Ataxia telangiectasia

Evaluation of radiosensitivity in cultured skin fibroblasts as a diagnostic test

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SUMMARY   The sensitivity to x-ray inactivation of cultured skin fibroblasts from clinically confirmed and suspected cases of ataxia telangiectasia was compared with that of cultures from normal subjects. The results confirm previous observations of an association between ataxia telangiectasia and enhanced *in vitro* radiosensitivity, and also suggest that clonal survival of x-irradiated cultures of skin fibroblasts is a valuable technique for confirming the diagnosis of the disease.

The early clinical diagnosis of ataxia telangiectasia (AT) is often difficult to make (Boder, 1975). While ataxia is usually present from an early age, the other principal features of this syndrome may not be apparent until much later. Although the diagnosis can be supported by the finding of abnormal humoral and cell-mediated immunity (Peterson *et al.*, 1963), raised levels of alpha-fetoprotein (Waldmann and McIntire, 1972), and cytogenetic abnormalities (Hecht *et al.*, 1966), it is basically on clinical grounds that the diagnosis is made.

Patients with AT appear to be abnormally sensitive to the effects of x-rays (Gotoff *et al* 1967; Morgan *et al.*, 1968; Cunliffe *et al.*, 1975). Following these radiotherapeutic observations, Taylor *et al.* (1975) found that cultured skin fibroblasts from AT patients showed enhanced sensitivity to the inactivating effects of γ-rays. The radiosensitivity of AT is also manifest in the high frequency of chromosome abnormalities after *in vitro* irradiation of lymphocytes from patients (Taylor *et al.*, 1976). Biochemical studies with cultured fibroblasts have provided evidence that, in some strains, the radiosensitivity of AT is associated with deficiencies in enzyme systems that repair damaged DNA (Paterson *et al.*, 1976) and it has been suggested that DNA repair-deficiency represents the primary defect in AT (Taylor *et al.*, 1976; Paterson *et al.*, 1976).

We have studied the clonal survival of x-irradiated cultures of skin fibroblasts from clinically 'definite' cases of AT, from patients in whom the diagnosis was thought probable but who lacked oculocutaneous

telangiectasia, and from patients in whom the clinical diagnosis was thought possible but unlikely. The results of these studies suggest that quantitative clonal survival techniques that measure the radiosensitivity of freshly-isolated human cells (Cox and Masson, 1974) are valuable in confirming the diagnosis of AT.

Patients and methods

Patients. Group I: 3 patients (from a group of 15) with a definite clinical diagnosis of AT. Group II: 4 patients with a probable clinical diagnosis of AT, although there was no oculocutaneous telangiectasia (Table 1A). Group III: 4 patients in whom the clinical diagnosis of AT was considered 'possible' but improbable (Table 1B).

Skin biopsies. After initial cleansing of the skin, a small area of the flexor surface of a forearm was anaesthetised with an intradermal injection of $0 \cdot 5$–$1 \cdot 0$ ml 1% lignocaine. An area of skin 1–2 mm diameter was excised and the sample transferred to 10 ml sterile cell culture medium (see below). The wound was covered with a dry dressing and complete healing occurred within a week.

Initiation and maintenance of fibroblast cultures. Skin biopsies were cut into small fragments using sterile scalpels and the fragments (5–10) were anchored to the growth surface of 25 cm² plastic culture flasks with plasma clots. Culture medium (8 ml) was added and the skin fragments were incubated at 37° in 95% air plus 5% CO_2 for up to 8 weeks. The culture medium in all experiments was Eagle's Minimal Essential Medium containing 50 IU penicillin, 50 µg/ml streptomycin, and supplemented with 10%

Table 1 *Summary of relevant clinical data in 4 'probable' and 4 'possible' cases of ataxia telangiectasia (AT)*

Patients	Age (years)	Sex	Family history	Ataxia	Chorea	Oculomotor apraxia	Infective problems	Other relevant clinical findings
A: Probables								
AT6LO	4·5	M	—	+	+	—	+	—
AT1LO	11	M	—	+	+	+	—	Spastic diplegia
AT11LO	5	F	—	+	—	—	+	—
AT8LO	14	M	—	+	+	—	—	Possible early ocular telangiectasia
B: Possibles								
AT9LO	2·5	F	Parental consanguinity	+	—	+	—	—
AT7LO	10	F	—	+	—	+	—	Mild spastic diplegia; low IQ
AT4LO	4	M	—	+	+	+	—	Acute onset of ataxia at age 6 m
AT12LO	2·5	F	Parental consanguinity; sister with a similar condition	+	—	—	—	Spastic diplegia

+ = present; — = absent

fetal calf serum. Culture medium in primary cultures was renewed weekly. When the outgrowth of cells from each skin fragment exceeded 1 cm diameter, the cells were removed from the flask by a 5 minute incubation at 37° in a solution of 0·1% trypsin plus 0·4 mg/ml EDTA. The cells were then resuspended in fresh medium and reinoculated into fresh flasks ($5 \cdot 10^5$ cells/25 cm² flask containing 8 ml medium). These secondary cultures were maintained in exponential growth by regular subculture.

Estimation of radiosensitivity of fibroblast cultures. The sensitivity of fibroblast cultures to 250 kV x-rays was estimated using clonal survival of single cells from an irradiated population as a measure of radiosensitivity (Cox and Masson, 1974, 1975). Cells were irradiated with x-ray doses of up to 450 rad (dose rate 50–250 rad/min) 20 hours after attachment to the growth surface of 9 cm diameter plastic Petri dishes. Estimates of radiosensitivity were made in quadruplicate on cells from the second and fourth subculture using a feeder layer technique described previously (Cox and Masson, 1974). Each experiment was made up of two series of Petri dishes: series I, where the number of cells on dishes was based upon the radiosensitivity of normal human fibroblasts (Cox and Masson, 1974, 1975); and series II, where the number of cells on dishes was based on the known radiosensitivity of fibroblasts from AT patients (Taylor *et al.*, 1975). After x-irradiation, dishes each received 10 ml fresh culture medium and were incubated in 95% air plus 5% CO_2 at 37° for 14–18 days; after this time clones of surviving cells were stained with 0·25% azur A dye and counted using a low power microscope. The fraction of cells surviving each dose of radiation was calculated with respect to the cloning efficiency of unirradiated cells.

Results

Growth of fibroblast cultures. Incubation of skin fragments for 4 to 6 weeks usually produced sufficient outgrowth of fibroblasts to allow sub-culture. In 2 cases (AT5LO and AT8LO), incubation for 8 weeks was necessary. The population doubling time of secondary fibroblast cultures from patients varied; those cultures later found to be radiosensitive generally grew more slowly and had a lower

Table 2 *Cloning efficiency and radiosensitivity of early passage fibroblast cultures from normal humans and patients with confirmed or suspected ataxia telangiectasia*

Subject*	Sex	Mean cloning efficiency (%)	Radiosensitivity Mean x-ray Do† ± SE (rad)
Normal	M & F	Range 40–90	Range 100–160
Definite AT			
AT2LO	F	18	40 ± 1
AT3LO	F	5	49 ± 1
AT5LO	M	7	48 ± 4
Probable AT			
AT1LO	M	57	80 ± 2
AT6LO	M	20	50 ± 2
AT8LO	M	2	78 ± 3
AT11LO	F	30	39 ± 1
Possible AT			
AT4LO	M	95	126 ± 2
AT7LO	F	65	122 ± 2
AT9LO	F	46	104 ± 6
AT12LO	F	58	123 ± 2

*Normal human fibroblast cultures were initiated from (a) fetal lung and skin (male and female, 8–14 weeks, 10 samples); (b) adolescent skin (male 15 yr, 1 sample); and (c) adult skin (male 26 yr, 1 sample). For details of AT patients see text and Table 1.

†Calculated by linear regression analysis of data shown in Figs. 1–3. Range of Do values for normal humans includes x-ray survival data from other laboratories (Albertini and DeMars, 1973; Weichselbaum *et al.*, 1976); x-ray survival curves for normals are not shown.

cloning efficiency than those of normal radio-sensitivity (Table 2). Extended division cycles (Elmore and Swift, 1976) and low cloning efficiencies (Hoar, 1975) have been noted in previous studies with cultured AT fibroblasts.

Radiosensitivity of fibroblast cultures. The observations on cell survival in fibroblast cultures after different doses of x-rays are shown in Figs. 1–3, each of which also shows the normal range of response. The 3 cases of definite AT are clearly abnormal. None of the 4 possible cases is at all abnormal.

Data on cell survival of the type shown in Figs. 1–3 can be fitted to the classical exponential survival equation: surviving fraction = $e^{-\lambda D}$, where λ is a constant and D is the dose of radiation. A common convention is to characterise such curves by $D_O = 1/\lambda$ = the radiation dose required to reduce the number of surviving cells to 0·37 of the initial number. The more sensitive the cells the smaller the value of D_O.
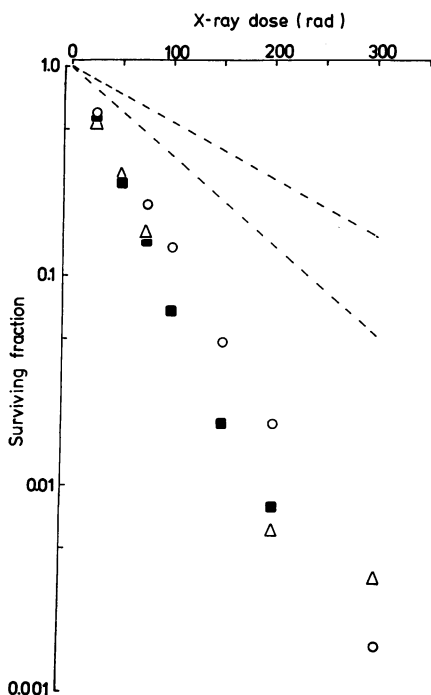
D_O values for our 3 group I (definite AT) patients were in the range 30–50 rad (Fig. 1, Table 2), approximately three times smaller than the value for normals and similar to those of 3 AT cases studied by Taylor *et al.* (1975). Of our 4 group II (probable AT) patients, 2 gave D_O values comparable with those of definite cases and 2 gave D_O values intermediate between definite cases and normals (Fig. 2, Table 2). The 4 patients in group III (possible AT) all gave D_O values in the normal range (Fig. 3, Table 2).

When a culture was found to be extremely radiosensitive (D_O 30–50 rad) the Petri dishes in series I of the experiment (number of cells seeded based on normal radiosensitivity) contained few surviving clones. Conversely, cultures with normal radiosensitivity produced confluent growth on Petri dishes in series II (number of cells seeded based on extreme radiosensitivity). For cultures of intermediate radiosensitivity, it was possible to count surviving clones in both series of Petri dishes.
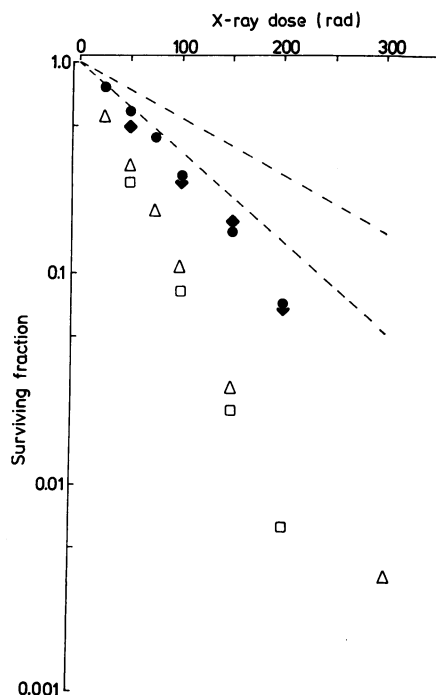

Fig. 1   X-*ray dose-response for inactivation of fibroblast cultures from group I (definite AT) patients.* ■ = *AT2LO;* O = *AT3LO;* △ = *AT5LO. Broken lines represent survival range of normal control cultures (see Table 2).*

Fig. 2   X-*ray dose-response for inactivation of fibroblast cultures from group II (probable AT) patients.* ● = *AT1LO;* △ = *AT6LO;* ◆ = *AT8LO;* □ = *AT11LO. Broken lines as in Fig. 1.*
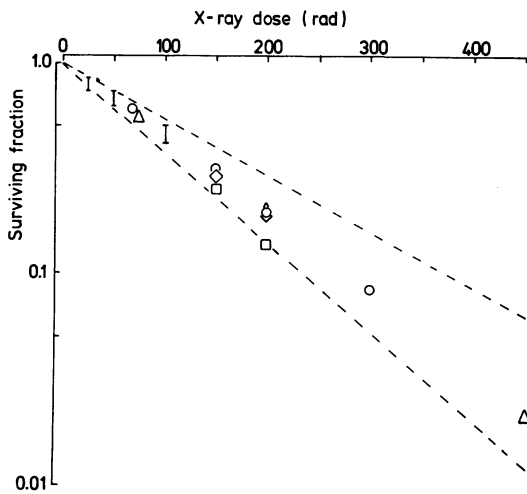
X-ray dose (rad)

Fig. 3 X-ray dose-response for inactivation of fibroblast cultures from group III (possible AT) patients. O = AT4LO; △ = AT7LO; □ = AT9LO; ◊ = AT12LO. Bars at 25, 50, and 100 rad give range of survival values for all cultures. Broken lines as in Fig. 1.

Discussion

The clinical features of AT have been extensively reviewed (Boder, 1975). Boder and Sedgewick (1963) included 101 cases in their comprehensive study. Our clinical finding for 15 cases of definite AT accord closely with those of Boder and Sedgewick but due to the late appearance of characteristic oculocutaneous telangiectasia the correct diagnosis in many of these cases was not made until late in the first decade of life. Pyramidal tract involvement in these patients, and chorea sufficiently severe to mask the ataxia in 3 other patients, also served to delay correct diagnoses. 2 of the 15 patients presented with the problems of severe immune deficiency and only later were the clinical features of AT identified.

Defects in humoral and cellular immunity are very common in patients with AT, but not invariable. Raised alpha-fetoprotein levels have been shown in a group of 20 patients with AT (Waldmann and McIntire, 1972) and is undoubtedly a valuable diagnostic feature in suspected cases. Spontaneous *in vivo* cytogenetic abnormalities are well recognised in AT (Hecht *et al.*, 1966; Polani, 1976) and the *in vitro* finding of chromatid type aberrations in the first metaphase of G₀ irradiated lymphocytes from AT patients (Taylor *et al.*, 1976) may provide the basis of a useful laboratory test for diagnosis of the disease.

Taylor *et al.* (1975) showed that the raised radiosensitivity of cultured fibroblasts from AT patients clearly distinguished them from cultures of normal subjects. The data presented here confirm this and in addition allow comment upon the diagnostic value of clonal survival techniques for AT.

Patients in whom the diagnosis of AT was incomplete (groups II and III) provided examples of extreme ('classical' AT), intermediate, and normal radiosensitivity. In 2 group II patients extreme radiosensitivity provided a provisional diagnosis of AT. Radiosensitivity intermediate between this extreme level and the normal, as in our other group II patients, suggests genetic heterogeneity in AT, a possibility that accords with biochemical evidence (Paterson *et al.*, 1976) and also with our clinical impression of phenotypic variability in the disease. An example of genetic heterogeneity associated with different sensitivities of cultured fibroblasts to a toxic agent may be found in the ultraviolet light-sensitive human syndrome, xeroderma pigmentosum (XP). In this genetic disease the UV sensitivities of fibroblast cultures from some genetically distinct patients (XP variants) are either normal (Cleaver, 1972) or intermediate between those of classical XP and those of normal (Robbins *et al.*, 1976).

Recently it has been found that individuals with genetic diseases other than AT may also show a degree of x-ray sensitivity (Weichselbaum *et al.*, 1977; C. F. Arlett, personal communication). Consequently, until further clinical evidence is available and biochemical studies have been made, it is not possible to assign the intermediate x-ray sensitivities of AT1LO and AT8LO to (a) a genetically distinct form of AT, (b) the moderating effect of other genes, or (c) a different defect also associated with enhanced radiosensitivity. X-ray sensitivity, as measured by clonal survival of cultured cells, is a gross phenotype and is probably influenced by a number of genes. AT may therefore be only one of a number of hereditary defects associated with some degree of radiosensitivity. If so, a spectrum of different radiosensitivities is to be expected in the human population and inevitably there will be some overlap in sensitivity between different subpopulations in the spectrum. Consequently, the technique described here cannot be regarded as a definitive test for AT.

We have preliminary evidence that radiosensitivity of cultured fibroblasts in early passage represents a stable genetic trait in normal humans. Provided that this also applies to AT patients then clonal survival of irradiated fibroblast cultures is a valuable diagnostic test for suspected cases of AT and will allow an early presumptive diagnosis of AT to be made in cases exhibiting extreme radiosensitivity.

The existence of intermediate x-ray sensitivities and the comparisons already made between AT and XP argue that patients failing to show *in vitro* radiosensitivity (e.g. our group III patients) should not, on these grounds alone, be excluded as possible cases of AT. We further suggest that any patient presenting early in life with severe immune deficiency might also be helpfully studied in this way.

Compared to cytogenetic analysis of radiosensitivity, the cell survival technique reported here offers two main advantages for diagnosis of AT. (1) It does not require any special operator training beyond that normally used for the culture of cells. (2) It is less subjective since macroscopical cell colonies are scored rather than microscopical and often complex chromosome configurations. The disadvantage of the cell survival technique is that in its present form it requires a large number of cells ($\sim 2 \times 10^6$) and takes a minimum of 7 weeks to complete. Consequently, cytogenetic analyses of the type used by Taylor *et al.* (1976) may be more suitable for antenatal diagnosis of AT in cultured amniotic cells.

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