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Genetics

Genetic heterogeneity in ataxia-telangiectasia studied by cell fusion

(DNA synthesis/x-rays/complementation analysis)

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ABSTRACT **The effect of x-rays on the rate of semiconservative DNA replication was investigated by autoradiography in single cells obtained from normal individuals and from patients having ataxia-telangiectasia (AT). In the five AT cell strains studied, the rate of DNA synthesis was inhibited to a lesser extent than in two normal cell strains. By using this abnormal regulation of DNA replication in AT cells as a marker, an experimental procedure was developed that allowed genetic complementation analysis of AT. After Sendai virus-induced fusion of AT cells, the grains were counted over binucleate cells with both nuclei in S phase. In some cases, the inhibition of DNA synthesis caused by x-rays in the heterodikaryons was more pronounced than that in the parental homodikaryons and was comparable to that in normal binucleate cells, indicating complementation. By using this approach, the five AT cell strains that were investigated could be assigned to three complementation groups. The data suggest that extensive genetic heterogeneity exists in AT.**

Ataxia-telangiectasia (AT) is a rare, inherited, cancer-prone syndrome clinically characterized by progressive neurological degeneration and immunological incompetence. The patients show a greatly exaggerated response to therapeutic treatment with x-rays (for a review, see ref. 1).

Cultured cells obtained from individuals suffering from the disorder consistently show an increased frequency of spontaneous and radiogenic chromosomal aberrations and hypersensitivity, in terms of cellular survival, to various types of ionizing radiation and to bleomycin (for recent reviews, see refs. 2 and 3). Another consistent characteristic of AT cells that was recently discovered in various laboratories (4–9) is that, in AT cells exposed to ionizing radiation, the rate of semiconservative DNA replication is inhibited to a lesser extent than in normal cells. Still very little is known about the molecular defect underlying these cellular abnormalities.

Besides the consistently observed characteristics of AT cells, varying responses to ionizing radiation in different AT cell strains are shown with respect to some other parameters. Most relevant of these is the capacity to perform repair DNA synthesis after exposure to γ-rays, which is impaired in some AT cell strains, whereas others behave normally (10–12). The existence of these two categories ("excision-deficient" and "excision-proficient") suggests the possibility of genetic heterogeneity in AT. The first genetic complementation studies of AT were carried out by Paterson *et al.* (12). In this analysis, the recovery of a normal level of γ-ray-induced repair DNA synthesis after fusion of AT cells was used as a criterion for complementation. Therefore, these investigations that show the existence of two complementation groups were necessarily restricted to the category of "excision-deficient" AT cell strains. Because the diminished inhibition of DNA replication after radiation exposure is a common characteristic of all AT cell strains, the use of this phenomenon as an indicator in complementation analysis should allow a genetic survey of "excision-proficient" AT cells as well.

This report describes the development of an experimental procedure for genetic analysis of AT by using such an approach. The results of these studies indicate that an extensive genetic heterogeneity exists in AT.

MATERIALS AND METHODS

Cell Strains and Culture Conditions. Fibroblast cell strains from AT patients were obtained from A. M. R. Taylor (Birmingham, England; AT262 and AT3BI, two different biopsies from the same patient), C. F. Arlett (Brighton, England; AT4BI and AT5BI), M. Ikenaga (Osaka, Japan; AT1OS), and J. Zaremba (Warsaw, Poland; AT1PWA). Cell strain AT3BI is defective in γ-ray-induced repair DNA synthesis, and the others are normal in this respect (refs. 10–12; unpublished data). Cells were cultured in Ham's F10 medium supplemented with 2 mM glutamine, 7.5% fetal bovine serum, 7.5% newborn bovine serum, and with penicillin and streptomycin (each, 100 units/ml). The strains were screened for mycoplasma contamination at least monthly with propidium iodide fluorescence.

Cell Fusion Procedure. Confluent cultures were trypsinized and split into two subcultures in medium containing carboxylated polystyrene microspheres ("beads") of 0.78- or 1.83-μm diameter (Polysciences, Philadelphia, nos. 7766 and 7769; final concentration, 1.8×10^8 and 0.8×10^8 beads per ml, respectively). The cells were grown at 37°C for 3 days to allow efficient uptake of the beads. Cells preloaded with different types of beads were fused by using inactivated Sendai virus (13) and were seeded on coverslips in 3-cm Petri dishes (2×10^5 nuclei per dish). When attached, they were rinsed twice with warm phosphate-buffered saline (Dulbecco variant) and cultured further at 37°C in complete medium additionally buffered with 20 mM Hepes (pH 7.4).

Inhibition of DNA Synthesis. Twenty-four hours after fusion, the cells were exposed to 300-kV x-rays in Hepes-buffered medium at room temperature (175 rad min^{-1}) and were incubated further with fresh medium at 37°C. They were pulse-labeled at indicated times for 60 min in Hepes-buffered medium containing [*methyl*-^{3}H]thymidine (46 Ci/mmol, 0.2 μCi/ml; 1 Ci = 3.7×10^{10} becquerels), rinsed twice with ice-cold phosphate-buffered saline, and fixed with Bouin's fixative. Autoradiography was performed with Ilford K-2 dipping fluid. The exposure time was 3–5 days. The grains over at least 50 S-phase nuclei were counted. Only nuclei with more than three grains were considered labeled. The relative rate of DNA replication was defined as the ratio of the mean grain numbers in irradiated and sham-irradiated cells.

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Abbreviation: AT, ataxia-telangiectasia.

RESULTS

DNA Replication in Unfused Cells. In order to investigate the rate of DNA synthesis, the cells were labeled with [^{3}H]thymidine for 60 min after exposure to x-irradiation. The amount of radioactivity incorporated by cells in S phase was determined autoradiographically. Fig. 1 shows the results from an experiment in which the kinetics of DNA replication were investigated in normal and AT cells exposed to x-rays. For this purpose, the cells were pulse-labeled at different times after irradiation with a dose of 2 krad. In both AT and normal cells, an initial decrease in the relative rate of DNA synthesis wassobserved, followed by a gradual recovery starting at about 4 hr after x-ray exposure. At all times after irradiation, the inhibition of DNA replication in two AT cell strains was less pronounced than in normal cells. One of the AT strains was defective in γ-ray-induced repair DNA synthesis (AT3BI), whereas the other (AT4BI) was normal in that respect. When the grain numbers were arranged in histograms, unimodal profiles were observed in all cases, which indicates that the diminished inhibition of DNA synthesis in AT is not caused by an abnormal subpopulation of cells.

Because the extent of the inhibition of DNA synthesis and its difference between AT and normal cells were maximal between 2 and 3 hr after irradiation, this time-point of labeling (see arrow in Fig. 1) was chosen for a study of the effect of x-rays on other AT cell strains. The results of this experiment (Fig. 2) indicate that the rate of DNA replication was depressed in the AT cells to a significantly lesser extent than in normal cells after exposure to doses of x-rays up to at least 4 krads. All five AT cell strains tested behaved similarly in this respect. These data indicate that autoradiographic analysis of single cells is a suitable method for the detection of differences in the relative rates of DNA replication as observed in x-irradiated AT and normal cells.

DNA Replication in Fused Cells. For genetic complementation analysis, the inhibition of DNA synthesis after x-irradiation was studied in fused AT cells. One day after fusion, the cells were irradiated and pulse-labeled 2 hr later. In the autoradiograms, the grains were counted over unfused cells in S phase and over binucleate cells that contained two S-phase nuclei.

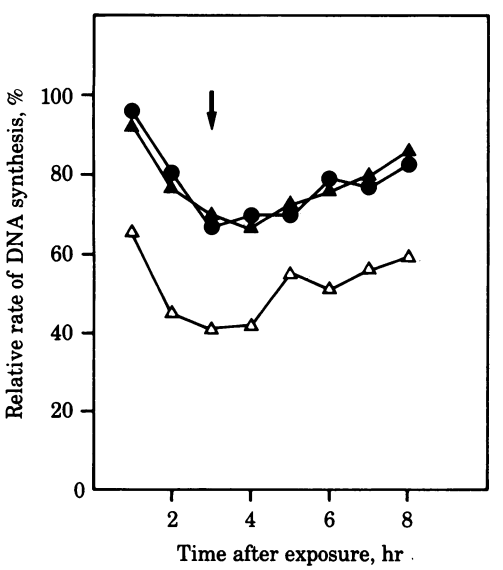

FIG. 1. Kinetics of DNA replication after exposure to 2 krad of x-rays. After irradiation, the cells were cultured for different periods in nonradiative medium, followed by 60 min in medium with [^{3}H]-thymidine, and were fixed at the times indicated in the abscissa. △, Normal cells (C5RO); ▲, AT3BI; ●, AT4BI.

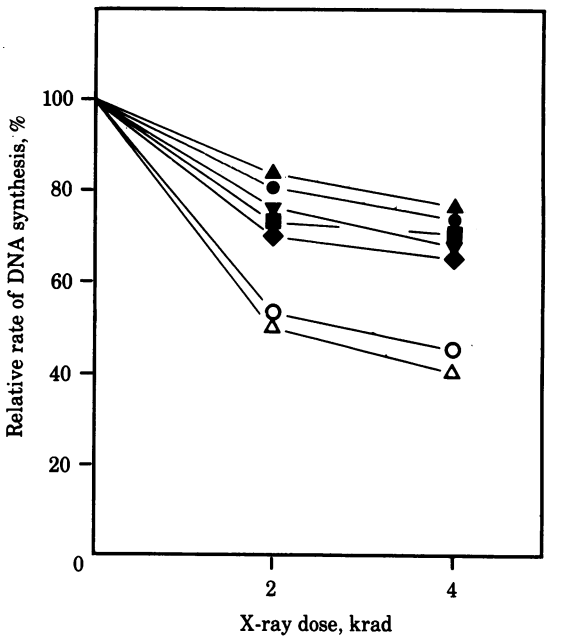

FIG. 2. Inhibition of DNA replication in various cell strains. Labeling with [^{3}H]thymidine started at 2 hr after x-ray exposure. Normal cells: C3RO (○) and C5RO (△). AT cells: AT3BI (▲), AT4BI (■), AT5BI (▼), AT1OS (●), and AT1PWA (◆).

Before fusion, the parental cells were preloaded with polystyrene beads of different sizes, which allowed an easy microscopical identification of the various types of mononucleate and binucleate cells that were present on the same slide. Fig. 3 shows the results of such an experiment, in which the "excision-deficient" AT cell strain AT3BI containing 1.8-μm beads was fused with "excision-proficient" AT4BI cells containing 0.8-μm beads. In a parallel incubation, normal C5RO cells not preloaded with beads were fused. Similar relative rates of DNA synthesis were seen in mononuclear cells and homodikaryons, but the inhibition in the normal cells was more pronounced than in the AT cells.

It appears that the relative rate of DNA synthesis after exposure to x-rays is not affected by the presence of the beads and is also not influenced by cell fusion. The inhibition of DNA synthesis in the AT heterodikaryons (AT3BI/AT4BI) was much more pronounced than in the parental homodikaryons and was comparable to that in normal homodikaryons. These results demonstrate that AT3BI and AT4BI cells complement each other with respect to their defects in the regulation of DNA replication after exposure to ionizing radiation.

Similar studies were carried out by fusion of other pairs of AT cell strains (Table 1). In the control cultures that had not been exposed to x-rays, the mean grain number over AT heterodikaryons was very near to the average of the grain numbers over the two parental homodikaryons. But after x-ray exposure, a difference in some cases between these two values ("observed" and "expected") was evident, resulting in a relative rate of DNA synthesis in heterodikaryons that was comparable to that in normal binucleate cells. In other cases, the radioactivity in the nuclei of the irradiated AT heterodikaryons was not statistically different from the mean of the two parental homodikaryons, indicating the absence of complementation.

No complementation occurred after fusion of AT3BI with AT262. This fusion served as a control, because these two strains were from different biopsies from the same patient. AT262 cells proliferated more slowly in culture than did AT3BI cells, and

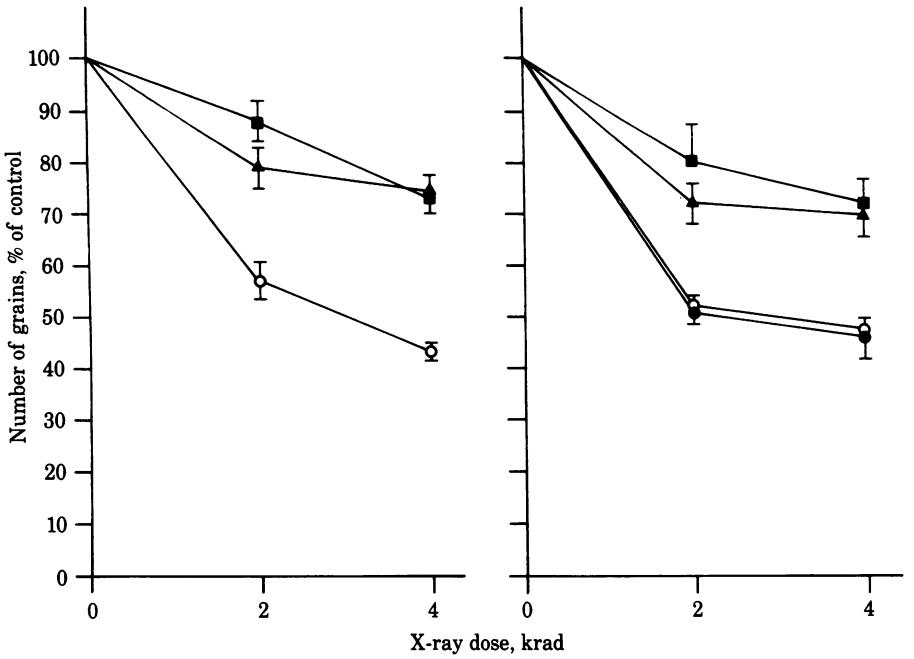

FIG. 3. Inhibition of DNA synthesis in different types of cells obtained by fusion. Conditions were as described in Fig. 2. (*Left*) Mononucleate cells. (*Right*) Binucleate cells. ○, C5RO (normal cells); ▲, AT3BI; ■, AT4BI; and ●, heterodikaryons AT3BI/AT4BI.

they were morphologically distinct—e.g., the nuclear diameter in AT262 was larger.

AT3BI cells complemented the "excision-proficient" AT cells AT4BI (the experiment in Table 1 is an independent repeat of that in Fig. 3) and AT5BI. Because AT4BI also complemented AT5BI, it follows that AT3BI, AT4BI, and AT5BI are in three different complementation groups. The absence of complementation between AT5BI and AT1OS and between AT4BI and

Table 1. Number of grains over binucleate cells after exposure to x-rays

Exp.	X-ray dose, krad	Ataxia-telangiectasia: Homodikaryons, Large beads	Ataxia-telangiectasia: Homodikaryons, Small beads	Ataxia-telangiectasia: Heterodikaryons, Observed	Ataxia-telangiectasia: Heterodikaryons, Expected*	Normal cells	Complementation
1		AT3BI	AT4BI			C5RO	Yes
	0	61.0 ± 3.7	45.6 ± 3.2	56.6 ± 2.7	53.3	55.2 ± 3.2	
	4	26.4 ± 1.4 (44%)	21.4 ± 1.1 (47%)	16.9 ± 1.4 (29%)	23.9 (45%)	13.8 ± 1.1 (25%)	
2		AT3BI	AT262				No
	4	67.5 ± 4.4	106.1 ± 5.6	89.3 ± 4.6	86.8	—	
3		AT3BI	AT5BI			C5RO	Yes
	0	111.9 ± 10.0	108.8 ± 8.6	111.1 ± 5.3	109.8	77.0 ± 3.7	
	2	83.3 ± 5.2 (74%)	81.5 ± 5.0 (75%)	65.9 ± 3.3 (59%)	82.4 (75%)	44.1 ± 2.2 (57%)	
	4	72.2 ± 2.8 (65%)	62.7 ± 2.9 (56%)	46.5 ± 2.2 (42%)	67.5 (62%)	35.2 ± 1.5 (46%)	
		AT1OS	AT5BI				No
	4	75.9 ± 2.8	60.6 ± 2.5	68.8 ± 1.7	68.3	—	
4		AT5BI	AT4BI			C5RO	Yes
	0	84.0 ± 6.8	92.1 ± 7.0	89.4 ± 5.9	88.1	110.0 ± 10.1	
	2	66.1 ± 5.0 (79%)	69.1 ± 5.2 (75%)	55.5 ± 3.9 (62%)	67.6 (77%)	65.8 ± 4.9 (60%)	
	4	58.8 ± 4.2 (70%)	62.6 ± 5.1 (68%)	47.3 ± 3.0 (51%)	60.7 (69%)	54.1 ± 3.8 (49%)	
5		AT1OS	AT4BI			C5RO	Yes
	0	—	—	—	—	62.5 ± 3.8	
	4	60.7 ± 3.9	49.8 ± 3.1	37.2 ± 2.0	55.2	31.8 ± 1.2 (51%)	
		AT4BI	AT1PWA				No
	4	45.1 ± 2.7	55.2 ± 3.4	50.4 ± 2.9	50.1	—	
		AT1OS	AT1PWA				Yes
	4	60.3 ± 2.3	64.1 ± 3.0	44.6 ± 2.3	62.2	—	

The average grain number per nucleus was determined in each binucleate cell. The given data represent the mean ± SEM of at least 25 binucleate cells. Numbers in parentheses indicate the relative rate of DNA replication.

* The "expected" value represents the calculated mean of the grain numbers obtained in the two parental homodikaryons.

AT1PWA indicates that AT1PWA is in the same group as AT4BI and that AT1OS belongs to the same group as AT5BI. Complementation occurred after fusion of AT1PWA with AT1OS, which is consistent with this group assignment.

We conclude from these results that the five AT cell strains tested fall into three separate complementation groups; two of these contain "excision-proficient" AT cell strains and another contains "excision-deficient" cells.

DISCUSSION

Our group (6, 9) and several other laboratories (4, 5, 7, 8) have shown that the inhibition of DNA synthesis in AT cells exposed to ionizing radiation is significantly less pronounced than in normal cells. In these studies, the rate of DNA synthesis was estimated by the amount of tritiated thymidine that had been incorporated by whole cell cultures as measured by liquid scintillation counting. Complementation analysis with this technique is unreliable because of the presence of a significant portion of unfused cells in the cultures and the inhibition of entry into the S phase in multinucleate cells (13). For these reasons, we have chosen to measure the rate of DNA replication at the single-cell level by performing autoradiography and counting the grains above the nuclei in S phase. This procedure is similar to that followed by Rudé and Friedberg, who studied the inhibition of DNA synthesis by UV light in xeroderma pigmentosum (14).

The data obtained by autoradiographic analysis of unfused cells confirm the results from the previous studies of AT (4–9) and show that, after x-ray exposure, the rate of DNA synthesis in single AT cells is inhibited to a lesser extent than in normal cells. The kinetics of DNA replication in x-irradiated cells were very similar to those we found with liquid scintillation assay (9). This similarity suggests that the abnormal inhibition of DNA synthesis in AT found by liquid scintillation measurements is caused by an effect only on DNA-synthesizing cells and cannot be explained by an increased efficiency of entry into the S phase by AT cells upon radiation exposure.

The study of the inhibition of DNA replication in x-irradiated fused cells shows that complementation occurred after fusion of some pairs of AT strains, whereas this was not the case in other pairs. The absence of complementation after fusion of AT262 with AT3BI cells demonstrates that the experimental results were not influenced by differences in morphological characteristics or growth potential of the cell strains used. Therefore, we consider the inhibition of DNA synthesis after x-ray exposure a suitable parameter in genetic complementation analysis of AT. This procedure can be applied for a broad genetic survey of AT cell strains because the abnormality in the inhibition of DNA synthesis is a consistent feature of cultured AT cells. The five AT cell strains investigated in our study could be assigned to three different complementation groups.

To combine our genetic data with those obtained by Paterson *et al.* (12), we also carried out fusions with AT2BE cells, which were shown by these authors to be genetically different from AT3BI. Unfortunately, the proliferation of this cell strain under our experimental conditions was very poor, resulting in an insufficient amount of S-phase nuclei in the preparations. But because our results with AT3BI indicate that differences in the level of repair DNA synthesis may have a genetic basis, it seems probable that also AT2BE cells are genetically different from the "excision-proficient" cells. Therefore, our data and those of Paterson *et al.* (12) indicate that four different complementation groups exist in AT (Table 2). We suggest a provisional nomenclature using the letters A–D. The fact that, in seven different AT cell strains, four complementation groups were identified suggests that an extensive genetic heterogeneity may exist in AT, comparable to that observed in xeroderma pigmentosum (15).

Table 2. Complementation groups in ataxia-telangiectasia

Group	Our data	Paterson *et al.**
A	AT3BI	AT3BI, AT1BE
B	—	AT2BE
C	AT4BI, AT1PWA	—
D	AT5BI, AT1OS	—

* Ref. 12.

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