Reduced Capacity to Repair Irradiated Adenovirus in Fibroblasts from Xeroderma Pigmentosum Heterozygotes

Andrew J. Rainbow

Abbreviations used are: XP, xeroderma pigmentosum; HCR, host cell reactivation; Vag, viral structural antigen; Ad 2, adenovirus type 2; D37, dose necessary to reduce survival to 37% of that for unirradiated virus.

ABSTRACT

Xeroderma pigmentosum (XP) is one of a number of autosomal recessive syndromes in humans characterized by a marked predisposition to cancer. Fibroblasts from these patients show a defect in DNA repair. The XP heterozygotes also show elevated skin cancer incidence, but reports concerning their DNA repair capacity are conflicting. In this study, the DNA repair capacity of four XP heterozygotes was examined using a sensitive host cell reactivation technique. Unirradiated and irradiated suspensions of adenovirus type 2 (Ad 2) were assayed for their ability to form viral structural antigens in fibroblasts from XP heterozygotes, XP homozygotes, and normals. A reduced host cell reactivation (of viral structural antigen production) for both ultraviolet- and γ-irradiated Ad 2 was detected in four XP heterozygotes representing three different complementation groups as well as their XP homozygous children. The doses necessary to reduce the survival of viral structural antigen production by irradiated Ad 2 to 37% in the XP heterozygotes strains were expressed as a percentage of that obtained in normal strains and ranged from 55 to 82% for ultraviolet-irradiated Ad 2 and 71 to 79% for γ-irradiated Ad 2. These results add further support to a direct relationship between cancer proneness and DNA repair defects and show the merits of this host cell reactivation technique in identifying XP heterozygotes. Identification of XP heterozygotes is of considerable public health interest not only in genetic counseling but also in the prevention of cancer.

INTRODUCTION

XP2 is an autosomal recessive syndrome in humans characterized by a marked predisposition to develop skin cancers on sun-exposed areas (9, 24, 26). Cell lines developed from skin biopsies of these individuals are defective in their ability to repair damage induced by UV irradiation (3, 4), γ-irradiation under anoxic conditions (28), and certain chemical mutagens and carcinogens (6, 29, 31). Typical XP strains show a defect in their ability to perform excision repair of UV-induced cyclobutane pyrimidine dimers (7, 30), whereas XP variant strains show normal excision but some defect in the postreplication repair of UV-induced DNA damage (13). Both typical and variant XP cell as well as some of the heterozygous carriers show reduced levels of photoreactivating enzyme (32). Although the XP homozygotes are rare, Swift has calculated the frequency of XP heterozygous carriers to be about 0.5% of the population (15). The XP heterozygotes are not at an increased risk of dying from cancer, but they do appear to have a higher incidence of skin cancers than do noncarriers of the disease. Since defects of DNA repair have been correlated with several inherited diseases causing cancer proneness (27), the repair capacity of the XP heterozygote is of considerable interest.

HCR of Vag production for irradiated adenovirus has been shown to be a sensitive assay for the repair capacity of human fibroblasts capable of detecting reduced repair rates for radiation-damaged DNA (12,18-21). The HCR technique used has been described previously (21). Unirradiated and irradiated suspensions of Ad 2 were assayed for their ability to form Vag in human fibroblast cells infected in monolayer. Ad 2 is a double-stranded DNA virus which infects cultured human fibroblasts and replicates in the nucleus, forming large quantities of viral structural proteins (14) which can be readily detected by immunofluorescent staining. Since viral DNA synthesis is a requirement for the production of Vag, the HCR examined in this way is a reflection of the DNA repair capacity required for DNA synthesis as well as the transcription and translation necessary for the synthesis of structural proteins. Assuming that the XP heterozygote expresses one normal and one “XP gene” and that the XP gene product affects a rate-limiting step in the repair of UV-induced lesions, then, provided the level of XP gene product is insufficient to saturate the substrate presented to it, we would expect a reduced repair rate in the XP heterozygote.

Previous studies on the excision repair capacity of XP heterozygotes using a number of different techniques (2, 5, 8, 10, 11, 25) have yielded conflicting findings. By examining the HCR of Vag production for irradiated adenovirus, a reduced repair for both UV- and γ-irradiated DNA in fibroblasts from 4 XP heterozygotes representing different XP complementation groups (26) has been detected.

MATERIALS AND METHODS

Cells and Virus. Stock monolayer cultures of diploid human fibroblasts were grown in screw-cap bottles (Falcon Plastics, Oxnard, Calif.) and placed in a CO2 incubator at 37° and 90 to 100% humidity. The growth medium was α-minimum essential medium (No. 410-2000; Grand Island Biological Company, Grand Island, N. Y.) supplemented with 10% fetal calf serum together with 1% penicillin-streptomycin (No. 600-5140; Grand Island Biological Company). The normal fibroblast strains A1, A2, A6, and RE were kindly supplied by Dr. Samuel Goldstein, Departments of Medicine and Biochemistry, McMaster University, Hamilton, Ontario, Canada. The normal strain GM23 was obtained from the American Type Tissue Culture Collection, Rockville, Md. (1). The normal strain GM23 was obtained

1 Supported by the National Cancer Institute of Canada.

2 The abbreviations used are: XP, xeroderma pigmentosum; HCR, host cell reactivation; Vag, viral structural antigen; Ad 2, adenovirus type 2; D37, dose necessary to reduce survival to 37% of that for unirradiated virus.
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from the Human Genetics Cell Repository, Camden, N. J. Cell cultures were generally confluent at 7 to 9 days following a split ratio of 1:3.

The preparation of purified Ad 2 has been described (22). Purified stock virus, generally containing approximately 10^{12} particles/ml, was suspended in Tris-buffered saline (NaCl, 8 g/liter; KCl, 0.38 g/liter; Na_{2}HPO_{4}, 0.1 g/liter; glucose, 1 g/liter; Tris, 3.63 g/liter at pH 7.4) plus 20% glycerol and stored at −45°.

Irradiation. The method of γ-irradiation was essentially the same as that described previously (16). One-ml samples of stock Ad 2 were kept at dry-ice temperature (−75°) during irradiation at a dose rate of 1.6 Mrads/hr using a ^{60}Co source.

The method of UV irradiation has also been described (17). Stock virus was diluted 2- to 3-fold in a minimum essential medium, and 1 ml of viral suspension was irradiated in a 35-mm-diameter Petri dish (Falcon Plastics) and kept on ice, with constant swirling during the irradiation. Dosimetry of the UV germicidal lamp (G8T; General Electric Company, Cleveland, Ohio) was determined using a J-225 short-wave UV meter (Ultra-Violet Products, Inc., San Gabriel, Calif.). The incident dose rate under these conditions was about 6 watts/sq m.

Previous reports have shown that, after these conditions of UV irradiation, the fraction of thymine present as dimer is 1.9 × 10^{-5} sq m/J at doses up to 2.4 × 10^{3} J/sq m (23).

Production of Antiserum. Antiserum against Ad 2 structural proteins (Vag) was obtained by immunizing rabbits with purified Ad 2. The Ad 2 antigen was prepared by mixing about 2 × 10^{11} purified Ad 2 particles suspended in 1 ml Tris-buffered saline (NaCl 8 g/liter; KCl, 0.38 g/liter, Na_{2}HPO_{4}, 0.1 g/liter; glucose, 1 g/liter, Tris, 3.63 g/liter at pH 7.4) with 1 ml of Freund’s complete adjuvant (No. 660-572; Grand Island Biological Company). Initially, 1 ml of the Ad 2 antigen suspension was injected into a rabbit i.p. and 1 ml was injected i.m. Subsequent injections consisting of 0.5 ml i.p. and 0.5 ml i.m. were made at 3 and 5 weeks. The blood was harvested on the sixth week, and the antiserum was collected and sterilized by filtration. Antiserum was generally diluted 20-fold in phosphate-buffered saline (NaCl, 8 g/liter; KCl, 0.2 g/liter, Na_{2}HPO_{4}, 1.15 g/liter; KH_{2}PO_{4}, 0.2 g/liter at pH 7.5) before use.

Experimental Procedure. Nonirradiated and irradiated suspensions of Ad 2 were assayed for their ability to form Vag in human fibroblasts. For the experiments, monolayers of cells grown in 8-well chamber slides (Lab-Tek Products, Naperville, Ill.) were infected with either irradiated or nonirradiated Ad 2 as described previously (21). Three serial dilutions of the virus were used to infect each slide. The dilution series for nonirradiated and irradiated virus were prepared separately, with nonirradiated virus diluted to a greater extent than the irradiated virus.

Duplicate wells were used for each viral dilution with the 2 additional wells serving as uninfected controls. Following viral adsorption for 2 hr, infected cells were incubated in growth medium. At 48 hr after infection, the monolayers were fixed in cold acetone:ethanol (1:1), incubated in the presence of rabbit Ad 2 antiserum for 30 min at 37°, and then incubated for the same time with fluorescein-conjugated sheep anti-rabbit globulin (No. 660-352; Grand Island Biological Company). All slides for a particular experiment were stained with the same batch of diluted antisera under identical conditions and scored for Vag positive cells by a single observer. For each slide, the number of fluorescing centers was counted in duplicate wells at 3 serial dilutions of the virus, and the data points were fitted to a straight line using least-squares analysis. Taking into account the dilution factor, the slope of the line was then used as a quantitative measure of Vag formation for each treatment of the virus. UV survival of this viral function was then taken as a ratio of Vag formation for UV-irradiated virus as compared to that for nonirradiated virus.

RESULTS

Vag Production in Ad 2-infected Fibroblasts. Fig. 1 shows a photomicrograph of Ad 2-infected fibroblasts stained by indirect immunofluorescence for Vag at 48 hr after infection. It can be seen that fibroblasts producing Vag are readily scored above the dark background of nonproducing cells. Counts of Vag-positive cells obtained by different experienced independent observers for the same slides resulted in surviving fractions for Vag which were within 10% and usually much better than this.

UV Irradiation of Ad 2. Typical results for the infection of fibroblasts from normals and XP heterozygotes are shown in Chart 1. It can be seen from Chart 1, left that there were no consistent differences in the frequency of Vag positive cells between the 4 XP heterozygotes and the 2 normal strains at 48 hr after infection with nonirradiated virus. However, the frequency of Vag-positive cells was considerably lower in the XP heterozygotes than in the normal strains following infection with UV-irradiated virus (1.2 × 10^{3} J/sq m) as shown in Chart 1, right.

Chart 2 shows UV survival data in 2 normal strains, the 4 XP heterozygotes strains, and 3 of their XP homozygous children. Survival points in each of the normal and XP heterozygous strains were fitted to a straight line using least-squares analysis to obtain the D_{37} values as shown in Table 1. D_{37} values in the XP homozygous strains were obtained from the initial portion of the survival curves. It can be seen that the survival of Vag production for UV-irradiated Ad 2 was significantly less in the XP heterozygotes than in the normal strains, reflecting a reduced repair capacity for UV-damaged Ad 2 DNA in the XP heterozygotes. The D_{37} values obtained for the XP heterozygotes and XP homozygote strains were expressed as a per-
Reduced HCR of Ad 2 in XP Heterozygotes

The results of this study indicate a reduced repair of radiation-induced DNA damage for 4 heterozygotes from 3 different XP complementation groups and demonstrate the high sensitivity of this HCR technique in the detection of cellular DNA repair deficiencies.

Previous studies on the repair capacity of XP heterozygotes showed conflicting results. Using the UV-induced incorporation of 5-bromodeoxyuridine into nonreplicated DNA, Cleaver (5) found about 45% normal repair replication in 2 XP heterozygotes, about 80% normal in another, and 100% in another. Using the same technique, Kleijer et al. (11) detected about 55 and 70% normal repair replication in 2 XP heterozygotes, respectively, whereas 2 other heterozygotes showed normal levels. Using autoradiographic analysis, Bootsma et al. (2) showed 100% normal UV-induced uptake of labeled thymidine in 2 XP heterozygotes, whereas Kleijer et al. (11) showed 100% values for 3 XP heterozygotes [2 of which were the same as those used by Bootsma et al. (2)] and about 78, 71, 83, and 91%, respectively, for 4 others. Using autoradiographic analysis, Giannelli and Pawsey (10) compared the UV-induced unscheduled DNA synthesis in heterokaryons between fibroblasts from XP homozygotes and XP heterozygotes with that in heterokaryons between XP homozygotes and normal cells.

Table 1

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<th>Fibroblast strain</th>
<th>UV (J/sq m)</th>
<th>D$_{37}$ (Krad)</th>
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<td>RE</td>
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<td>ND$^c$</td>
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<td>XP5BE</td>
<td>20$^b$</td>
<td>340 ± 10</td>
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a Mean ± S.E.
b Obtained from the initial portion of the survival curve only.
c ND, not determined in this experiment.

Percentage of the average value for the normal strains used in the same experiment. This percentage of HCR value for Vag formation of irradiated Ad 2 was taken as a measure of the repair capacity of the XP strains. The percentage of HCR values obtained for a number of experiments are shown in Table 2. Average percentage of HCR values for UV-irradiated Ad 2 in the 9 normal strains used ranged from 91 to 106%. A similar range of percentage of HCR values for UV-irradiated Ad 2 in the normal strains has been reported previously (12). The percentage of HCR values for UV-irradiated Ad 2 in the 4 XP heterozygous strains ranged from 55 to 82%, with a mean of 65%.

UV-irradiation of Ad 2. Recent results from this laboratory have shown a reduced survival for Vag formation of UV-irradiated Ad 2 in XP homozygous strains as compared to normal fibroblast strains (21). It was, therefore, considered of interest to examine the survival of UV-irradiated Ad 2 in the XP heterozygous strains. UV-irradiation data are shown in Chart 3 and Table 1. It can be seen that the survival of Vag production for UV-irradiated Ad 2 was significantly less in the XP heterozygotes than in the normal strains, indicating a reduced repair capacity for UV-damaged DNA in the XP heterozygotes. As seen from Table 2, the percentage of HCR values for Vag formation of UV-irradiated Ad 2 in the XP heterozygous strains ranged from 71 to 79%, with a mean of 73%. The percentage of HCR values in the normal strains ranged from 93 to 111%.

DISCUSSION

Chart 1. Frequency of Vag-positive cells following infection of 4 XP heterozygotes and 2 normal fibroblast strains. Ad 2 was prepared, diluted, irradiated, and used to infect fibroblast monolayers grown in 8-well chamber slides at 3 serial dilutions as described previously (20, 21). The dilution series for nonirradiated and irradiated virus was prepared separately. Virus was adsorbed for 2 hr with either unirradiated (left) or UV-irradiated virus (1.2 x 10$^4$ J/sq m) (right). At 48 hr after infection, cells were fixed in cold acetone:methanol (1:1), incubated in the presence of Ad 2 antisera for 30 min, and then incubated for the same time with fluorescein-conjugated sheep anti-rabbit globulin. Positive cells were scored for duplicate wells at 3 serial dilutions of the virus for each treatment of the virus. Each point is the average of 2 chambers, the counts for each chamber generally being within 10% of each other. The total number of cells in each well was approximately 4 x 10$^4$. Normal fibroblast strains: CRL1254; V, CRL1165; A, CRL1167; M, CRL1159.

Chart 2. UV survival curves for Vag formation of Ad 2 in fibroblasts from XP heterozygotes, XP homozygotes, and normals. The frequency of Vag positive cells was determined in duplicate at 3 serial dilutions for each treatment of the virus, and the data points were fitted to a straight line using least-squares analysis in order to obtain each survival point. Symbols for the normal and XP heterozygous strains are as in Chart 1. XP homozygous strains: O, XP12BE; V, XP2BE, A, XP5BE.
### Table 2

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# ACKNOWLEDGMENTS

I thank Margaret Howes for carrying out the experiments.

# REFERENCES

5. Cleaver, J. E. DNA repair with purines and pyrimidines in radiation- and UV-irradiated Ad 2 on 4 XP heterozygous strains, 3 of which (CRL1159, CRL1165, and CRL1254) were found in the present study to have a reduced HCR of Vag production for irradiated Ad 2. Presumably, this difference results from the fact that the HCR of Vag formation measured the repair of viral DNA over a fixed time interval (48 hr), and this gives a measure of repair rate, whereas in the plaque assay deficiencies in the repair rate of the viral DNA can still result in eventual plaque formation when scored 16 to 18 days after infection.

Although it remains to be seen whether this HCR method is capable of detecting repair deficiencies in all the other known heterozygous carriers, it appears at the present time to be a relatively simple technique with a good potential for identifying the XP heterozygotes. Since the XP heterozygous carriers constitute a cancer-prone population, identification of these individuals in the population is of considerable value not only in genetic counseling but also in the prevention of cancer.

They used 3 different XP heterozygotes and were able to show a reduced unscheduled DNA synthesis using 2 of them but not when using the third. Presumably, the latter result was due to a difference in complementation group between the heterozygote and the XP homozygote which formed the heterokaryons. More recently, Ritter (25) has used a Micrococcus luteus endonuclease assay to measure the repair of UV-induced pyrimidine dimers in 5 obligate XP heterozygote fibroblasts. After 30 J/sq m of germicidal light, repair rates for the XP heterozygotes ranged from 40 to 70% of rates found in the normal controls.

Day (8) has shown normal HCR of plaque formation for UV-irradiated Ad 2 on 4 XP heterozygous strains, 3 of which (CRL1159, CRL1165, and CRL1254) were found in the present study to have a reduced HCR of Vag production for irradiated Ad 2. Presumably, this difference results from the fact that the HCR of Vag formation measured the repair of viral DNA over a fixed time interval (48 hr), and this gives a measure of repair rate, whereas in the plaque assay deficiencies in the repair rate of the viral DNA can still result in eventual plaque formation when scored 16 to 18 days after infection.
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