Aberrant DNA topoisomerase II activity, radiosensitivity and inherited susceptibility to cancer

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Summary Inherited susceptibility to a wide variety of neoplasias (Li-Fraumeni syndrome), has been shown in studies of one cancer-prone family, to have an intriguing association with an aberrant c-raf-1 gene and inheritance of a radioreistant phenotype in many types of non-cancerous skin fibroblasts. This association together with observations that DNA topoisomerases, when defective, can introduce errors into DNA and that these enzymes are perturbed in vitro by serine/threonine kinases similar toraf encoded proteins, prompted investigation of DNA topoisomerase activity of the family's fibroblasts. Since radiosensitivity was transferred to murine cells (NIH-3T3) when the aberrant c-raf-1 gene from this family was transfected, we also examined transformants containing this and other oncogenes. K-ras/myc and E1a-transformed cells were examined, the former because the family's skin fibroblasts also have 3–8-fold elevated myc expression (not apparently relevant to radiosensitivity) and the latter because ras, like myc, conveys radioresistance. The family members' fibroblasts and the three transfected murine lines, showed a similar perturbation of a spermide and ATP-dependent DNA catenation activity (typical of DNA topoisomerase II). There was a significant positive correlation (r = 0.93; P = 0.0026) between the degree of activation of topoisomerase II and one measure of radioreistance (the D{sub}0 value). Relaxation of DNA supercoiling (topoisomerase I activity and other DNA nicking enzymes) was not abnormal. Cytotoxicity assays and evaluation of the influence of topoisomerase II inhibitors on DNA/protein complex formation, corroborated the existence of a qualitative topoisomerase II defect in the family's cells and transfectants. Although the contention that the qualitative topoisomerase II abnormalities observed here may be associated with malformation is highly speculative, these findings may be relevant to the mechanism of oncogenesis, not only in this family, but with raf and ras type oncogenes.

DNA topoisomerases regulate the topology of DNA. Their roles in normal cells and disease states have been widely reviewed (Osheroff, 1989; Epstein, 1988). We have suggested (Francis et al., 1987a,b), on the basis of several lines of evidence, that DNA topoisomerases might be directly involved in oncogenesis. First, these enzymes can introduce errors into DNA, particularly (but not exclusively) when perturbed by inhibitors and activators. Their malfunction has been implicated in mutation (Overbye et al., 1982; Pommier et al., 1985), sister chromatid exchanges (Pommier et al., 1985; Dillehay et al., 1987; Renault et al., 1987), illegitimate recombination (Bae et al., 1988), chromosome stickiness (Renault et al., 1987; Gaulden et al., 1987), fragmentation of DNA (Jaxel et al., 1988) and tumour promotion (Kaneko & Horikoshi, 1987). The breadth of these observations, including mutant studies and recombination assays, where no extraneous agents were used (Overbye et al., 1982; Bae et al., 1988), and the range of perturbing agents eliciting errors, suggests that this is an inherent property of these enzymes, much exacerbated by a variety of perturbations. Second, the type II enzyme is involved in cellular differentiation (Francis et al., 1987b). Third, the function of both type I and II topoisomerases is perturbed by oncogene-derived and cellular protein kinases, including tyrosine kinases (Tse-Dinh et al., 1984) and serine/threonine kinases (Durban et al., 1983; Rottmann et al., 1987). The hypothetical link that perturbed topoisomerase action provides between oncogene activation, defective differentiation and a tendency to acquire further genetic changes is provocative, since the latter two functional abnormalities are so frequently found together in pre-neoplastic states.

In the cancer family syndrome described by Li and Fraumeni (1969) susceptibility to many types of neoplasia is inherited in a dominant fashion, including: sarcomas, cancers of the breast and other tissues, neurological tumours and both lymphoid and myeloid leukemias. Many individuals in six generations of a large kindred had more than one primary cancer (Blattner et al., 1979). Thus the mechanism of oncogenesis (although unlikely to be identical in each pedigree (Little et al., 1987)) may be relevant to many forms of non-familial neoplasms. Radiation resistance has been demonstrated in the non cancerous skin fibroblasts from family members (Bech-Hansen et al., 1981), but this finding is not common to all Li-Fraumeni families (Little et al., 1987). The consistently normal non-cancerous radiosensitive cells were found to have an apparent activation of the c-raf-1 gene and a 3–8-fold elevation in the expression of c-myc (Chang et al., 1987). The transfer of either the family's c-raf-1 gene, the genes of other serine/threonine kinases or ras, into murine cells conveyed the radioreistant phenotype, but the myc, fes and abl oncogenes failed to do so (Chang et al., 1987; Pirollo et al., 1989; Sklar, 1988).

There is a potential, albeit speculative, link between mutability, radiosensitivity and perturbed topoisomerase activity. Bacterial mutants lacking a type I topoisomerase gene are hypersensitive to DNA damage but resistant to mutation (Sternlanz et al., 1981; Overbye et al., 1982). Chromatin structure, particularly, but not exclusively, 'openness', is known to influence mammalian cell DNA repair (Bohr, 1988). This may account for observations apparently linking topoisomerase activity to repair capacity, despite failure to detect direct involvement in repair in some systems (see below).

Since serine/threonine kinases activate topoisomerases (Durban et al., 1983; Rottman et al., 1987), this particular Li-Fraumeni cancer family could conceivably be the converse of the bacterial mutants with increased activity and/or deranged regulation of the topoisomerases making cells radiosensitive but more prone to mutation. Susceptibility to a wide range of cancers is consistent with such a mechanism, since mutation introduced by malfunctioning topoisomerases could affect many genomic sites.

For these reasons we have studied DNA topoisomerase activity in non-cancerous fibroblasts from members of a Li-Fraumeni family. Studies of NIH-3T3 transformants and a radiosensitive ataxia-telangiectasia fibroblast line were used to investigate the relationship between perturbation of DNA topoisomerase, oncogene activation/expression and radiosistance.

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Materials and methods

Genealogy
A full genealogy of the family is not included because this is published elsewhere (Blattner et al., 1979) and that of the branch of the family under study was given previously (Chang et al., 1987).

Cell lines
Nine fibroblast lines were examined. Details of their origins are given elsewhere (Chang et al., 1987; Pirollo et al., 1989). Five were human lines, from the proband, great uncle and father of the cancer-prone family, an unaffected spouse and an unrelated ataxia-telangiectasia patient (ATSB1, kindly supplied by Dr M. Paterson to Dr E. Chang). Four were murine NIH-3T3 lines: one containing a truncated c-raf-1 gene from a family member (Pirollo et al., 1989); a line containing both v-raf and c-myc to simulate the co-existing defects in the family's fibroblasts; and a line containing activated ras (EJ). The recipient NIH-3T3 cells were used as a control.

Cell culture
Lines were grown in Ham's F12 medium with 0.12% w/v sodium bicarbonate, 0.27% w/v anhydrous glucose (adjusted to pH 7.2), 2 mM L-glutamine, 1 mM sodium pyruvate, 1000 IU ml-1 penicillin, 100 µg ml-1 streptomycin solution and 10% heat inactivated fetal calf serum (FCS). Experiments were performed on the mouse lines at confluence and 24 h after splitting (sub-confluent with approximately 60-80% coverage of the flask). The human lines were estimated firstly at confluence, at 24 h post splitting (approximately 50-60% coverage), and thirdly 5 days post splitting having been refed 24 h prior to assay (approximately 50-90% coverage).

Topoisomerase assays
The assay exploits the ability of topoisomerase II to catenate (join by strand passing) supercoiled circular DNA (the plasmid pBR322). The supercoiled substrate plasmid is converted to catenates, relaxed plasmid and a small fraction is linearised in this reaction. Only catenation is relatively specific for topoisomerase II, the relaxation of supercoiling and linearisation cannot be solely attributed to type II enzyme since topoisomerase I and any DNA nicking enzyme (e.g. endonucleases) can perform this reaction. Since no topoisomerase II assay of crude extracts can be assumed to be specific, the characteristics of the catenation reaction were also evaluated (see Results and Figure 2 below).

Trypaninised fibroblasts were diluted in 10 ml Ham's F12 and counted using a Coulter FN. They were centrifuged at 400 g for 6 min and the pellet resuspended in RPMI 1640 with 10% FCS at either 1.2 x 106 or 1.2 x 107 cells ml-1, depending on the range of extract concentrations to be tested. The cells were recovered by centrifugation at 400 g for 6 min, 200 µl of the ice cold cytoplasmatic lysing mix was added (10 mM Tris PO4 pH 6.75, 1 mM 2-mercaptoethanol, 0.1 mM NaCl, EDTA, 0.2 mM EGTA, 10% glycerol (v/v), 0.5% Triton X-100 (v/v) 0.5% Nonidet P-40 (v/v), 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM dithiothreitol (DTT), 10 mM epsilon aminocaproic acid), left on ice for 5 min, centrifuged at 1,000 g for 8 min and the supernatant reserved on ice (cytoplasmatic extract).

The pellet was washed (10 mM Tris HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl2, 1 mM PMSF, 1 mM DTT and 1 mM NaCl), centrifuged at 1,000 g for 8 min, the supernatant discarded and the pellet dissolved in 25 µl of nuclear lysing solution (10 mM Tris HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl2, 1 mM PMSF, 1 mM DTT and 1 mM NaCl), centrifuged at 10,000 g for 5 min. Polyethylene glycol 6000 (BDH) dissolved in nuclear wash solution was added to a final concentration of 18% and the resulting solution left for a further 15 min, then centrifuged at 9,000 g for 15 min and the supernatant reserved on ice as the nuclear extract. The final nuclear NaCl concentration was 0.25 M.

Serial dilutions of extracts were made in the appropriate lysis buffer with a highest concentration equivalent to 3 x 10⁸ murine cells, 3 x 10⁵ human cells in the cytoplasmatic extract and a two-fold higher concentration for the nuclear extract. These were mixed with 5 µl of the reaction mixture (20 mM Tris HCl pH 8.1, 10 mM MgCl2, 20 mM KCl, 0.5 mM Na3 EDTA, 30 µg ml-1 bovine serum albumin, 1 mM DTT, 15% glycerol (v/v), 10 mM spermidine, 10 mM ATP and 20 µg ml-1 PBR 322 (BCL, supercoiled form) and incubated for 1 h at 33°C. The amount of extract used is expressed as the equivalent number of cells. Captoproin, the lactone form (Sigma), 100 µM stock in dimethylsulphoxide or appropriate diluent controls was added calculated. In selected experiments spermidine and ATP were omitted from the reaction mixture. The reactions were stopped by adding 2.5 µl (0.1% SDS, 15 mM EDTA) and 2.5 µl orange G. The products were electrophoresed through 1% agarose gels with 1 µg ml-1 ethidium bromide in tris borate EDTA buffer pH 8.3 for 1 h at 3 V cm⁻¹. The various topological forms (catenanes, relaxed, linearised, supercoiled) were measured by scanning densitometry.

Cytotoxicity assays
Human fibroblasts growing in log phase were seeded at 2 x 10⁴ cells 100 µl⁻¹ in 96-well microtitre plates (Nunc). This low cell density was chosen to preclude the possibility of controls achieving confluence while survivors of inhibition can continue to grow. They were incubated for 48 h at 37°C and 5% CO2 in the presence of the DNA topoisomerase II inhibitory agents, diluted in tissue culture medium (100 µl), VP16-213, VM26 (both kind gifts of Bristol-Myers Inc., Syracuse, NY, USA), mAMSA (NSC 249992 provided by the Drug Synthesis and Chemistry Branch, NCI) and the less inhibitory analogue of the latter oAMSA (NSC 156306) as well as the DNA topoisomerase I inhibitor camptothecin (Sigma). We also assessed Adriamycin (Farmitalia), which although it inhibits topoisomerase II has additional mechanisms of cytotoxicity including free radical generation (Young et al., 1981). Cytosine arabinoside (Upjohn) and thioguanine (Sigma), neither of which inhibit topoisomerase II were also examined. All the drugs were freshly dissolved at 10⁻² M in appropriate solvents, which were used as diluent controls: the epipodophyllotoxins and amside derivatives in DMSO (BDH Spectroscal grade), the thioguanine in 0.1 N NaOH and the rest in water. The dose range tested was 10⁻⁹ to 10⁻² M. After 48 h, cell numbers were estimated using essentially the method of Finlay et al. (1984) using methylene blue staining to assess total cell mass. Robust regression analysis using least absolute deviation (with a robust constant of 1.0) was performed using a proprietary computer algorithm (NCSS copyright of J.L. Hintze) to calculate ID₅₀ values from pooled results of quadruplicate cultures, at five drug doses, from 2-3 preparations for each drug.

Cytotoxicity assays on murine lines were performed on log phase cells which were seeded at 0.5-1 x 10⁵ in 96-well microtitre plates (Nunc). They were incubated at 37°C and 5% CO2 for 16 h and then the appropriate amount of drug or diluent was added as for the human lines. The range of doses tested was from 10⁻¹¹ to 10⁻⁴ M. After 4 days, viable cell numbers were estimated by the method of Alley et al. (1979) and analysed by similar methods to those used for the human lines (ID₅₀ values were calculated from 12 datum points for each of 8 drug doses).

Radiation resistance
The radiation resistance was estimated previously (Buch-Hansen et al., 1981; Pirollo et al., 1989). The Dq values were not previously calculated but were included in this study because they measure an additional facet to the D₅₀
values (it should be appreciated all three parameters measure different features of the response). The Dq value (the quasi-threshold dose) is defined as the intersection of the extrapolation of the terminal linear portion of the radiation survival curve and the 100% survival line (Hall, 1988); the Dq value is the radiation dose required to reduce survival to 10%. We have re-estimated the Dq values since the earlier publication (to ensure against changes in the cell lines) and use the most recent estimates here, because there were some minor differences.

**SDS/KCl precipitation of DNA/protein complexes**

This was performed essentially by the method of Trask et al. (1984). Cells in exponential growth were exposed to 10⁻³ M VM26 (freshly prepared as a 10⁻² M stock solution dissolved in dimethylsulphoxide) or 10⁻³ M novobiocin for 70 min. They were trypsinised, washed in serum-containing and then serum-free medium, then resuspended in 2 ml of serum-free RPMI 1640 to which 0.2 ml of 10% SDS, 25 ml of buffer A (10 mM Tris-HCI pH 7.5, 2% bovine serum albumin, 1% SDS) and 2.5 ml of 2.5 M KCl were added sequentially. This solution was incubated on ice for 20 min and then spun at 300g for 10 min, the supernatant discarded and the pellet resuspended twice in a wash solution buffer B (10 mM Tris-HCI pH 7.5, 100 mM KCl, 1 mM EDTA).

After centrifugation (300 g), the pellet was resuspended in 16 ml of buffer C (10 mM Tris-HCI pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA) at 37°C and ethanol precipitated. The solution was centrifuged at 10,000 g for 10 min, resuspended in TE pH 7.4 and the protein digested at 37°C overnight with proteinase K at 400 µg ml⁻¹. The solution was then phenol-chloroformed, ethanol precipitated and the DNA analysed by gel electrophoresis and densitometry as above.

**Results**

Both the family's fibroblast lines and the NIH-3T3 transfectants have a similar perturbation of the dose response relationship for cytoplasmic catenation activity (Figure 1), with higher thresholds, but steeper slopes and significantly (P < 0.05) elevated activity levels at high cell extract concentrations, with respect to control. Note there are different scales in the a and b panels and that the logarithmic scale is interrupted to indicate the value for no extract (0 cells). Table 1 gives the statistical analyses. Like all other topo-isomerase assays, this assay when used with crude cell extracts cannot be assumed to be specific for topo-isomerase II. We therefore confirmed that the catenation activity (Figure 2a) was dependent on spermidine and was reduced when exogenous ATP was omitted (Figure 2b), characteristics of type II DNA topo-isomerases. Inhibition of catenation by intercalators cannot be used to confirm that catenation activity is solely due to topo-isomerase II because, as found by previous workers (Zwelling et al., 1988) mAMSA incompletely inhibits this activity. In our assay this may in part reflect the interference of polyacations on the drug/DNA/enzyme interaction (Pommier et al., 1989). Catenation activity was not significantly reduced by the topo-isomerase I inhibitor camptothecin at 10⁻³ to 10⁻⁴ M (126 ± 27% and 91 ± 7% of control respectively in three experiments). Although combinations of nuclease and ligase or topo-isomerase I plus nuclease could simulate catenation, the former is unlikely because spermidine and EDTA inhibit nuclease (Krasnow and Cozarelli, 1982) and the latter because it would be inhibited by camptothecin.

Nuclear catenation activity was highly variable being low or undetectable at confluence and much higher in rapidly dividing cells. It was thus not possible (since the lines have different growth rates) to make reliable comparisons between lines. In 'log phase' (albeit with different growth rates)- there was no significant difference between control nuclear extracts (NIH-3T3 and 'spouse') and murine transfectants and 'family' nuclear extracts (34.9 ± 17.5% versus 26.6 ± 9.0% and 1.4 ± 0.8% versus 6.0 ± 5.5% catenanes, per cent total plasmid respectively). However, all but major differences could be obscured by the differences in growth rates, since the activities range from undetectable at confluence to much higher levels in rapid growth. We did, however, observe that in confluent cultures of murine cells there was residual nuclear activity in transformants (2.5 ± 0.7%) whereas in NIH-3T3 there was no catenation. However, it must be emphasised that we cannot exclude differences in the efficiency of density arrest in these lines, hence this result may not have a simple interpretation. Human confluent lines were uninformative, having no detectable activity in tests or controls. This is in agreement with the findings of others for untransformed fibroblasts (Davies et al., 1989).

Nuclear and cytoplasmic extracts showed no consistent difference in relaxation of DNA supercoiling between tests and controls (Figure 3a and b), none of the family's cells and only two transfectants were different from controls. Note again there are different scales in the a and b panels and that the logarithmic scale is interrupted to indicate the value for no extract (0 cells). Relaxation of supercoiling does not
correlate with catenation activity in this assay system, nor is it dependent on ATP or spermidine (unpublished observations). Camptothecin $10^{-5}$ to $10^{-4}$ M, had a variable effect on the ratio of supercoiled to relaxed plasmid (data not shown) suggesting that topoisomerase I and other DNA nicking activities make a variable contribution to DNA relaxation.

Ataxia telangiectasia (AT) contrasts with the Li-Fraumeni syndrome in that there is a DNA repair defect associated with a radiosensitive rather than a radioreistant phenotype (Debenham et al., 1987). An untransformed fibroblast cell line from an AT patient (AT5BI) was therefore compared with the lines from the Li-Fraumeni syndrome family. In contrast to the family’s fibroblasts, AT DNA catenation activity was reduced with respect to control, being only detectable at the highest cell extract concentration tested ($3 \times 10^5$ cells per assay). Activity at this extract dose was not significantly different from the spouse’s cells at a tenfold lower dose (Table I). Relaxation of supercoiling in the AT line was not significantly different from controls (Figure 3a).

In order to confirm the apparent perturbation of the family’s DNA topoisomerase II enzyme activity, we first evaluated the response of fibroblasts from two family members, the spouse and the AT patient to a panel of cytotoxic drugs. These included those agents known to target topoisomerase I and II, and neither enzyme. Figure 4 shows the differences in responses of the fibroblasts to the panel. The family members’ fibroblasts (filled symbols) showed consistently a 1 to 3 orders of magnitude increase in resistance to drugs targeting topoisomerase II (VP16, VM26 and m-AMSA). ID$_{50}$ values are shown because they demonstrated the differences between the lines most clearly (this minimised the number of points falling outside the interpolated, $10^{-9}$ to $10^{-5}$ M, dose range where results cannot be ranked). With o-AMSA there was, as anticipated for a less inhibitory structural analogue, a circa 1-2 log increase in ID$_{50}$ over that seen for m-AMSA. Adriamycin and camptothecin showed no consistent difference between the family and non-family members’ fibroblasts. The family’s cells were more sensitive to cytosine arabinoside than the controls and less sensitive to 6-thioguanine.

Similar experiments for VM26, m-AMSA, o-AMSA and cytosine arabinoside confirmed these results, albeit with less marked differences, between the ras-3T3 and NIH-3T3 cells. ras-3T3 were resistant to m-AMSA and VM26 in comparison with NIH-3T3 (ID$_{50}$ $1.6 \times 10^{-4}$ M versus $7.2 \times 10^{-9}$ M and $3.0 \times 10^{-5}$ M versus $7.8 \times 10^{-10}$ M respectively). Both ras-3T3 and NIH-3T3 were at least 2 orders of magnitude less sensitive to o-AMSA than m-AMSA (ID$_{50}$ $1.3 \times 10^{-4}$ M and $3.2 \times 10^{-6}$ M respectively). As with the family lines, ras-3T3 was also relatively sensitive to cytosine arabinoside (ID$_{50}$ $2.3 \times 10^{-8}$ M versus $6.0 \times 10^{-7}$ M). The ras/myc-3T3 double transfecant did not behave like the ras-3T3 with m-AMSA, having a greater sensitivity than control (ID$_{50}$ $1.8 \times 10^{-8}$ M), but showed a similar resistance to VM26 (ID$_{50}$ $3.4 \times 10^{-9}$ M).
versus protein diluent, formant mechanism higher complexes line, lines enzyme complexes been hand, linked increases topoisomerase novobiocin and antisense (Osheroff, 1989). Novobiocin, like VP16 usually increases the amount of precipitated complexes by trapping topoisomerase II at the stage where the enzyme is covalently linked to DNA (Osheroff, 1989). Novobiocin, on the other hand, usually reduces the amount of complexes and this has been suggested to be due to it having a different inhibitory mechanism (stimulatory effects have sometimes been observed, but these are uncommon). In contrast to NIH-3T3 which shows this expected pattern, with more complexes in the presence of VM26 than novobiocin, all three transformants failed to show this pattern (Figure 5b). Theraf andraf/myc lines actually showed a reversal of the usual pattern with higher complexes with novobiocin than with VM26 (227 ± 92 versus 48.5 ± 8.2% diluent control respectively for theraf-line, means ± s.e.m. for three independent experiments, and 122–127% versus 62.8% for theraf/myc-line). The ras transformant showed a small but significant increment (137 ± 5% diluent control, mean ± s.e.m. of three experiments) in DNA/protein complex formation when treated with VM26 but no significant change when treated with novobiocin (156 ± 101% diluent control, mean ± s.e.m. three experiments).

Although debated (see below), an influence of DNA topoisomerase on sublethal repair provides a potential link between ras andraf activity and radioresistance. We therefore examined the relationship between the extent of activation of topoisomerase II and the extent of radioresistance (assessed as Dq values). There was a significant positive correlation (Figure 6) between the percent increase (with respect to appropriate control) in topoisomerase II activity at high cell extract concentration (mean values from Table I) and the percentual increase in Dq value. Dq values (legend to Figure 6) and Dq values (published previously) which reflect

### Table I Cytoplasmic catenation activity (catenanes % total plasmid)

| Line       | Extract concentration (cell equivalent × 10²) |
|------------|---------------------------------------------|
|            | 0.03 | 0.1 | 0.3 | 1.0 | 3.0 |
| Proband    | 0    | 0   | 0   | 0.9±0.6 | 14.4±2.6 | 24.7±4.9 |
| Great uncle| 0    | 0   | 0   | 0.6±0.3 | 0      | 11.1±2.6 |
| Father     | 0    | 0   | 0   | 0    | 0.9±0.6 | 2.5±1.2  |
| Spouse     | 0    | 0   | 0.3±0.2 | 2.2±1.9 | 1.6±0.5 |
| AT patient | 0    | 0   | 0.3±0.2 | 2.2±1.9 | 1.6±0.5 |

Results are mean ± s.e.m.

**Figure 4** Growth inhibitory concentrations for a panel of cytotoxic agents (for abbreviations see text). Results are ID₅₀ values, obtained by regression analysis of pooled experiments from proband (■), great uncle (▼), spouse (○) and AT line (□).

**Figure 5** Influence of novobiocin and VM26 on SDS/KCl precipitation of DNA/protein complexes. (a) The amount of protein-linked DNA recovered expressed as per cent diluent control (novobiocin = white columns; VM26 = black columns). (b) Per cent difference VM26-novobiocin ± s.e.m.
different features of radiation response were not significantly correlated with topoisomerase activity.

Discussion

Cells from three members of a cancer-prone family with the Li-Fraumeni syndrome had a similar disturbance of dose-response curves for cytoplasmic extract catenation activity. The key features: higher thresholds, steeper slopes and significantly elevated activity levels with respect to controls at higher extract concentrations, were reproduced by transfection of the family's c-raf-1 oncogene in NIH-3T3 cells. The perturbation of catenation activity is not consistent with there merely being differences in the same enzymatic activity between the cell lines, but indicates modulation of the topoisomerase II activity by an additional factor or factors in test and/or control extracts. Since c-raf-1 encodes a serine/threonine kinase and this class of enzyme is known to activate the topoisomerases (Durban et al., 1983; Rottmann et al., 1987), the activated c-raf-1 gene is the obvious candidate for such a factor. Topoisomerase I activity and other DNA nicking agents, detected by relaxation of DNA supercoiling, were not disturbed and the contrasting results with the topoisomerase I inhibitor camptothecin and the topoisomerase II inhibitors in the cytotoxicity test are consistent with these enzyme assay results.

The differential effect of the topoisomerase II inhibitors VP-16, VM-26 and m-AMSA on the family's cells and controls corroborates the evidence for a functional disturbance of topoisomerase II (the difference between the effects of o-AMSA and m-AMSA is consistent with their inhibitory action in the cytotoxicity assay being related to their anti-topoisomerase action). Although resistant to epipodophyllotoxins, the family's cells do not exhibit classical multi-drug resistance since they are not significantly resistant to adriamycin (Ueda et al., 1987). The discrepancy between the results for the two antimetabolites cytosine arabinoside and 6-thioguanine is obscure. Toxicity testing of the murine raf-transfected corroborated these results, including the sensitivity to cytosine arabinoside. The double raf/myc-3T3 transfected did not mirror this behaviour, but this could reflect compound effects of the presence of the two oncogenes in murine cells that do not faithfully reflect the situation in the family's cells.

The SDS/KCL precipitation experiments (Figure 5) provide further evidence of functional abnormality of topoisomerase II activity and suggest abnormal interactions with two topoisomerase II inhibitors with different mechanisms of action, VM26 and novobiocin. This potentially provides an explanation for the reduced sensitivity to topoisomerase II targeting epipodophyllotoxins and intercalators. Both the cytotoxicity data and these inhibitors studies extend the observation of abnormal findings from phosphorylation to nuclear topoisomerase II, since the end-point is the formation of complexes with, or damage of, DNA. Serine threonine kinase-mediated phosphorylation activates topoisomerase II (Durban et al., 1983; Rottmann et al., 1987), which at first sight might be expected to potentiate drugs acting via this enzyme. However, the consequences of phosphorylation vis a vis drug/enzyme/DNA interactions are unknown.

The opposing findings for AT and the family cells (Table II) extend not only to radiation resistance, to the radiation induced DNA synthesis delay (Paterson et al., 1985; Houldsworth & Lavin, 1980), apparent topoisomerase II activity (at high cytoplasmic extract concentrations), but also to sensitivity to topoisomerase II targeting cytotoxics, to which the family's cells are resistant and AT cells are reportedly hypersensitive (Henner & Blazka, 1986). This reiterates the counter-intuitive relationship between topoisomerase II activity and drug sensitivity, but, as with the family's cells, in AT topoisomerase II is probably not simply quantitatively changed but qualitatively abnormal with normal or even increased protein levels (Singh & Lavin, 1989). Our finding of low topoisomerase II activity, which corroborates observations of Mohamed et al. (1987) and Singh et al. (1988), contrasts with those of Smith and Makinson (1989) and Davies et al. (1989), who found increased activity in the transformed AT5BIVA line, also in one of two untransformed AT fibroblast lines, but low enzyme content in two lymphoblastoid cell lines. This may reflect methodological differences in the way various assay systems detect qualitatively abnormal enzyme. The linkage between the phenomena illustrated in Table II is certainly intriguing, it extends to cell line mutants (Evans et al., 1989) and leads us to speculate that the primary lesion in the two syndromes interferes with a cellular 'machine' that is involved in regulating/producing these individual cell features.

If one allows that the perturbations of DNA topoisomerase II activity (both activation and inhibition) associated with observed enhancement in mutation, illegitimate recombination and the introduction of other errors may be causally related, and that an aberrant c-raf-1 gene may behave like other serine-threonine kinases which have been observed to phosphorylate and hence aberrantly activate DNA topoisomerase II, this provides a readily testable hypothesis to account for the family's cells having a higher than usual mutation rate. We are currently examining phosphorylation status and error rate directly. That both activation and inhibition of the enzyme can increase error proneness, potentially reconciles the paradox of two syndromes (AT and Li-Fraumeni) with opposing findings for topoisomerase activity, sensitivity to topoisomerase inhibitors and contrasting response to radiation damage (Table II), but each having increased mutability.

Table II: Reciprocal relationship between cell properties

| Topoisomerase activity | Ataxia telangiectasia | Li-Fraumeni family |
|------------------------|-----------------------|-------------------|
| Radiosensitivity       | increased<sup>a</sup> | reduced<sup>b</sup> |
| Cytoplasmic topoisomerase II activity | decreased<sup>a</sup> | increased<sup>d</sup> |
| Nuclear topoisomerase II activity | varied | n.a. |
| Sensitivity to cytotoxic action of topoisomerase II inhibitors | increased<sup>a</sup> | decreased<sup>d</sup> |

<sup>a</sup>Increased radiosensitivity in association with sensitivity to topoisomerase II inhibitors (cleavable complex stabilising type), and also been observed in Chinese hamster ovary cell mutants (Robson et al., 1987; Elkind et al., 1988).<sup>b</sup>Radioresistance is associated with resistance to topoisomerase II inhibitors in murine lymphoma lines (Evans et al., 1989).<sup>c</sup>In this but not all assay systems. <sup>d</sup>At high cell extract concentrations.
The presence of coexisting defects in myc and raf in the family's cells could provide a greatly increased probability for a diverse range of tumours of many types, and could reflect the complex nature of the pedigree with cancers in all four ancestral lines. The raf and myc oncogenes are known to interact synergistically in oncogenesis in experimental systems (Rapp et al., 1988).

The influence of ras and raf transfection on radiosensitivity and our finding that both induced an identical perturbation of the topoisomerase II dose response curve, suggests that this could be associated with the radioreistant phenotype (co-ordinate findings for ras and raf may be due to ras being upstream of raf in the same signal transduction pathway (Rapp et al., 1988)). Support for this suggested association comes from (1) the significant correlation observed between the extent of topoisomerase II activation and radiation resistance (D2 values); (2) the contrasting effects of serine/threonine kinases (raf and mos), tyrosine kinases (fos and abl) and myc on radiosensitivity (Pirrollo et al., 1989), given that the latter do not activate topoisomerases; (3) the association between disturbed response to topoisomerase II inhibitors and radiosensitivity/sensitivity observed in cell mutants (see footnote to Table II); and (4) the association between a radiosensitive phenotype and apparently decreased/perturbed topoisomerase II activity found in the same studies in AT. Given the debate concerning the evidence for a role of topoisomerases in DNA repair, speculation concerning a causal relationship between the derangement of topoisomerase II activity and radiosensitivity must be extremely tentative. However, controversy mostly concerns UV-induced excision repair and the use of novobiocin, an agent incorrectly assumed to be a specific inhibitor of the enzyme (Downes & Johnson, 1988). It is premature to rule out involvement of topoisomerase in all repair processes. First, as mentioned above, the association in AT of radiosensitivity and qualitatively abnormal topoisomerase II (hypersensitive to topoisomerase II targeting cytotoxic agents (Henner & Balzka, 1986)) is provocative. The repair defect in AT has not yet been elucidated, but misrepair (Debenham et al., 1987) rather than lack of ligation (Lehmann, 1982) of double stranded DNA breaks has been implicated. Misrepair could be a consequence of deranged topoisomerase II activity (Bae et al., 1988). Secondly, the failure of topoisomerase II inhibitors (other than novobiocin which has pleiotropic effects) to inhibit repair in some systems (Downes et al., 1987; Synder et al., 1987), is not universal (Dressler & Robinson-Hill, 1987). In any case, such results do not rule out an indirect involve-

ment of topoisomerase II related to its influence on chromatin structure, since this may not be sufficiently rapidly affected by the inhibitors in a large enough proportion of the genome to be revealed in short term experiments. Topoisomerases I and II act within and around transcriptionally active genes (Wu et al., 1988) and such genes do have an enhanced repair capacity (Bohr, 1988). Topoisomerase may be involved in transitions of chromatin structure rather than in maintaining the 'active' chromatin configuration, since the enzyme is not found in DNAase hypersensitive, transcriptionally competent sites after transcription has subsided (Muller et al., 1987). This notion of involvement in transitions is consistent with the observation that novobiocin has its major effect in the repair of inactive rather than active chromatin (Bohr & Hanawalt, 1986).

The association of ras and a radioreistant phenotype is not limited to the Li-Fraumeni kindred but has been observed in head and neck cancer (Kasid et al., 1987) and with ras and amplified myc in small cell lung carcinoma (Carney et al., 1983; Rapp et al., 1988). Since radiosensitivity occurs with other serine/threonine kinases and ras too, our finding may have widespread implications. If topoisomerase II radiosensitivity is due to the aberrant topoisomerase this work could have direct clinical application, since this abnormality is potentially reversible. While the familial contribution to oncogenesis appears to be limited (Fraumeni, 1982), the study of cancer families and the mechanism of their inherited susceptibility to many different forms of cancer should provide insights into some key mechanisms of oncogenesis.

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References

ALLEY, M.C.; SCUDIERO, D.A.; MONKS, A. & 7 others (1988). Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. Cancer Res., 48, 589.

BAE, Y.S.; KAWASAKI, I.; IKEDA, H. & LIU, F. (1988). Illegitimate recombination mediated by calf thymus topoisomerase II in vitro. Proc. Natl Acad. Sci. USA, 85, 2076.

BECH-HANSEN, N.T.; SELL, B.M.; LAMPKIN, B.C. & 4 others (1981). Transmission of an in vitro radiosensitivity in a cancer-prone family. Lancet, 1, 1335.

BLATTNER, W.A.; MCGUIRE, D.B.; MULVHILL, J.J.; LAMPKIN, B.C.; HANANIAN, J.; FRAUENI, J.F. (1979). Genealogy of cancer in a family. J. Am. Med. Assoc., 241, 259.

BOHR, V.A.; HANAWALT, P.C. (1986). Novobiocin does not inhibit DNA repair in an active gene. Carcinogenesis, 7, 1917.

BOHR, V.A. (1988). DNA repair and transcriptional activity in genes. J. Cell Sci., 90, 175.

CARNEY, D.N.; MITCHELL, J.P. & KINSELLA, T.J. (1983). In vitro radiation and chemotherpay sensitivity of established cell lines of human small cell lung cancer and its large cell morphological variants. Cancer Res., 43, 2806.

CHANG, E.H.; PIROLLO, K.F.; ZOU, Z.Q. & 7 others (1987). Oncogenes in radiosensitive, noncancerous skin fibroblasts from a cancer prone family. Science, 237, 1036.

DAVIES, S.M.; HARRIS, A.L. & HICKSON, I.D. (1989). Overproduction of topoisomerase II in an ataxia telangiectasia fibroblast line: comparison with a topoisomerase II-overproducing hamster cell mutant. Nucleic Acids Res., 17, 1337.

DEBENHAM, P.G.; WEBB, M.B.T.; JONES, N.J. & COX, R. (1987). Molecular studies on the nature of the repair defect in ataxia telangiectasia and their implications for radiobiology. J. Cell Sci., 6, 177.

DILLEHAY, L.E.; DENSTMAN, S.C. & WILLIAMS, J.R. (1987). Cell cycle dependence of sister chromatid exchange induction by DNA topoisomerase II inhibitors in Chinese hamster V79 cells. Cancer Res., 47, 206.

DOWNES, C.S.; MULLINGER, A.M. & JOHNSON, R.T. (1987). Action of etoposide (VP-16-213) on human cells: no evidence for topoisomerase II involvement in excision repair of UV-induced damage, nor for mitochondrial hypersensitivity in ataxia telangiectasia. Carcinogenesis, 8, 1613.

DOWNES, C.S. & JOHNSON, R.T. (1988). DNA topoisomerase and DNA repair. BioEssays, 8, 179.

DRESSLER, S.L. & ROBINSON-HILL, R.M. (1987). Direct inhibitor of UV-induced DNA excision repair in human cells by novobiocin, coumermycin and nalidixic acid. Carcinogenesis, 8, 813.

DURBAN, E.; MILLS, J.S.; ROLL, D. & BUSCH, H. (1983). Phosphorylation of purified Novikoff hepatoma topoisomerase I. Biochim. Biophys. Res. Commun., 111, 897.

ELKIND, M.M.; UTSUMI, H. & KOSAKA, T. (1988). Inhibitors of topoisomerase II and their action in repair-competent and repair-deficient Chinese hamster cells. J Cell Biochem., Suppl. 12A, 286.

EPSTEIN, R.J. (1988). Topoisomerase in human disease. Lancet, 1, 521.

EVANS, H.H.; RICANATI, M.; HORGAN, M.F. & JAROSLAV, M. (1989). Relationship between topoisomerase II and radiosensitivity in mouse L5178Y lymphoma strains. Mutation Res., 217, 53.

FINCK, G.F.; BAGULEY, B.C. & WILSON, W.R. (1984). A semiautomated microculture technique for investigating growth inhibitory effects of cytotoxic compounds on experimentally growing carcinoma cells. Anal. Biochem., 139, 272.
FRANCIS, G.E. (1987). Leukaemogenesis: a postulated mechanism involving tyrosine protein kinase and DNA topoisomerase. Med. Hypoth., 22, 223.

FRANCIS, G.E., BERNEY, J.J., NORTH, P.S. & others (1987). Evidence for the involvement of DNA topoisomerase II in neutrophil-granulocyte differentiation. Leukemia, 1, 653.

GAULDEN, D. (1987). Hypothesis: some mutations directly alter specific chromosomal proteins (DNA topoisomerase II and peripheral proteins) to produce chromosome stickiness, which causes chromosome aberrations. Mutagenesis, 2, 357.

HALL, E.J. (1988). Radiobiology for the Radiologist, Lippincott: Philadelphia.

HENNER, W.D. & BLAZKA, M.E. (1986). Hypersensitivity of cultured ataxia-telangiectasia cells to etoposide. J. Natl Cancer Inst., 76, 1007.

HOULDSWORTH, J. & LAVIN, M.F. (1980). Effects of ionizing radiation on DNA synthesis in ataxia-telangiectasia cells. Nucleic Acids Res., 8, 3709.

JAXEWSKY, T.J., KURAJIC, J.J., PORTÉMER, C., MIRABAUE, G., PANIEL, J. & DUGUET, M. (1988). Topoisomerase inhibitors induce irreversible fragmentation of replicated DNA in concanavalin A-stimulated splenocytes. Biochemistry, 27, 95.

KANEKO, M. & HOKRISHI, J. (1987). Topoisomerase inhibitors suppressed lipothecolic acid-induced promotion of transformation in BALB/C. Br. J. Cancer, 56, 614.

KASID, U., PFEIFER, A., WIECHSELBAUM, R.R., DRITSCHEL, A. & MARK, G.E. (1987). The raf oncogene is associated with a radiation-resistant human laryngeal tumour. Science, 237, 1039.

KRAUS, P.T. & COZARKI, N.R. (1982). Catenation of DNA rings by topoisomerases: mechanism of control by spermidine. J. Biol. Chem., 257, 2687.

LEHMANN, A.R. (1982). The cellular and molecular responses of ataxia-telangiectasia cells to DNA damage. In Ataxia-telangiectasia, Bridges, B.A. & Harden, D.G. (eds), p. 83. Oxford University Press.

LI, F.P. & FRAUMENI, J.F. (1969). Soft-tissue sarcomas, breast cancer, and other neoplasms, a familial syndrome. Ann. Intern. Med., 71, 747.

LITTLE, J.B., NOVE, J., DAHLBERG, W.K., TROLO, P., NICHOLS, W.W. & STRONG, L.C. (1987). Normal cytotoxic response of skin fibroblasts from patients with Li-Fraumeni familial cancer syndrome to DNA-damaging agents. Cancer Res., 47, 4229.

MAHAMED, R., SINGH, S.P., KUMAR, S. & LAVIN, M.F. (1987). A defect in DNA topoisomerase II activity in ataxia-telangiectasia cells. Biochem. Biophys. Res. Commun., 149, 233.

MULLAR, M.T. (1987). Eukaryotic topoisomerase I and II activity in chromatid: mapping catalytic sites during cell differentiation. Leukemia, 1, 827.

OSHEROFF, N. (1989). Biochemical basis for the interaction of type I and II topoisomerase with DNA. Pharm. Ther., 41, 223.

OVERBY, K.M., BASU, S.K. & MARGOLIN, P. (1982). Loss of DNA topoisomerase I activity alters many cellular functions in salmonella typhimurium. Cold Spring Harbor Symp. Quant. Biol., 47, 785.

PATTERSON, M.C., GENTNER, N.E., MIDDLESTADT, M.V., MIRAZYS, R. & WEINFELD, M. (1985). Hereditary and familial disorders linking prostates with abnormal carcinogen response and faulty DNA metabolism. In Epidemiology and Quantification of Environmental Risk in Humans from Radiation and Other Agents, Castellani, A. (ed.), p. 235. Plenum: New York.

PIRILLO, K.F., GARNER, R., YUAN, S.Y., LI, L., BLATTNER, W.A. & CHANG, E.H. (1989). Raf involvement in the simultaneous genetic transfer of the radioresistant and transforming phenotypes. Int. J. Radiat. Biol., 55, 783.