Hypersensitivity to DNA-damaging agents in cultured cells from patients with Usher's syndrome and Duchenne muscular dystrophy

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SUMMARY Lymphoblastoid lines from nine Usher’s syndrome (recessively inherited retinitis pigmentosa and congenital sensorineural deafness) patients (representing eight kindreds) and from ten Duchenne muscular dystrophy patients (representing seven kindreds) showed a small but statistically significant hypersensitivity to the lethal effects of X-rays, as measured by the cellular ability to exclude the vital dye trypan blue, when compared with lines from 26 normal control subjects. Fibroblast lines from the Usher’s syndrome patients, treated with X-rays or the radiomimetic, DNA-damaging chemical N-methyl-N'-nitro-N-nitrosoguanidine, also showed a statistically significant hypersensitivity when compared to normal fibroblast lines. These findings are consistent with the possibility that defective DNA repair mechanisms may be involved in the pathogenesis of these degenerative diseases.

The term "abiotrophy" was introduced by WR Gowers in 1902 to signify the premature death of excitable tissues occurring in the absence of any histopathological evidence of the aetiology.¹ Gowers included primary neuronal degenerations and the muscular dystrophies among the abiotrophies. In 1919, E Treacher Collins classified retinitis pigmentosa, with its premature degeneration of photoreceptors, as an abiotrophy.² The concept of abiotrophy has recently been reviewed.³

The cause of the premature death of excitable tissue in these genetic disorders has long been unknown. We have suggested that the abiotrophic degeneration of neural, retinal, and muscle cells might result from the accumulation of damaged DNA.⁴³¹³ This DNA-damage hypothesis is based primarily on information obtained from the study of the autosomal recessive, sunlight-sensitivity disease xeroderma pigmentosum, one form of which involves a primary neuronal degeneration.⁴ Cultured cells from patients with xeroderma pigmentosum are hypersensitive to the lethal effects of ultraviolet radiation and ultraviolet-mimetic chemicals because of inherited defects in the repair of DNA.⁴¹⁴ Fibroblasts and lymphoblastoid lines from xeroderma pigmentosum patients with an early onset of neuronal degeneration are the most sensitive to the lethal effects of ultraviolet radiation, while cells from patients with later onset neurodegeneration or without neuronal degeneration are less sensitive.⁵⁵⁶ Cells from patients with ataxia telangiectasia, another inherited primary neuronal degeneration,¹⁵ are hypersensitive to ionizing radiation⁶¹⁴¹⁶⁻¹⁹ and to the radiomimetic chemical N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)¹⁸²⁰ because of a defect in either DNA repair systems¹⁴¹⁸²⁰ or some other system leading to secondary failure of DNA repair.²¹⁻²³ More
recently, cultured cells from patients with the following abiotropic neurodegenerative diseases have also been reported to be hypersensitive to the X-ray-type of DNA-damaging agent: Huntington’s disease,9 10 16 17 24 25 Friedreich’s ataxia,26 familial dysautonomia,10 Alzheimer’s disease,11 and Parkinson’s disease.11 We have also found hypersensitivity to MNNG in a group of nine fibroblast lines from patients with muscular dystrophy (one limb-girdle dystrophy, two Becker’s muscular dystrophy, three myotonic dystrophy, and three Duchenne muscular dystrophy lines).27 We now report that cells from patients with Usher’s syndrome (recessively inherited retinitis pigmentosa and congenital sensorineural deafness) are hypersensitive to X-rays and to MNNG. We also report that lymphoblastoid lines from patients with Duchenne muscular dystrophy are hypersensitive to X-rays.

**Methods**

**Patients**

In this study we evaluated lymphoblastoid lines from 26 apparently healthy normal control donors, nine patients (representing eight kindreds) with Usher’s syndrome, nine patients (representing seven kindreds) with dominantly inherited retinitis pigmentosa unassociated with any neurological abnormalities, and ten patients (representing seven kindreds) with Duchenne muscular dystrophy. Fibroblast lines were studied from 18 normal control donors, seven patients (representing six kindreds) with Usher’s syndrome, and two patients with ataxia telangiectasia. Identification and features of the cell donors are presented in the legend of fig 1 and in table 1. Skin biopsy and

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**Usher’s syndrome**

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**Ataxia telangiectasia**

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*The $D_0$ is the dose in rads of X-rays or the $\mu$M concentration of MNNG reducing survival from any point on the exponential portion of the survival curve to 37% of that point. The $D_{10}$ and $D_{01}$ are the doses reducing colony-forming ability to 10% and 1%, respectively. The $D_{0}$ values in series IV and V for the normal lines have been published previously.27† Lines GM 3889 and GM 3891 are from brothers.
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![Graph](image)

**Fig 1** Survival of lymphoblastoid lines treated with 100 rads of X-rays. Each symbol represents the mean viability ratio of at least six replicate experiments on a line with the exception of three Duchenne muscular dystrophy, one Usher's syndrome, and two dominant retinitis pigmentosa symbols each of which represents the average of the viability ratios of lines derived from a pair of afflicted family members. The viability ratio of each afflicted member of a pair from a kindred was within 2% of the average ratio of the pair. Each horizontal bar represents the mean ratio for its group. The p value is for comparison with the 26 normal control lines. Omission of the highest normal control line, whose viability ratio of 0-73 increases the variance of the normal control group, would result in a mean ratio for the normal control lines of 0-528 and in lower p values for the hypersensitive patient groups (p = 0.003 for the eight Usher's syndrome and 0.004 for the seven Duchenne dystrophy kindreds). Duchenne dystrophy lines: AG 3929, GM 3780, 3782, RB 4100, 5014, 5113, 5115, 5124, 5126 and NIH line C. Usher's syndrome lines: GM 3853, 3892, RB 4361, 5062, 5064, 5204, 5207, 5333 and 5360. Dominant retinitis pigmentosa lines: GM 3823, 3834, 3836, 3894, 3907, 3911, RB 4562, 5058 and 5060.

venepuncture were performed after obtaining informed consent of the donors. Lines with "GM" and "RB" prefixes were obtained from the Institute for Medical Research, Camden, NJ. Lines with "CRL" prefixes were from the American Type Culture Collection, Rockville, MD.

**Survival of lymphoblastoid lines**

Epstein-Barr virus-transformed peripheral blood lymphocytes were cultured, irradiated with X-rays, and evaluated as described previously except that the lymphoblastoid lines were not centrifuged prior to treatment with X-rays but were simply diluted with culture medium to the required cell concentration. The post-irradiation viability ratio of a cell line was calculated by dividing the concentration of viable (trypan-blue-excluding) cells in an irradiated culture on the third day after irradiation by the concentration of viable cells in an unirradiated culture of the same line on the same day. A limitation of this dye exclusion assay is that it is not automated and requires visual scoring of viable cells. Therefore, to avoid observer bias, counting of viable cells was performed using coded samples, and at least six replicate experiments were done on each line except that one normal line had only five replicate experiments. Even though the dye exclusion assay involves scoring single cells, results with the assay correlate well with colony-forming ability of the lymphoblastoid lines. For the 26 normal control lines used in this study and for the 16 genetic control lines reported on previously, there was no correlation between the post-irradiation viability ratio and either donor age or growth rate of unirradiated lines. For the statistical comparison of groups of lines, analysis of variance methods were used. One-sided p values are reported.

**Survival of fibroblast lines**

With the exception of the two ataxia telangiectasia fibroblast lines used in the X-ray experiments, all fibroblast lines were coded so that the personnel performing and evaluating the experiments did not know the identity of the lines. There was no correlation between the post-treatment survival parameters of the fibroblast lines and either donor age or plating efficiency of the untreated lines. Fibroblast lines for X-ray treatment were cultured and irradiated as described previously. Fibroblast lines for the N-methyl-N'-nitro-N-nitosoguanidine experiments were cultured and treated with MNNG after the method of Scudiero and exactly as in the previous MNNG series I-III. The serum concentrations referred to therein as 10% and 20% were actually 8½% and 16½%, respectively. We present here the results on all the coded normal and Usher's syndrome lines studied in MNNG series IV and V.

Fibroblast colony-forming ability after treatment was calculated by dividing the colony-forming efficiency (that is, "plating efficiency") of a line at a given treatment dose by the colony-forming efficiency of that line's untreated cells in the same experiment. If the latter was less than 1%, the experiment was discarded. At least four colonies had to be present at a given treatment dose for tabulation. The average plating efficiency of the Usher's syndrome lines when untreated was not significantly different from that of the normal lines. Two to ten replicate experiments were performed with each cell line for X-rays, and at least four with each cell line for MNNG (except that one normal line from MNNG series V had only one experiment and another had three). In each experiment a straight line was fit to the logarithms of the colony-forming ability at all X-ray doses and at 6 μM and higher MNNG doses using...
the method of least squares. From each straight line the Y-axis intercept and the $D_0$, $D_{10}$, and $D_{37}$ survival parameters were obtained by computer as described in detail previously.²⁹ The $D_0$ is the negative inverse of the slope of the exponential portion of the survival curve and is the treatment dose which reduces the colony-forming ability from any point on the exponential portion of the survival curve to 37% of that point. The $D_{10}$ and $D_{37}$ values are the doses which reduce the colony-forming ability to 10% and 1%, respectively. The methods used to estimate survival curves and to compare lines and groups of lines have been described previously.²⁹ One-sided $p$ values are reported.

**Results**

**SURVIVAL OF LYMPHOBLASTOID LINES**

Figure 1 shows the post-X-ray viability ratios of the lymphoblastoid lines. The mean viability ratio (±SE) of 0.54 (±0.010) for the 26 normal control lines (first column) was not significantly different from that of 0.55 (±0.030) for the seven dominant retinitis pigmentosa kindreds (last column). The mean viability ratio of 0.49 (±0.010) for the eight Usher’s syndrome kindreds (third column) was significantly less than the mean ratio of the normal control lines ($p = 0.009$) and of the seven dominant retinitis pigmentosa lines ($p = 0.029$). The mean viability ratio of 0.49 (±0.009) for the seven Duchenne muscular dystrophy kindreds (second column) was significantly less ($p = 0.012$) than the mean ratio of the normal control lines.

**SURVIVAL OF FIBROBLAST LINES**

Figure 2 shows the post-X-ray survival of the nine normal fibroblast lines (shaded area), seven Usher’s syndrome lines (open symbols), and the two ataxia telangiectasia lines (solid symbols). Table 1 presents the post-X-ray survival parameters for these lines. The normal lines had mean $D_0$ and $D_{10}$ values of 138 (range 104–175) and 342 (range 302–409), respectively. The two ataxia telangiectasia lines (fig 2) were very sensitive to the X-rays and had mean $D_0$ and $D_{10}$ values of 53 and 138, respectively. The seven Usher’s syndrome lines represented six kindreds, four of which had lymphoblastoid lines studied. These Usher’s syndrome lines had survival curves which extended from the lowest part of the normal zone to well below that zone (fig 2). Their mean $D_0$ and $D_{10}$ values (table 1) of 115 (range 99–133) and 272 (range 201–324), respectively, were significantly less than the corresponding mean values for the nine normal lines ($p = 0.007$ and 0.001, respectively). The two Usher’s syndrome siblings, GM 3889 and GM 3891, had similar survival curves (fig 2; table 1). With their average $D_0$ and $D_{10}$ values representing a single kindred, the mean $D_0$ (116) and $D_{10}$ (272) values for the six Usher’s syndrome kindreds were still significantly less than the corresponding values of the nine normal lines ($p = 0.015$ and 0.002, respectively).

The post-MNNG survival parameters of the five normal lines in MNNG series IV (table 1) had mean $D_0$ and $D_{10}$ values of 2.8 (range 2.3–3.4) and 16.1 (range 13.8–18.9), respectively, and were not significantly different from those obtained for these lines when they had been studied and presented previously in series III.¹⁰ The Usher’s syndrome line RB 5205, from one of the lymphoblastoid line donors, had $D_0$ and $D_{10}$ values of 1.8 and 11.0, respectively, which were significantly below those of the five normal lines ($p = 0.02$ and 0.05, respectively). The eight additional normal lines of MNNG series V had mean $D_0$ and $D_{10}$ values, respectively, of 2.7 (range 2.1–3.1) and 14.2 (range 12.3–16.4) (table 1). The three new Usher’s syndrome lines of series V were from three of the Usher’s syndrome.

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**Fig 2** Post-X-ray survival of normal and Usher’s syndrome fibroblast lines. Each plotted point for a cell line is the geometric mean of the post-X-ray colony-forming ability obtained at the indicated dose from the replicate experiments performed. The straight line depicting the exponential portion of each survival curve corresponds to the $D_{10}$ and $D_{20}$ estimates reported in the table. Identification of cell lines is presented in table 1. Usher’s syndrome lines GM 3889 and GM 3891 are from siblings. The shaded normal zone encompasses the survival curves for the nine normal lines.
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lymphoblastoid line kindreds. Their mean \(D_\alpha\) and \(D_{0,1}\) values of 2-3 and 12-4, respectively, were significantly less than the corresponding mean values of the normal lines (\(p = 0.023\) and 0.006, respectively). The summary \(p\) values for the mean \(D_\alpha\) and \(D_{0,1}\) values of these three Usher’s syndrome lines and the Usher’s syndrome line of series IV were \(p = 0.013\) and 0.009, respectively, demonstrating that these four Usher’s syndrome lines were hypersensitive to MNNG.

Discussion

Usher’s syndrome is the first disease with retinitis pigmentosa demonstrated to have enhanced sensitivity to ionising radiation or to a radiomimetic chemical. Usher’s syndrome cells are hypersensitive in the lymphoblastoid (fig 1) and fibroblast-X-ray (fig 2; table 1) assays and the fibroblast-MNNG assay (table 1). While there is overlap in all three assays between the post-treatment survival of the Usher’s syndrome lines and that of the normal lines, the hypersensitivity found is statistically significant. A similar degree of overlap with the lower normal limit has been reported in ionising-radiation survival assays for the following radiosensitive disorders: Huntington’s disease,17 24 25 Alzheimer’s disease,11 Parkinson’s disease,11 Friedreich’s ataxia,26 and the heterozygous state of ataxia telangiectasia.10 18 A possible reason for such overlap is that the lethal damage responsible for the hypersensitivity represents but a small portion of the total lethal lesions induced by the ionising radiation. A more detailed discussion of this possible mechanism and of its relationship to defective processes for repairing damaged DNA is presented below.

Several diseases with sensorineural deafness other than Usher’s syndrome are also associated with hypersensitivity to DNA-damaging agents: xeroderma pigmentosum6 14 and Cockayne’s syndrome14 30 31 cells are hypersensitive to the ultraviolet-type of DNA-damaging agent, and Friedreich’s ataxia26 cells to the ionising-radiation-type of DNA-damaging agent. Both xeroderma pigmentosum and Cockayne’s syndrome cells have demonstrated inherited defects in DNA repair—fibroblasts from these diseases are unable to repair normally ultraviolet-irradiated viruses in host-cell reactivation experiments.12 32—34 Friedreich’s ataxia may also be a disease with defective DNA repair; recently, Lewis and coworkers have shown that Friedreich’s ataxia fibroblasts have hypersensitivity to ionising radiation,26 moderately impaired potentially lethal DNA damage repair,26 and inhibition of post-irradiation DNA synthesis.35

The \(D_\alpha\) values (mean 138; range 104–175) of our irradiated normal fibroblast lines are similar to the values reported for normal lines by Chamberlain and Lewis26 (mean 150; range 125–168) and by Cox and Masson26 (mean 122; range 98–160). It is of interest that the mean post-irradiation \(D_\alpha\) value for the Friedreich’s ataxia fibroblasts reported by Chamberlain and Lewis26 was 83% of the mean value for their normal lines, while the mean post-irradiation \(D_\alpha\) of our Usher’s syndrome fibroblast lines also was 83% of the mean of our normal lines (table 1). If Usher’s syndrome and Friedreich’s ataxia are disorders with defective repair of DNA, it should be possible to find other DNA-damaging agents which will induce a relatively larger differential in post-treatment survival than that induced by ionising radiation or MNNG. We have used X-rays and MNNG because of the hypersensitivity of ataxia telangiectasia fibroblasts to these agents;14 16 18 20 however, for the reasons discussed by Chamberlain and Lewis,26 the molecular abnormality of ataxia telangiectasia is probably different from that of Friedreich’s ataxia and, therefore, of Usher’s syndrome. In this regard, it is important to note that neither deafness nor retinitis pigmentosa occurs in ataxia telangiectasia.13 15

Many patients with the ultraviolet-sensitive disorder Cockayne’s syndrome have a retinitis pigmentosa,37 and Cockayne’s syndrome cells are hypersensitive to the lethal effects of ultraviolet radiation14 30 31 and have abnormal host-cell reactivation of ultraviolet-irradiated viruses.12 33 34 In contrast, cells from patients with Usher’s syndrome, which is not clinically a sunlight sensitive disorder, can repair ultraviolet-radiation-induced DNA damage normally: the three Usher’s syndrome lymphoblastoid lines we tested had normal post-ultraviolet irradiation survival (unpublished data), and Usher’s syndrome fibroblasts have normal host-cell reactivation of ultraviolet irradiated \(H\) \(s\) \(m\) \(i\) \(p\) \(e\) \(x\) \(p\) \(e\) virus.12 Thus, Usher’s syndrome is the first retinal dystrophy shown to have hypersensitivity to the X-ray-type of DNA-damaging agent. However, not all diseases with retinitis pigmentosa are associated with hypersensitivity to these DNA-damaging agents.

The group of lymphoblastoid lines from patients with dominantly inherited retinitis pigmentosa had normal survival after treatment with X-rays (fig 1), and the one dominant retinitis pigmentosa lymphoblastoid line tested had normal survival after treatment with ultraviolet radiation (unpublished data). Similarly, not all primary neuronal degenerations have a hypersensitivity to the X-ray-type of DNA-damaging agent: groups of fibroblast lines from eight patients classified as having motor neuron disease26 and from five patients with spinal muscular atrophy38 had normal survival after treatment with
ionising radiation and MNNG, respectively, and a group of five lymphoblastoid lines from patients with amyotrophic lateral sclerosis had normal post-X-ray survival (unpublished data).

It is important to note that the absence of hypersensitivity to the lethal effect of a specific DNA-damaging agent does not necessarily indicate that the disease in question has no inherited defect in the repair of DNA. Thus, the lymphoblastoid line tested from one member of the xeroderma pigmentosum complementation group E kindred, as well as the fibroblast line from the other member, had post-ultraviolet irradiation survival in the normal range despite the fact that this kindred has well-documented defects in nucleotide excision repair and in host-cell reactivation of ultraviolet irradiated virus and purified viral DNA. Therefore, before concluding that dominantly inherited retinitis pigmentosa has no abnormal responses to DNA-damaging agents, other types of agents and tests will have to be utilised.

The presence of hypersensitivity to X-rays in the Duchenne muscular dystrophy lymphoblastoid lines (fig 1) is consistent with our finding of low post-MNNG survival in three Duchenne muscular dystrophy fibroblast lines. Thus, the association between hypersensitivity of fibroblasts to MNNG and hypersensitivity of lymphoblastoid lines to X-rays demonstrated for neurodegenerations and for Usher's syndrome is applicable also to the Duchenne form of muscular dystrophy.

We have demonstrated in Huntington's disease, Alzheimer disease, Parkinson's disease, Usher's syndrome, and Duchenne muscular dystrophy that the hypersensitivity is specific for the ionising-radiation type of DNA-damaging agent, since there is normal survival after treatment with 254-nm ultraviolet radiation in fibroblasts and/or lymphoblastoid lines from patients with these disorders. Although the molecular basis for the in vitro hypersensitivity to X-rays and MNNG of these cells is unknown, we hypothesise that X-rays and MNNG produce their lethal effect in vitro by damaging DNA. Exposure of cells to either of these agents in vitro results in a myriad of different types of lesions in DNA. Some are not lethal even if unrepaird; others are potentially lethal lesions. In normal cells a certain fraction of these potentially lethal lesions are not repaired. The fraction of these lesions not repaired by the patients' cells may be slightly higher due, perhaps, to the inability of the patients' cells to repair an infrequent type of normally repairable lesion. The resulting slight increase in the number of lethal lesions remaining in the patients' cells could cause the small (but significant) hypersensitivity we have demonstrated (figs 1 and 2; table 1). Presumably, greater differences between disease and normal cells would be evident in vitro if more selective DNA-damaging agents were used.

In vivo, the DNA of post-mitotic excitable tissue is constantly being damaged by intracellular metabolites and spontaneous hydrolytic reactions. Due to differences in the intracellular or extracellular environment, the types and quantities of this DNA damage would differ among different excitable tissues and between different neuronal populations. In normal cells the damaged DNA is readily repaired, but in these diseases characterised by hypersensitivity (table 2) there is defective repair of a type of potentially lethal lesion. The excitable tissue which undergoes premature death would be that in which the unrepairable type of lesion represents a relatively large proportion of the potentially lethal lesions induced. The different clinical patterns of these various disorders would result from different defective repair processes or from different mutations in the same repair process. The ultraviolet-type of DNA damage accumulates in xeroderma pigmentosum, while the X-ray-type of DNA damage accumulates in radiosensitive neurodegenerations, Usher's syndrome, and Duchenne muscular dystrophy. Examination of the DNA from Duchenne dystrophy muscle cells in various stages of dystrophic change should reveal increasing levels of accumulated DNA damage. Since samples of skeletal muscle can be safely and abundantly obtained, it should be possible to test our hypothesis.

### Table 2 Degenerative disorders of excitable tissue with cellular hypersensitivity to DNA-damaging agents

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*While Cockayne's syndrome is primarily a demyelinating disease, the degeneration of photoreceptors in Cockayne's syndrome appears to be a primary retinal degeneration.

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*J Neurol Neurosurg Psychiatry* 1984 47: 391-398
doi: 10.1136/jnnp.47.4.391

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