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GAMMA-RAY INDUCED INHIBITION OF DNA SYNTHESIS IN ATAXIA TELANGIECTASIA FIBROBLASTS IS A FUNCTION OF EXCISION REPAIR CAPACITY

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SUMMARY: The extent of the deficiency in γ-ray induced DNA repair synthesis in an ataxia telangiectasia (AT) human fibroblast strain was found to show no oxygen enhancement, consistent with a defect in the repair of base damage. Repair deficiency, but not repair proficiency, in AT cells was accompanied by a lack of inhibition of DNA synthesis (replicon initiation) after either γ-rays or the radiomimetic drug bleomycin. Experiments with 4-nitroquinoline 1-oxide indicated that lack of inhibition was specific for radiogenic-type damage. Thus excision repair, perhaps by DNA strand incision or chromatin modification, appears to halt replicon initiation in irradiated repair proficient cells whereas in repair defective AT strains this putatively important biological function is inoperative.

INTRODUCTION

Ataxia telangiectasia (AT) is a multisystem disorder in man (for review see ref. 1). Affected individuals are cancer prone and also show striking clinical hypersensitivity to conventional radiotherapy administered for the treatment of malignancies . In keeping with the clinical findings, AT fibroblasts demonstrate consistent hypersensitivity *in vitro* to ionizing radiation, as measured by reduced colony forming ability (2,3) and enhanced frequencies of chromosome aberrations (4) relative to normal fibroblasts. AT cells also show hypersensitivity to DNA damaging chemicals (1) which have radiomimetic [e.g. the antibiotic bleomycin, (5,6)]or partially radiomimetic properties [e.g. 4-nitroquinoline 1-oxide, 4NQO, (1,7)].

A molecular basis for the radiosensitivity of some AT strains has been ascribed to the slow removal of radiogenic DNA base or sugar damage assayed by its sensitivity to incising activities present in crude extracts of

ABBREVIATIONS: AT, ataxia telangiectasia; 4NQO, 4-nitroquinoline 1-oxide; exr^+ and exr^-, excision repair proficient and deficient; PBS, phosphate buffered saline; UDS unscheduled DNA synthesis.

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Micrococcus luteus cells (1,8). Defective excision repair in AT is associated with reduced levels of hypoxic γ-ray induced repair synthesis (1,3,8,9). However, no consistent DNA repair defect has been found in all AT strains despite their relatively uniform sensitivity to ionizing radiation. Since AT strains show genetic heterogeneity in their responses to chemicals, which induce a relatively more narrow spectrum of lesions in cellular DNA, it has been proposed (1) that different DNA repair defects in AT can lead to uniform radiosensitivity because of the wide spectrum of potentially lethal DNA lesions induced by ionizing radiation.

Here we have examined the possibility that the inhibitory action of DNA damage on de novo DNA synthesis may be a critical molecular anomaly in AT cells. The inhibition of DNA synthesis was measured in both excision repair proficient (exr^+) and excision repair defective (exr^-) AT strains as classified on the basis of γ-ray induced repair synthesis.

MATERIALS AND METHODS

Cell Strains and Cell Culture: Our experiments were conducted with five diploid fibroblasts strain derived from skin biopsies of human (three normal and five AT) donors. Two of the normal control strains, GM 38 (9 yr old female) and GM 498 (3 yr old male), were purchased from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ, while the third control strain, 2650T (46 yr old male), was obtained from Meloy Laboratories, Springfield, VA. One AT strain, AT2BE (CRL 1343, 7 yr old female patient), was purchased from the American Type Culture Collection, Rockville, MD, and the second AT strain, AT4BI (6 yr old male patient) was kindly supplied by Dr. A.M.R. Taylor, University of Birmingham, Birmingham, U.K. The procedures used in the manipulation of fibroblast monolayer cultures have been described in detail previously (3).

Measurement of Unscheduled DNA Synthesis (UDS): UDS is an autoradiographic measure of the uptake of radioactive DNA precursors by non-S phase cells as a consequence of repair processes. Fibroblasts were plated at approximately 4×10^5 cells per 9 cm diameter plastic Petri dish (Lux Scientific Corp., Newbury Park, CA) each containing six plastic 25 mm diameter Thermanox coverslips (Lux). Following an 18-hr incubation period, cells undergoing semiconservative DNA replication, were labelled by incubation for 1 hr in medium containing 10 μCi ml^{-1} [methyl-^{3}H] thymidine (stock specific activity, 41 Ci $mmol^{-1}$). Cultures were then washed with phosphate buffered saline (PBS) and irradiated (4ml PBS per dish, maintained on ice) with 50 krad ^{60}Co γ-rays at a dose rate of 15-17 krad min^{-1}, using a Gammacell 220 unit (Atomic Energy of Canada Limited, Ottawa, Canada). Irradiations were carried out in equilibrium with air or under hypoxic conditions achieved by passing a stream of N_2 gas (99.8% pure, <10 ppm O_2; Air Products, Bramptom, Ontario, Canada) over the surface of the plate for 15 min prior to and during irradiation. Irradiated and sham-irradiated control coverslip cultures were then incubated in medium

containing 10 μCi ml^{-1} [methyl-^{3}H] thymidine (stock specific activity 41 Ci $mmol^{-1}$) for periods up to 6 hr. Cells on the coverslips were washed in PBS and fixed in 3:1 methanol:acetic acid mixture. Stained preparations were dip-coated in nuclear track emulsion (NTB-2; Kodak Canada Inc., Toronto, Canada) followed by an exposure period of 17 days.

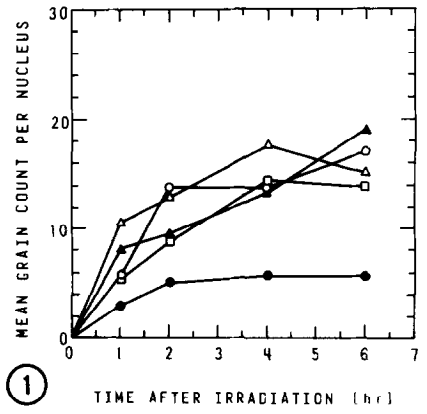
Measurement of DNA Synthesis Inhibition: Cells were cultured in 6cm plastic dishes (~2 x 10^5 cells per dish at time of treatment), incubated for 18 hr in medium containing 0.4 μC ml^{-1} [methyl-^{14}C] thymidine (stock specific activity,40.8 mCi $mmol^{-1}$) and incubated for an additional 1 hr in growth medium. The cultures were treated in one of three ways: i) Exposed to oxic ^{60}Co γ-rays (using Gammabeam 150C; Atomic Energy of Canada Limited) at various dose rates for an isoexposure period of 500 sec during which each dish (containing 2 ml PBS) was held at ambient temperature; ii) Exposed for 1 hr at 37°C to various concentrations of bleomycin sulphate (Sigma Chemical Company, St. Louis, MO,) in culture medium lacking serum but supplemented with 10 mM Hepes buffer; (iii) Exposed to various concentrations of 4NQO (K&K Laboratories, Plainview, NY) under the same conditions as for bleomycin. Following treatment or sham-treatment, the cultures were washed and incubated for 1 hr in growth medium containing 10 μCi ml^{-1} [methyl-^{3}H] thymidine (stock specific activity 52.2 Ci $mmol^{-1}$). At given times, each culture was washed with PBS and 1 ml lysis solution added [1.5 gm 4-aminosalicylic acid-sodium salt (BDH Chemicals Ltd., Poole, England), 0.25 gm tri-iso-propylnaphthalene sulphonic acid-sodium salt (Eastman Kodak Co., Rochester, NY), 1.5 ml 2-butanol, and 23.5 ml distilled water]. DNA in lysate samples was precipitated in ice-cold 10% TCA and collected on Whatman GF/C filters for liquid scintillation counting in toluene-Scintiprep 2 (Fisher Scientific, Toronto, Canada). Radioactivity in ^{3}H and ^{14}C channels was corrected for background and spill-over, and values expressed as disintegrations per minute. The ratio of ^{3}H/^{14}C values for treated and the corresponding untreated sample was taken as a measure of the inhibition of DNA synthesis.

RESULTS

1. Gamma-ray Induced UDS Although γ-ray induced repair synthesis has been measured previously in AT cells (3,8,9) the kinetics of the process and the effect of oxygenation conditions during irradiation have not been reported.

(a) Hypoxic Conditions: Fig. 1 compares the induction of UDS by 50 krad hypoxic γ-radiation [a non-saturating dose for repair synthesis, (3,8,9)] in normal and AT fibroblasts. In all strains the majority of UDS is expressed by the 2-4 hr period following irradiation. It is clear from the data that AT2BE is UDS defective (i.e. exr^-) whereas AT4BI expresses UDS levels similar to those in the three normal strains and is therefore UDS proficient (i.e. exr^+).

(b) Oxic Conditions: Fig. 2 gives the results of the same experimental protocol as in Fig. 1 but performed under oxic irradiation conditions. The kinetics for repair synthesis are similar to those under hypoxic conditions with the majority of UDS being expressed within the first four hours. The



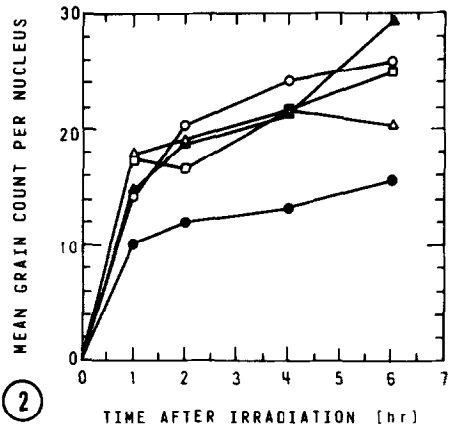


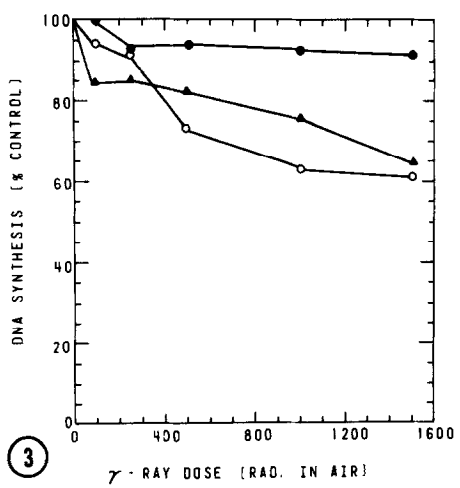
Fig. 1 Hypoxic γ-ray (50 krad) induced UDS in normal (○, GM 38; □, GM 498; △, 2650T) and AT (●, AT2BE; ▲, AT4BI) cells. Background corrected mean grain counts for 50 non-S phase nuclei (SE ≤ 10%).

Fig. 2 Oxic γ-ray (50 krad) induced UDS in normal and AT cells. See Fig. 1 for symbols and details.

results clearly demonstrate the repair deficiency of AT2BE and the repair proficiency of AT4BI. Oxia elevates UDS levels in all strains with mean oxygen enhancement ratios, calculated from mean grain counts of the four combined time points, being; 1.6 ± 0.2 (combined normals), 1.7 ± 0.2 (AT4BI) and 2.6 ± 0.2 (AT2BE). The significantly higher ($p<0.01$) oxygen enhancement ratio for AT2BE compared to all other strains examined is indicative of the proportionally greater repair deficiency under hypoxic compared to oxic conditions. However, it can be calculated, by combining all time points for AT2BE or the three normals, that the absolute repair deficiency shown by AT2BE is the same under oxic (7.5 ± 0.7 grains per nucleus) or hypoxic (7.5 ± 1.3 grains per nucleus) irradiation conditions.

2. Inhibition of DNA Synthesis

(a) Oxic γ-radiation: Fig. 3 shows that oxic γ-radiation inhibits DNA synthesis to a comparable extent in both a normal strain (GM 38) and the exr^+ AT strain (AT4BI) (65-70% of control level at 1500 rad). The exr^- strain (AT2BE) however appears essentially refractory to radiogenic inhibition (90-95% of control levels at 1500 rad). The inhibition curve for normal cells is



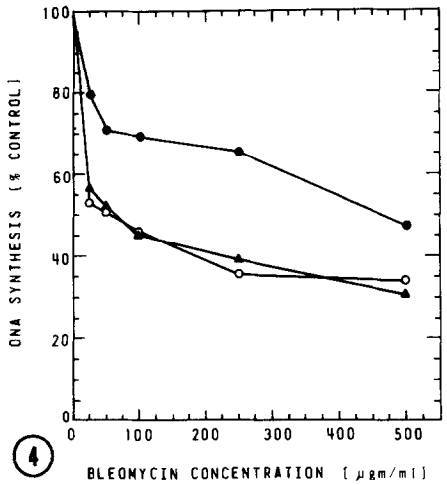


Fig. 3 Oxic γ-ray induced inhibition of DNA synthesis measured as incorporation of tritiated thymidine in treated cultures relative to controls. Percent control DNA synthesis is calculated from the ratio of $^{3}H/^{14}C$ values for treated and untreated cultures (see Materials and Methods). ○ , GM 38; ● , AT2BE; ▲ , AT4BI. Each point represents the mean of at least two determinations (SE $\leq$ 10%).

Fig. 4 Bleomycin induced inhibition of DNA synthesis. See Fig. 3 and Materials and Methods for details.

biphasic, as reported for other mammalian cells [for reviews see (10) and (11)], with an initially steep component up to 500 rad followed by a shallow component which falls to only 30% of control levels at 50 krad (data not shown). AT2BE appears to be resistant with respect to the initial steep component of the inhibition curve.

(b) Bleomycin: Fig. 4 shows that bleomycin has qualitatively the same effect as ionizing radiation. AT2BE shows resistance to the inhibitory effects of the drug only in the initial component of the biphasic response, while the response of AT4BI is very similar to that of the normal strain (GM 38).

(c) 4NQO: Fig. 5 indicates that 4NQO is a potent inhibitor of DNA synthesis. All three strains examined showed similar levels of inhibition of DNA synthesis.

DISCUSSION

This study reports the sensitivity of de novo DNA synthesis to inhibition by various agents, in two AT fibroblast strains each of different DNA repair

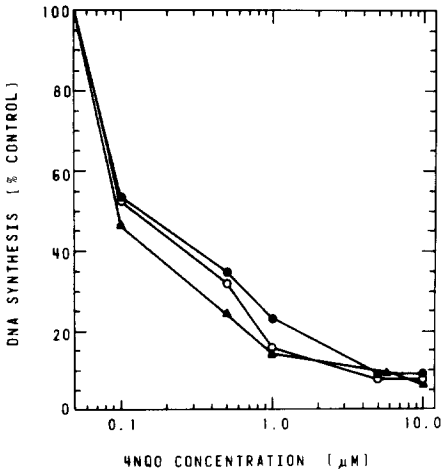


Fig. 5 4NQO induced inhibition of DNA synthesis. See Fig. 3 and Materials and Methods for details.

capacity with respect to radiogenic damage. The repair synthesis experiments indicate that in an exr^- AT strain (AT2BE) the absolute repair deficiency was the same under oxic or hypoxic irradiation conditions. This finding is in keeping with previous reports that certain AT strains are defective in the repair of DNA base (or sugar) damage (1,8,9), since the frequency of this class of lesions, unlike DNA strand breaks, is not appreciably affected by oxygenation conditions during γ-irradiation (12). Furthermore, we have observed that repair deficiency imparts resistance to the inhibitory effects of γ-radiation on de novo DNA synthesis. Resistance to inhibition was expressed in the initial steep component of the biphasic dose response curve. This is interpreted as resistance to the radiogenic inhibition of replicon initiation (13,14,15).

The radiomimetic drug bleomycin similarly yields a biphasic inhibition curve for DNA synthesis in normal and AT cells, with AT2BE cells again showing resistance with respect to the initial steep component. Failure to show inhibition appears to be related to the repair capacity for radiogenic or "ionizing radiation-like" DNA damage, since normal levels of inhibition occur in 4NQO treated AT2BE cells. 4NQO is primarily a "UV-mimetic" DNA damaging

agent, in the type of repair pathways which handle the majority of DNA lesions generated (16), and AT cells show normal repair capacities for both UV (1,8) and "UV-like" 4NQO induced DNA damage (7).

Both ionizing radiation and bleomycin induce DNA single and double strand breaks and it is these lesions which are thought to act as blocks to replicon initiation (17). However, AT strains show normal levels for induction and repair of strand breaks (including alkali-labile lesions) induced by ionizing radiation (2,8,18,19) or bleomycin (5,19). Moreover, replicon behaviour is normal in unirradiated repair proficient and deficient AT strains (20).

Since resistance to inhibition of DNA synthesis appears to be associated with the exr$^-$ phenotype in AT, we suggest that two principles are in operation. Firstly, the inhibitory action of ionizing radiation on replicon initiation in normal cells is due to a combined action of radiogenic DNA strand breaks and enzymatic DNA incisions which arise during the normal course of excision repair of base damage. Secondly, exr$^-$ AT cells (e.g. AT2BE) have reduced capacities for the enzymatic repair of base (or sugar) damage and consequently display reduced radiogenic inhibition of replicon initiation.

It has been suggested (10,17,21) that changes in higher orders of chromatin structure may influence replicon initiation events. Thus it is conceivable that certain AT cells are defective in lesion recognition functions which modify chromatin structure to permit the action of excision repair enzymes (which may therefore be present at normal intracellular levels in AT cells). In normal cells such lesion recognition steps would act to halt replicon initiation and consequently the template activity of damaged DNA, whereas in exr$^-$ AT cells this mode of inhibition of replicon initiation would beinefficient. It is unlikely that chromatin structure *per se* is different in unirradiated AT cells since it has been reported that bleomycin shows similar degradative properties (19,22) towards chromatin from AT and normal cells despite its preferential action on internucleosomal DNA (23).

Although this study provides no evidence of a direct correlation between cellular sensitivity and the inhibition of DNA synthesis in AT cells,the

premature replication of damaged DNA in exr^- AT cells may exacerbate the deleterious action of unrepaired DNA damage. We have previously suggested (1) that exr^+ AT strains may have currently undetected DNA repair defects and some exr^+ strains may therefore show anomalies in DNA synthesis following γ-irradiation depending on the nature of the defect and the critical lesions involved. Consequently DNA synthesis patterns may be useful markers in the study of genetic heterogeneity in both exr^- and exr^+ strains. Furthermore, since AT cells appear to suffer less X-ray induced mitotic delay than normal cells (24), initial excision repair events may also act to delay cell-cycle progression in normal cells, whereas certain AT cells would express reduced radiogenic cell-cycle delay and therefore a reduced pre-mitotic repair period. A putative defect in chromatin-associated functions which control cell-cycle progression may be etiologically involved in aberrant embryonic differentiation and predisposition to lymphoreticular neoplasia which characterize AT (1).

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