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Rejoining of DNA Double-Strand Breaks in Human Fibroblasts and Its Impairment in One Ataxia Telangiectasia and Two Fanconi Strains

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Using the technique of neutral elution through polycarbonate filters as a measure of DNA length, and hence of the number of double-strand breaks incurred as a result of radiation damage, we found that normal human fibroblasts rejoin 50% of all breaks within only 3 min (37°C). This fast rejoining was impaired in fibroblasts from one patient with Ataxia telangiectasia and in fibroblasts from two patients with Fanconi's anemia. Also the number of residual breaks after several hours of repair was higher than in control cells. Other cases with the same diseases were normal in their rejoining of double-strand breaks.

Key words: DNA repair, double-strand breaks, γ-ray irradiation, Ataxia telangiectasia, Fanconi's anemia, neutral filter elution

Ataxia telangiectasia (AT) and Fanconi's anemia (FA) are human autosomal recessive diseases, associated with a higher than normal incidence of cancer. Patients with AT suffer from neurological disorders, ocular and cutaneous telangiectasia, and immune deficiencies, and their cells are more sensitive to the effects of ionizing radiation and carcinogens [1–3] than cells from normal individuals. The symptoms of FA range from diverse congenital anomalies to a progressive insufficiency of the bone marrow leading to pancytopenia in the peripheral blood. Lymphocytes of patients with FA are also more sensitive to ionizing radiation and carcinogens. In addition, an increased sensitivity to bifunctional alkylating agents has been reported [4, 5]. In both diseases the frequency of spontaneous and induced chromosome aberrations is increased [6–8]. Since unrepaired double-strand breaks (DSB) are the most likely initial lesions ultimately generating chromosome aberrations, we examine here whether cells from such patients can repair DSB. Since earlier attempts to find a deficiency in the rejoining of DSB were unsuccessful in AT patients [9], we have used a new method, the neutral filter elution technique, to measure DSB. Evidence has been presented that the

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technique can detect DNA DSB produced by X-rays, bleomycin, and Hpa I restriction endonuclease [10]. It has the advantages of avoiding damage by shearing forces and of being able to detect different types of rejoining process [11]. Using this method, we were able to measure reduced rates and extents of DSB rejoining in one case of AT and in two cases of FA.

MATERIALS AND METHODS

Cells and Cell Labeling

The fibroblast strain "Munich" was derived from a normal child donor and obtained from Dr. R. Eife of the Universitäts-Kinderklinik München. The fibroblast strain "Berlin" was derived from a normal adult donor and obtained from Dr. K. Sperling of the Freie Universität Berlin. The fibroblasts AT2BE (CRL 1343), AT3BI, and AT5BI were derived from patients with Ataxia telangiectasia CRL 1343 was obtained from the American Type-Culture Collection, Rockville Maryland; AT3BI and AT5BI were kindly provided by Dr. D. Bootsma, Rotterdam. The fibroblast cells strains FA 1196, FA 1424, FA 3557, and FA Buse were from patients with Fanconi's anemia. FA 1196 was obtained from the American Type-Culture Collection. FA 1424, FA 3557, and FA Buse were kindly provided by Dr. K. Sperling, Berlin.

Cells were grown as monolayers in Dulbecco's medium supplemented with 20% fetal bovine serum, 1 μg penicillin, and 1 μg streptomycin/ml. [2-^{14}C]Thymidine was added to growing cells to an end concentration of 0.04 μCi/ml for several days and was removed 1 day before irradiation. At the time of irradiation, the cells were in exponential growth.

Irradiation

Irradiation was carried out in a ^{60}Co gamma source (Gammacell 220, Atomic Energy of Canada Ltd). This source yielded a dose rate of 33 Gy (3300 rad) per minute. The cells were irradiated as monolayers in fresh Dulbecco's medium at room temperature for 1.5 min. The cells were then incubated at 37°C in conditioned medium for the times indicated (humidified incubator, 95% air, 5% CO_2). When no incubation followed the irradiation, the cells were irradiated at 0°C.

Neutral Filter Elution

The method used is a modification of that described by Bradley and Kohn [10]. After incubation the medium was removed and the cells were treated with 0.25% trypsin in 0.5 mM Na_2 EDTA. An aliquot containing $3 \cdot 10^5$ cells was deposited on a polycarbonate filter of 2-μm pore size (Nucleopore), lysed at room temperature with a solution of 4.0 ml 0.05 M Tris, 0.05 M glycine, 0.025 M Na_2 EDTA, 2% w/v sodium dodecyl sulfate (SDS) and 0.5 mg/ml of freshly dissolved proteinase K (Merck) adjusted to pH 9.6. The lysis solution was allowed to flow slowly through the filter without suction. Then the eluting solution (which equals the above solution without proteinase K), was gently added, and the DNA was eluted using a pump at 0.03 ml/min. Fractions were collected at 90-min intervals and mixed with 10 ml of Instagel for scintillation counting. Radioactivity remaining on the filter was determined after treating the filters with 0.5 ml of 1 M

HCl at 80°C for 1 hr and then with 2.5 ml of 0.4 M NaOH. Radioactivity remaining in the funnel was recovered by three washes with 3 ml of 0.4 M NaOH, and the counts were added to those remaining on the filter.

RESULTS AND DISCUSSION

Normal Human Fibroblasts Repair DNA Double-Strand Breaks

The criterion for the existence of a double-strand break in DNA detected by neutral filter elution is the change in DNA length which determines the elution rate through polycarbonate filters at nondenaturing pH. The rate of elution increases as a function of radiation dose. The lowest dose producing a measureable response in the elution is 5 Gy (500 rad). Under aerobic conditions 5 Gy of γ-rays produce one DSB per 3.2×10^{10} daltons of DNA [11].

The fragmentation produced by 50 Gy of γ-irradiation in normal human fibroblasts was partially reversed by subsequent incubation of the cells at 37°C. The DNA elution profile returned very quickly to the control profile obtained with nonirradiated cells (Fig. 1). After 3 min about 50% of the γ-ray induced DSB

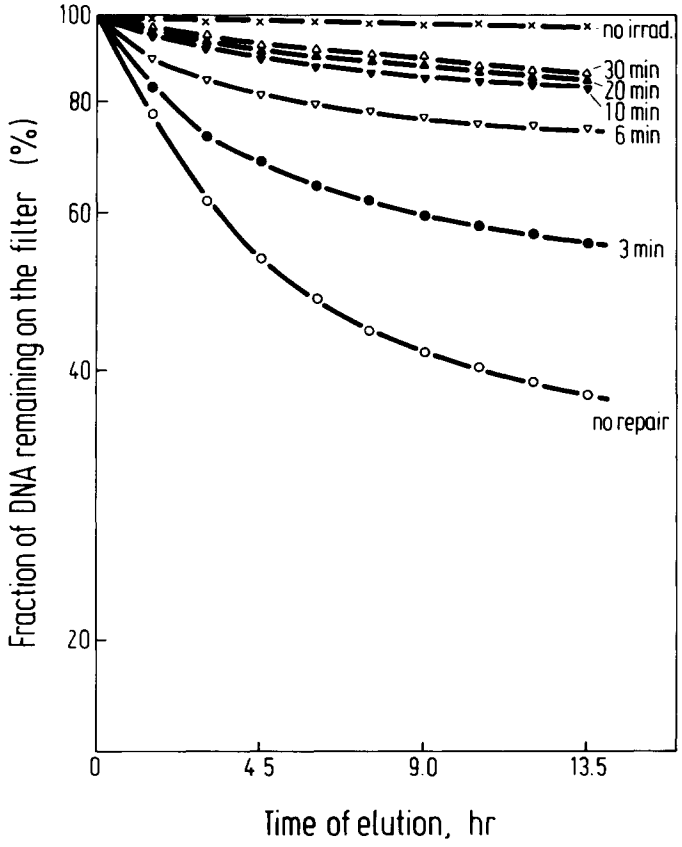

Fig. 1. Rejoining of DSB in normal fibroblasts. Monolayers of normal cells were irradiated with 50 Gy of γ-rays and incubated at 37°C for the time indicated. They were then lysed by SDS and proteinase K and analyzed by neutral elution.

had been rejoined. At 30 min, 90% were rejoined. About 5% remain unrejoined under these conditions (Table I). These data were similar in both the normal fibroblasts from a healthy child and those from a healthy adult (Fig. 2).

The rejoining kinetics in human cells are comparable with those obtained in hamster cells and bacteria [12] but faster. The time taken to rejoin half the DSB is 7.5 min in hamster cells and 8 min in bacteria. In bacteria it occurred at the same rate in the wild type as in a recombination deficient (recA) mutant. However, the extent of maximal rejoining was depressed in the recA mutant implying that recA may be involved in a slow rejoining process. The "fast" rejoining was suppressed at nonpermissive temperature in a mutant with a temperature sensitive ligase. Thus two kinds of DSB rejoining could be distinguished, a "fast" process of DNA ligation and a slower process involving other functions, possibly recombination.

TABLE I. Rejoining of DSB in Normal Fibroblasts and AT2BE Cells (CRL 1343) at Long Time Periods

	DSB rejoined (%)	
Repair incubation time	Normal fibroblasts	AT2BE
30 min	87.8 ± 1.7	81.3 ± 1.6
1 hr	90.0 ± 1.4	81.3 ± 5.3
2 hr	88.0 ± 1.0	82.9 ± 1.5
3 hr	89.6 ± 0.8	83.6 ± 4.4
4 hr	93.5 ± 0.9	89.6 ± 3.6

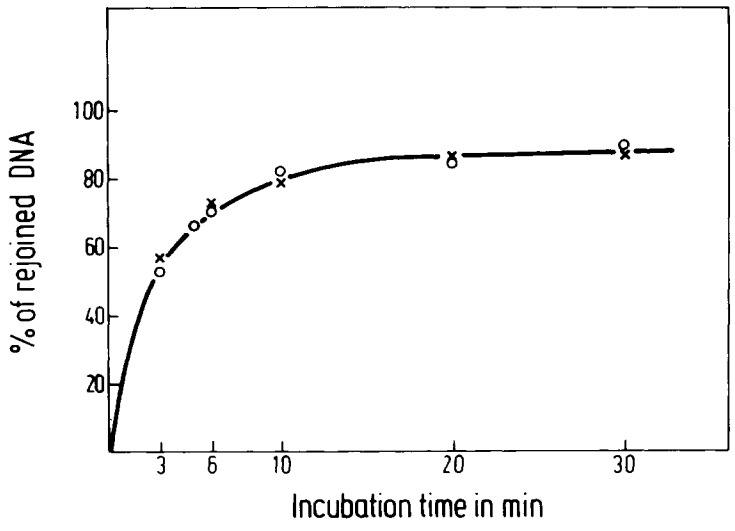

Fig. 2. Rate of DSB rejoining in normal fibroblasts irradiated with 50 Gy of γ-rays; (○), fibroblasts "Munich" from a normal child donor; (X), fibroblasts "Berlin" from a health adult. The percentage of rejoined DNA $= \frac{\log (MV(irr., rejoined / MV(irr., nonrejoined))}{\log (MV(control) / MV(irr., nonrejoined))}$.
MV = mean value of amount of DNA retained on filter during 15 hr of elution.

Recently two phases of recovery were observed in both plateau phase and exponentially growing human fibroblasts (AG 1518). The first (early) phase occurs at 2–10 min after irradiation, the second (late) at 30–90 min [13]. The early and late recovery processes seem to be of different types because they are suppressed at different temperatures. The two phases of rejoining may perhaps be related to the different phases of recovery. Further investigations will be undertaken to examine whether there is a correlation between the "fast" rejoining of DSB and the "early" recovery in human cells.

Ataxia Fibroblasts Differ in Their Ability to Rejoin DSB

The technique we have used measures both the extent of initial DNA degradation and the cells ability to rejoin the fragments. As expected, no spontaneous degradation was observed in the DNA of AT cells. The same level of DSB was induced by 50 Gy of γ-irradiation in AT and normal cells (data not shown).

After repair incubation, the DNA from one of the three AT strains investigated (AT2BE) eluted faster, indicating an impairment in DNA rejoining. Both the fast and the slow parts of the rejoining were affected (Fig. 3). The delay in the

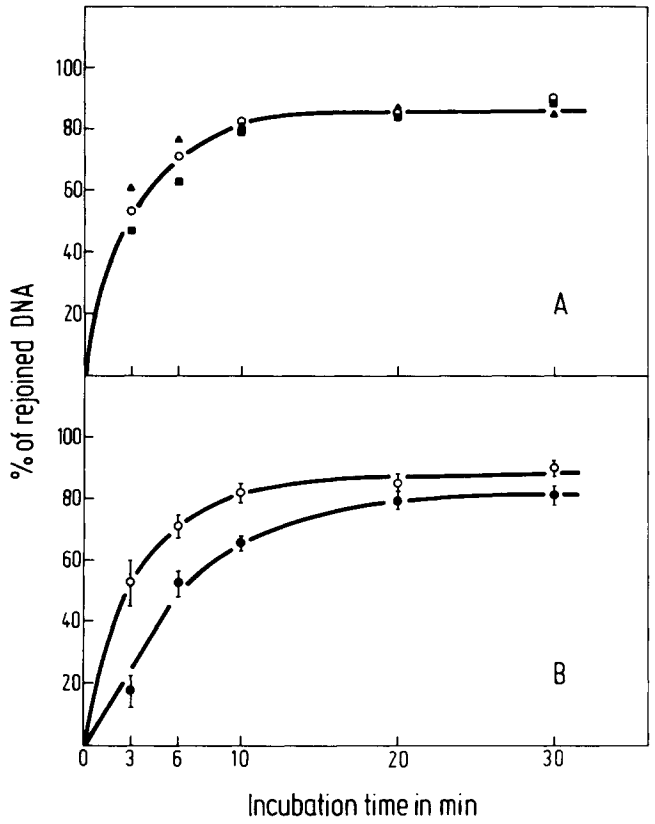

Fig. 3. Rate of DSB rejoining in normal and Ataxia telangiectasia fibroblasts. (A), (○), Normal fibroblasts "Munich". (▲), AT3BI; (■), AT5BI. (B), (○), Normal fibroblasts; (●), AT2BE (CRL 1343). Each point represents the mean value obtained from three experiments.

"fast" repair process may not be decisive for cell survival but reflects a deficiency in the repair machinery. Residual breaks as determined by our measurements (Table I) may relate, however, to lethal events [14, 15] and to the number of chromosome aberrations ultimately accumulated [7]. From cytogenetic data it has been suggested that only 5–10 % of the total DNA strand breaks need to remain unrepaired in AT, as compared to less than 1% in normal cells, in order to account for the difference in the chromosome aberrations [7]. The percentage of nonrejoined breaks in AT2BE cells after long incubation times was higher by 4–6% than in normal cells; significance was obtained using a pooled t- test ($P > 0.98$). The ability of AT2BE to rejoin DSB has not previously been investigated. In contrast, the rejoining of DNA single-strand breaks induced by X rays and bleomycin was analyzed by alkaline filter elution and found to be normal after 1–6 hr of incubation [16]. Nevertheless, in three independent experiments we observed a reduction in the rate and completion of double-strand resealing in AT2BE after irradiation with a dose of 50 Gy.

The other AT cells tested, AT3BI and AT5BI were normal in their rejoining kinetics (Fig. 3A).

While proficient in rejoining single-strand breaks, AT2BE and AT3BI both have a reduced capacity to remove alkali stable radioproducts and to carry out unscheduled DNA synthesis and repair replication. However, they were allocated to two distinct complementation groups [17, 18]. AT5BI was proficient in the rejoining of single-strand breaks and in excision repair. Our findings on DSB rejoining are consistent with the hypothesis that these cells belong to different complementation groups, as has been found by somatic cell hybridization [17].

Fanconi Fibroblasts With Reduced Ability to Rejoin DSB

In the four FA strains investigated no spontaneous degradation was observed and the same level of DSB was induced by 50 Gy of γ-irradiation (data not shown).

In two FA strains, FA CRL 1196 and FA Buse, the rejoining was normal (Fig. 4A), whereas in two other strains, FA 3557 and FA 1424, this process was impaired in both the fast and the slow phases. This shows that a heterogeneity in the rejoining capacity exists between the different FA strains. It is interesting to note that in cell fusion experiments FA 1424 and FA Buse complemented each other, with respect to mitomycin C sensitivity, whereas the hybrid cell FA 1424 $\times$ FA 3557 showed no complementation [19].

Bacterial experiments suggested that the "fast" rejoining process depended on ligase functions, and that recombination process plus ligase activity are involved in the slower phase of the rejoining kinetics [12]. A similar mechanism may exist in human cells since a decrease in DNA ligase activity has recently been observed in FA 1424 lymphocytes and fibroblasts [20]. This decrease has been related to the delayed rejoining of DNA fragments after UV irradiation and the accumulation of replicative intermediates.

All the deficient cells showed a prolonged cell cycle. Our experiments with hamster cells have shown that DSB rejoining ability is independent of the cell cycle [12]. Thus it seems unlikely that the differences in the rejoining kinetics of some AT and FA strains may be due to cell cycle abnormalities, but more probably to a deficiency in some enzyme activity involved in the rejoining of DSB.

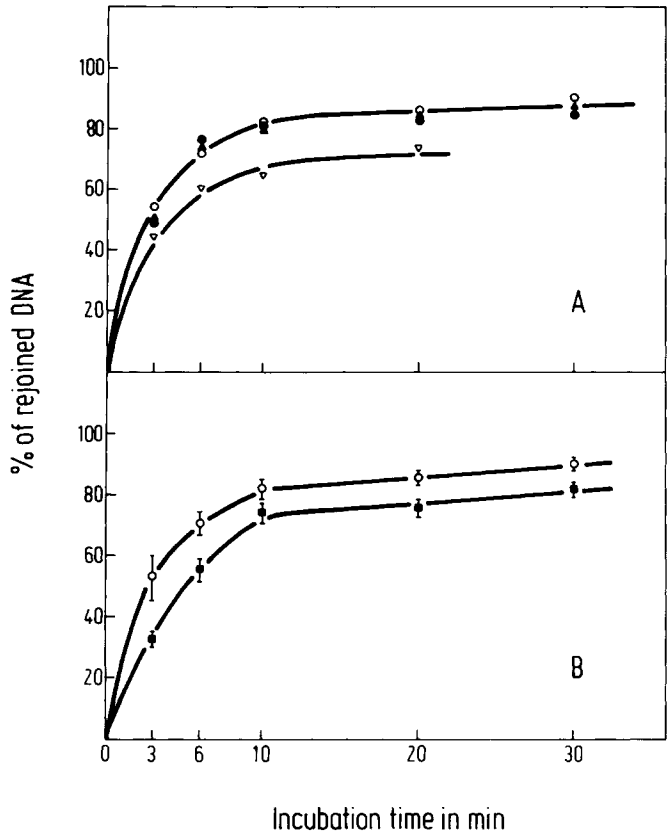

Fig. 4. Rate of DSB rejoining in normal and Fanconi fibroblasts. (A), (○), Normal fibroblasts "Munich"; (●), FA Buse; (▲), FA CRL 1196; (△), FA 3357. (B), (○), Normal fibroblasts "Munich"; (■), FA 1424. Except for those of FA 3557, each point represents the mean value obtained from three experiments.

The greater amount of aberrations in AT and FA cells than in normal cells may be the consequence of a higher percentage of unrepaired breaks critical for the cells. Very sensitive methods are required to detect such low numbers of unrepaired DNA double strand breaks.

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