a higher level of topo II activity relative to an untransformed normal human fibroblast and to a control untransformed AT fibroblast, AT4BI.

Western blots of a matched pair of transformed (VA13) and untransformed (WI38) normal fibroblasts are shown in Figure 7. For comparison, transformed AT5BIVA cells are included. While VA13 cells show an approximately 6-fold

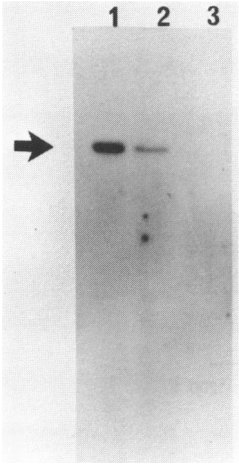

Figure 7
Western blot of nuclear extracts of AT5BIVA cells (Lane 1), VA13 cells (Lane 2) and WI38 cells (Lane 3) using specific topo II antibodies.

lower topo II content than AT5BIVA cells, the equivalent untransformed normal human line, WI38, contains an undetectable level of topo II. This result accords with the topo II activity measurements on VA13 and WI38 cells (see Fig. 4, section above). The level of topo II in nuclear extracts was consistently below detection by Western analysis in all of the untransformed normal and AT cells that we tested.

Comparison of AT and ADR-1 cells

ADR-1 is a Chinese hamster ovary (CHO) cell mutant which is hypersensitive to bleomycin and topo II inhibitors, and which overexpresses topo II protein by around 3-fold (19). Both of these features are seen in AT5BIVA cells. We wished to determine whether any abnormality of DNA synthesis inhibition after DNA damage is seen in ADR-1 cells. In AT lines, there is a failure to inhibit DNA synthesis after either ionizing radiation (6) or Adriamycin (8) treatment. It is clear from a comparison of ADR-1 and parental CHO-K1 cells that no abnormality in DNA synthesis inhibition is evident after X-ray (Fig. 8) or Adriamycin (data not shown) treatment of ADR-1 cells. It should be noted that ADR-1 cells show normal levels of resistance to X-irradiation (20).

Measurement of topo I protein content and activity

The striking hypersensitivity of AT5BIVA cells to camptothecin suggested a possible abnormality in topo I. We therefore measured the cellular level of topo I by Western blot analysis. The result (Fig. 9) shows that AT5BIVA

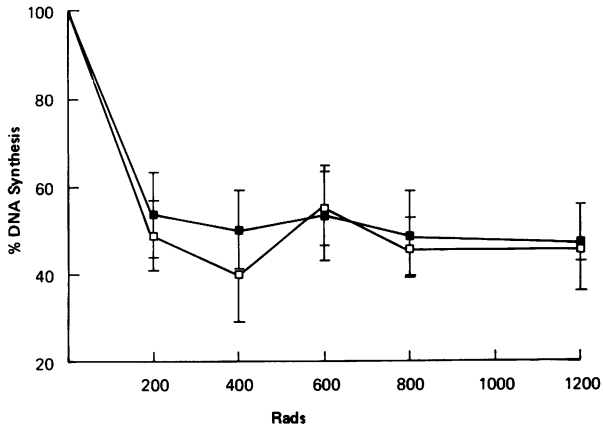

Figure 8
Inhibition of DNA synthesis in CHO-K1 (□) and ADR-1 (■) cells by X-irradiation. Results are expressed as the percentage of DNA synthesis relative to unirradiated controls. Points represent the mean of 3 independent experiments. Bars, SE.

cells show a 3 - 6-fold reduction in topo I compared with VA13 and MRC5-V1 cells. The levels of topo I enzyme activity, determined by ATP-independent plasmid relaxation, accord with the protein estimations by Western analysis (data not shown). Moreover, the topo I activity from AT5BIVA cells does not appear to be abnormally sensitive or resistant to the inhibitory effects of camptothecin in this assay (data not shown).

AT5BIVA cells overexpress topo II while underexpressing topo I protein. We

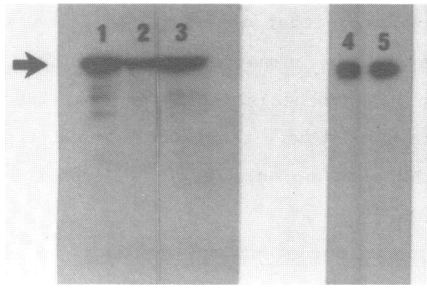

Figure 9
Western blot of nuclear extracts of VA13 (Lane 1), AT5BIVA (Lane 2), MRC5-V1 (Lane 3), ADR-1 (Lane 4) and CHO-K1 (Lane 5) cells, using specific topo I antibodies.

considered the possibility that an increase in the level of topo II inevitably leads to a reduction in topo I level. However, Figure 9 shows that ADR-1 cells express the same level of topo I protein as parental CHO-K1 cells, despite overexpressing topo II protein.

DISCUSSION

A possible role for DNA topoisomerases in the phenotype of AT cells has previously been suggested from both circumstantial and experimental evidence. A defect in some aspect of DNA metabolism is a consistent feature of AT cell lines. In particular, hypersensitivity to ionizing radiation and to several DNA damaging drugs is seen (4,8,21,22), as is a failure to inhibit DNA synthesis after radiation or chemical damage (6,7,8). The DNA topoisomerases, with their roles in DNA transcription, recombination and replication, are thus candidates for an involvement in these phenomena. Strong experimental evidence for a defect in topo II in AT cells has recently been provided by Singh *et al.* (23), who showed that topo II activity was up to 10-fold reduced in 5 complementation groups of AT lymphoblasts. The work described here indicates that this may not be a universal feature of AT cell lines. Indeed, AT5BIVA cells show a significant elevation in topo II enzyme activity and protein content compared to 2 normal control cell lines. Because AT5BIVA cells show all of the characteristic AT features, the observation that they overproduce topo II is evidence that the phenotype of AT cells is not the direct result of reduced topo II activity. This view is supported by our finding that another AT fibroblast cell line from a different genetic complementation group expresses essentially normal levels of topo II protein (unpublished results).

There are several possible explanations for the apparent relative difference in topo II activity between different fibroblast and lymphoblast cell lines. For example, topo II levels may vary even within a single complementation group and reflect inter-patient variation. In addition, there are obviously major differences between fibroblast and lymphoblast cells. In particular, growth characteristics (monolayer versus suspension growth) and mode of transformation (SV40 versus EBV) are factors that could be significant. These differences may also be compounded by variations in cell doubling time, which are known to affect topo II expression.

AT fibroblast lines appear to be sensitive to differing degrees to VP16 (21 and this work). The extreme VP16 sensitivity of AT5BIVA cells may result from the compound effect of intrinsic sensitivity due to the abnormal "AT" gene and overproduction of topo II. Similar results demonstrating increased sensitivity to VP16 in AT5BIVA have been shown by Smith et al. (26).

Since the involvement of one topo II dimer per DNA strand break is a requirement for intercalator-induced cell killing (12), a reduction in topo II level should protect cells against these drugs. This is what is observed when CHO cells are allowed to grow to confluency. The level of topo II drops and their resistance to intercalators increases (24). Similarly, we have previously shown that the CHO mutant, ADR-1, expresses elevated levels of topo II and is hypersensitive to intercalators relative to parental CHO-K1 cells (19). We conclude that the drug-hypersensitive phenotype of AT cells is not consistent with a reduction in topo II content, as suggested by Singh et al. (23). In the absence of other changes, we would predict that AT lymphoblastoid cells which express reduced topo II activity would be resistant to intercalators. However, the situation in AT is complicated by the possibility that the 'AT' gene mutation confers sensitivity to topo II inhibitors in the absence of an abnormality in topo II enzyme. Consistent with this possibility, Smith et al. (26) demonstrated hypersensitivity to VP16 in one AT lymphoblastoid cell line, but the response to intercalators was not studied. Whatever the underlying reason for the possible differences in topo II activity between AT fibroblast and lymphoblast lines, it is clear that both cell types are radiation sensitive and display radioresistant DNA synthesis.

Untransformed normal and AT cells have a significantly lower topo II level than their transformed counterparts. This confirms previous work in normal rat kidney cells showing that SV40 transformation alters topo II expression (18). The observation that untransformed AT5BI cells have a higher topo II activity than untransformed AT4BI and untransformed normal cells suggests that topo II overproduction is intrinsic to this cell line and is not an artefact of transformation. The level of topo II protein in untransformed cells from both normal and AT patients was below the level of detection by our Western analysis (this work and data not shown).

We have studied the mutant CHO cell line, ADR-1, which is phenotypically

similar to AT5BIVA cells, in an attempt to identify the features, if any, of the AT phenotype which might result from altered topo II. ADR-1 and AT5BIVA cells show hypersensitivity to bleomycin (5,20) and to the topo II inhibitors VP16 and m-AMSA (8, 21,26 and this work), and overproduce topo II by approximately the same extent (this work and 19). However, neither of the key features of AT cells, radiation sensitivity nor radioresistant DNA synthesis, are displayed by ADR-1. Although comparisons of this sort between rodent and human cells are necessarily imperfect, this result suggests that radiation sensitivity and radioresistant DNA synthesis are not the direct result of an overproduction of topo II.

AT5BIVA cells are very sensitive to the topo I inhibitor camptothecin. There is, however, a very significant variation in sensitivity between the two normal cell lines used in this work, MRC5-V1 and VA13. These normal cells express a similar level of topo I protein and have similar topo I activity, hence this difference in drug sensitivity is presumably mediated by a topo I-independent mechanism. Despite being very sensitive to camptothecin, AT5BIVA cells express a low level of topo I protein. If it is assumed that camptothecin acts via topo I in a manner analogous to that of the interaction of topo II inhibitors with topo II enzyme, it might have been expected that AT5BIVA cells would be relatively resistant to camptothecin. It is possible that an as yet unidentified defect in the processing of DNA damage caused by VP16 and camptothecin causes sensitivity to both these agents in AT5BIVA cells.

The fact that elevated topo II is mirrored by decreased topo I in AT5BIVA cells raised the possibility that the two enzymes are homeostatically regulated. In E. coli, changes in DNA topology brought about by an alteration in cellular level of topo I can cause an alteration in expression of DNA gyrase (the prokaryotic type II topoisomerase) (25). Measurement of topo I level in the hamster mutant, ADR-1, shows that its topo I content is similar to that in normal CHO cells, suggesting that a reduction in topo I is not an inevitable consequence of an elevation in topo II. However, the factors controlling expression of the topoisomerase genes are as yet unknown.

In conclusion, we have shown that AT5BIVA cells express a high level of topo II protein relative to 2 normal cell lines, and that this may contribute to the sensitivity to topo II inhibitors displayed by this AT line. The

AT5BIVA fibroblast cell line does not show the reduced topo II activity recently reported for AT lymphoblast cell lines. We would suggest, therefore, that reduced topo II activity is not essential for expression of the AT phenotype.

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