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Repair of potentially lethal and sublethal damage induced by neocarzinostatin in normal and ataxia-telangiectasia skin fibroblasts

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Neocarzinostatin is a radiomimetic antibiotic with a potent cytotoxic effect which elicits a hypersensitive response in human cells homozygous or heterozygous for the gene for ataxia-telangiectasia. The extent and the time course of potentially lethal damage repair and sublethal damage repair following neocarzinostatin treatment were investigated in human skin fibroblast strains and were found to be remarkably similar to those obtained following X-irradiation. Ataxia-telangiectasia homozygous cells essentially lacked potentially lethal damage repair, but were able to perform some degree of sublethal damage repair following neocarzinostatin treatment. Ataxia-telangiectasia heterozygous cells which show an intermediate degree of neocarzinostatin sensitivity could perform both processes but with somewhat reduced efficiency as compared to normal cells. These observations provide further evidence for a DNA repair defect in ataxia-telangiectasia cells.

The antitumor antibiotic neocarzinostatin (NCS) which is composed of a single polypeptide chain and a nonprotein chromophore is a potent cytotoxic agent in vitro. The NCS chromophore interacts directly with cellular DNA and induces extensive strand breakage accompanied by inhibition of DNA synthesis (see ref. 1 for review). There is probably some similarity between the DNA lesions induced by NCS, X-rays,and the radiomimetic glycopeptide bleomycin, since cells from patients with the autosomal recessive disorder ataxia-telangiectasia (A-T) are markedly hypersensitive to the cytotoxic effects of all three agents (2-6). Another typical response of A-T cells following treatment with these DNA breaking agents is reduced inhibition of DNA synthesis as compared to normal cells (2, 6-12). Although A-T cells efficiently rejoin DNA strand breaks induced by X-rays, bleomycin or NCS (2-4, 13), a defect in the repair of a certain radiogenic DNA lesion has been

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implicated in A-T. This is because the repair of potentially lethal and sublethal damage is severely reduced in these cells after treatment with X-irradiation (14-17). Potentially lethal damage repair (PLDR) is reflected by an increase in survival of plateau-phase cells, as compared to exponentially growing cells, when allowed to remain quiescent for the time between treatment with a DNA damaging agent and subculturing (14-21). Sublethal damage repair (SLDR) is observed in exponentially growing cells treated with a split dose of a DNA damaging agent, as compared to cells treated once with the same total dose (16, 21, 22).

In this study, the extent and time course of PLDR and SLDR were studied in human skin fibroblast strains treated with NCS. Additional evidence for a DNA repair defect in A-T cells was obtained by showing that following NCS treatment both PLDR and SLDR were defective in A-T homozygous cells and were reduced to some extent in A-T heterozygous cells.

MATERIALS AND METHODS

Cells. Skin fibroblast strains were established at the Department of Human Genetics, Hebrew University-Hadassah Medical Center, from skin biopsies donated voluntarily. The cells were maintained in Dulbecco's Modified Eagle's Medium (Grand Island Biological Co., N.Y.) supplemented with 15% fetal calf serum (Seralab, West Sussex, England) and were free of mycoplasma. Experiments were carried out with cells at passage levels 5-14 which were seeded in 5 cm tissue culture dishes.

NCS treatment. The medium was removed and 5 ml of phosphate buffered saline (PBS) containing the desired concentration of NCS (Kayaku Antibiotics Research Co. Ltd., Tokyo, Japan, Lot No. NC09S) were added to the cultures. After 15 or 30 min at 37°C, the treatment was terminated by removing the NCS solution and rinsing the monolayers twice with prewarmed PBS.

Measurement of PLDR. To obtain quiescent cultures, the cells were seeded at a 1:5 split ratio and were grown for 16 days with medium changes at 4, 8 and 12 days after seeding. On the 16th day, the level of residual DNA synthesis in these cultures was 0.2-0.4% of that in exponentially growing cultures containing an equal number of cells (data not shown). The medium was removed, the cells were treated with NCS, and the used medium was then returned to the cultures which were incubated at 37°C for the desired period. The monolayers were then trypsinized and the cells were inoculated into 5 cm tissue culture dishes containing 5 ml of Ham's F-10 medium (GIBCO), supplemented with 10% fetal calf serum and 10% horse serum (Seralab) at concentrations of 200-5000 cells/dish. Between four and eight dishes were seeded for each determination point. Following incubation for ten days at 37°C, the cultures were stained with 0.2% (w/v) crystal violet in 50% ethanol and colonies composed of at least 50 cells were recorded under a dissecting microscope. Survival curves were constructed, using a linear regression program.

Measurement of SLDR. Three days after cells were seeded at a 1:7 split ratio, the exponentially growing cultures were treated with either one dose of NCS or two doses separated by various incubation intervals at 37°C in fresh medium. Immediately following the last treatment, the cells were trypsinized and seeded as described for colony formation.

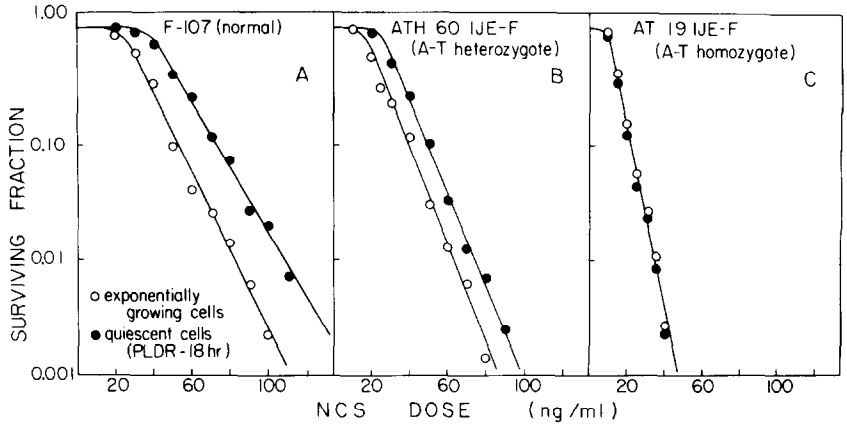


Fig. 1. Survival curves of exponentially growing and plateau-phase human fibroblasts treated with NCS. Plateau-phase cells were allowed to remain in quiescence for 18 hr between NCS treatment and subculturing.

RESULTS

PLDR: The survival curves obtained with quiescent normal human fibroblasts after an interval of 18 hr between NCS treatment and subculturing showed a pronounced increase in cellular survival as compared to exponentially growing cells subcultured immediately after treatment (Fig. 1A and Table 1). A-T heterozygous cells, previously shown to be moderately hypersensitive to NCS (5, 6) responded similarly (Fig. 1B and Table 1). The ratio between the average D_{37} values (treatment dose reducing cellular survival to 37%) obtained with and without PLDR was 1.46 for normal strains and 1.27 for heterozygous strains. The corresponding ratios of D_{10} values (treatment dose reducing cellular survival to 10%) were 1.41 for normal strains and 1.21 for A-T heterozygous strains. This shows that in relative terms, the process of PLDR was slightly less efficient in the A-T heterozygous cells than in normal cells. Identical survival curves were obtained with A-T homozygous cells with and without the PLDR procedure (Fig. 1C and Table 1), showing that in these cells this process was completely lacking.

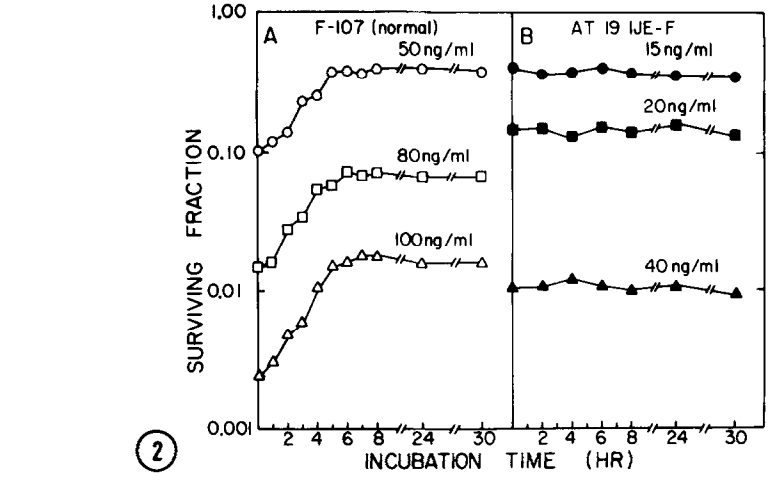
The time course of PLDR was followed in the normal strain F-107, using three different NCS doses: PLDR was essentially complete 5-6 hr after treatment (Fig. 2A). The deficiency in PLDR in A-T cells was again demonstrated in time course experiments carried out with strain AT 19 LJE-F (Fig. 2B).

SLDR: SLDR was assayed in exponentially growing cells treated with either a single

Table 1 Potentially lethal and sublethal damage repair in human fibroblast strains treated with neocarzinostatin[a].

Cell strains	Exponentially growing cells				Quiescent cells (PLDR interval:18 hr)	
	Single dose		Split dose (SLDR interval:18 hr)			
	D_{37}[b]	D_{10}[c]	D_{37}	D_{10}	D_{37}	D_{10}
Normal (n=2)	37.2 ± 2.1	53.1 ± 1.9	57.8 ± 3.1	79.3 ± 3.8	54.3 ± 3.0	75.1 ± 2.5
A-T heterozygotes (n=2)	26.6 ± 0.9	40.5 ± 2.4	34.9 ± 1.9	52.5 ± 1.6	33.9 ± 1.1	49.1 ± 2.8
A-T homozygotes (n=3)	15.3 ± 0.5	22.1 ± 0.7	17.5 ± 1.0	25.0 ± 1.3	15.1 ± 0.6	21.6 ± 0.8

(a) Each cell strain was tested twice or three times.
(b) NCS dose reducing cellular survival to 37% of control value (ng/ml).
(c) NCS dose reducing cellular survival to 10% of control value (ng/ml).



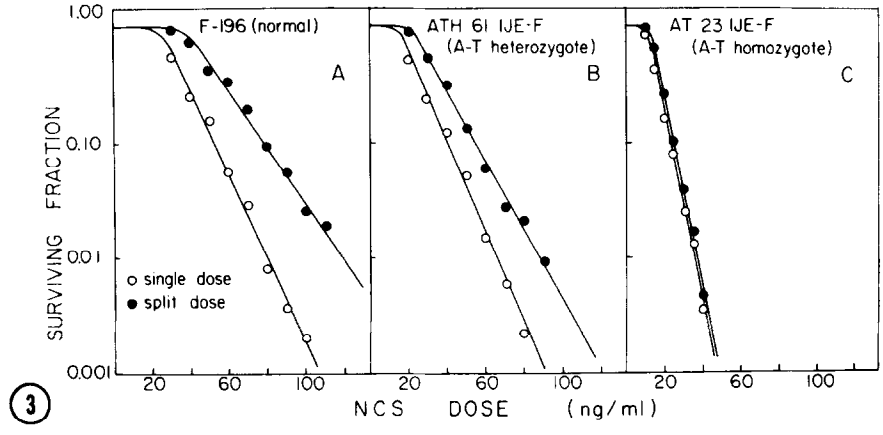


Fig. 2. Time course of PLDR in normal and A-T homozygous fibroblast strains. Plateau-phase cells were treated with the indicated doses of NCS and were incubated in quiescence for different time intervals before subculturing.

Fig. 3. Survival curves of exponentially growing human fibroblasts treated with NCS either once or with the same dose split in two halves. The time allowed for SLDR was 18 hr.

dose or with a split dose given with an interval of 18 hr. All three types of cells used in this study could perform SLDR, but with different efficiencies: the maximal efficiency of SLDR was found with normal cells, while a less pronounced extent of SLDR was observed in A-T heterozygous strains (Fig. 3A and B and Table 1). D_{37} ratios between split-dose and single dose treatments: 1.55 for normal cells and 1.31 for A-T heterozygous cells. The corresponding D_{10} ratios: 1.49 and 1.29. SLDR was found to be markedly deficient in A-T homozygous cells (Fig. 3C and Table 1), although not completely absent, as was the case with PLDR. Time course experiments showed that the

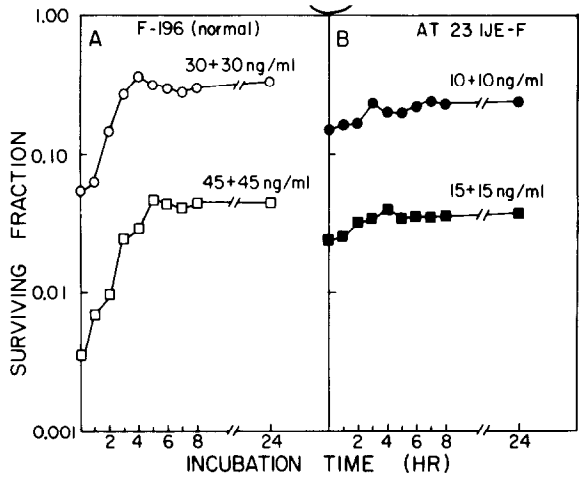


Fig. 4: Time course of SLDR in normal and A-T homozygous fibroblast strains. Exponentially growing cells were treated twice with the indicated doses with different time intervals between the treatments.

kinetics of SLDR performed by normal cells were basically similar to those of PLDR, and the process was completed by 4 hr following the first treatment (Fig. 4A). The extremely limited capacity of the A-T cells was again demonstrated (Fig. 4B).

DISCUSSION

The extent and time course of PLDR and SLDR observed in normal human cells within the first few hours following NCS treatment (Figs. 2 and 4) were very similar to those previously obtained with X-rays (14-22). This is taken as an indication for a similarity in DNA repair pathways operating after treatment with both agents. In both cases, the primary DNA lesion is the strand break. However, with X-rays most of the damage is induced by free radicals formed in the cells (23), while with NCS the chromophore interacts directly with the DNA at preferred DNA sequences, by intercalation, formation of covalent adducts, and possibly also by radical formation (1, 24, 25). The exact chemical nature of NCS-induced strand breaks is now being investigated (26, 27). The lack of PLDR in A-T homozygous cells after NCS treatment correlates with the results obtained with X-rays (14-17). On the other hand, we observed a limited degree of SLDR after treatment of these cells with NCS, while this process was absent in A-T homozygous cells treated with X-rays (16). This may reflect the ability of these cells to repair some DNA lesions not common to both agents, but even so, the extent of SLDR observed with the A-T

homozygous cells is remarkably low, as compared to normal cells. The somewhat reduced efficiency of both PLDR and SLDR in the A-T heterozygous cells again indicates the partial degree of DNA repair deficiency which is reflected also by their intermediate sensitivity to NCS (6).

It has been suggested that the increased radiosensitivity of A-T cells derives solely from a defect in regulation of DNA synthesis after irradiation and not from a DNA repair defect (9). The deficiency of PLDR observed in A-T homozygous cells indicates, however, a DNA repair defect. It should be noted that xeroderma pigmentosum cells, which are clearly repair deficient with regard to ultraviolet-induced DNA damage, also fail to perform PLDR after uv-irradiation (14).

Fornace et al. (28) found a correlation between the time course of X-ray-induced PLDR and rejoining of SSB in mouse C3H/10T½ embryo fibroblasts. The kinetics of NCS-induced PLDR do not correspond to those of the rejoining of SSB in NCS-treated cells (13), but rather seem to fit the time course of the rejoining of DSB (13). However, we previously found that A-T homozygous cells had a normal ability to rejoin both types of strand breaks after NCS treatment (13), as is the case with X-ray induced strand breaks (2-4). DNA strand breaks may indeed be efficiently rejoined in A-T cells, but subsequent processes, such as restoration of the correct spatial conformation of the DNA and chromatin at the site of the breakage, may be faulty. Thus the DNA breaks may be "rejoined" in A-T cells, but not completely "repaired". Such a defect may also be linked to the defective regulation of DNA synthesis in A-T cells after treatment with X-rays, bleomycin and neocarzinostatin.

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