Effects of X Irradiation on a Temperate Bacteriophage of *Haemophilus influenzae*

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The inactivation of bacteriophage HP1c1 by X rays in a complex medium was found to be exponential, with a D_0 (the X-ray exposure necessary to reduce the survival of the phage to 37%) of approximately 90 kR. Analysis of results of sucrose sedimentation of DNA from X-irradiated whole phage showed that the D_0 for intactness of single strands was about 105 kR, and for intactness of double strands, it was much higher. The D_0 for attachment of X-irradiated phage to the host was roughly estimated as about 1,100 kR. Loss of DNA from the phage occurred and was probably due to lysis of the phage by X irradiation, but the significance of the damage is not clear. The production of single-strand breaks approaches the rate of survival loss after X irradiation. However, single-strand breaks produced by UV irradiation, in the presence of H_2O_2 , equivalent to 215 kR of X rays, showed no lethal effect on the phage. Although UV-sensitive mutants of the host cell, *Haemophilus influenzae*, have been shown to reactivate UV-irradiated phage less than does the wild-type host cell, X-irradiated phage survive equally well on the mutants as on the wild type, a fact suggesting that other repair systems are involved in X-ray repair.

Ionizing radiation causes alterations in nucleic acids and in proteins. Due to the simplicity of the virus particle and its economy of structure, one might predict that almost any structural alteration would prevent successful completion of its life cycle, and that measurement of this ability to survive would be a sensitive reflector of X-ray damage. We have undertaken to demonstrate the types of X-ray-induced changes in the phage and to account for the inactivation of the phage in terms of these changes. Complicating such measurements is the ability of host cells to repair phage DNA.

Freifelder showed that damage to the protein of the head region of the phage may rupture the particle, permitting the escape of the DNA (6). Other alterations in the protein of the phage could conceivably prevent either the attachment of the phage to the host or the injection of the DNA into the host cell. Double-strand breaks in the DNA could also result in the incomplete injection of phage DNA as shown by observations that markers on the DNA distal to the double-strand breaks remained in the phage heads after injection (10).

The fact that single strands were broken and rejoined in the excision-repair process that removes UV-induced pyrimidine dimers attests to the presence of enzymes capable of repairing some kinds of single-strand breaks (17). It was also shown that T4 has a repair system (con-

trolled by genes x and y) for damage by ionizing radiations (2), that phage-coded enzymes are necessary for proper repair of UV damage, and that discontinuities in the DNA involving single-strand gaps are also repaired (3). The *uvrA* gene product makes single-strand nicks near base or sugar damage caused by X rays, and some of these breaks as well as those directly caused by X rays are repaired (22). Van der Schans and Bleichrodt (21) measured the contributions of the various X-ray-induced damages to DNA and found that nucleotide damage accounts for most of the inactivation (87%) and that double-strand breaks are lethal, whereas only 2% of single-strand breaks are lethal. Only one phage containing double-stranded DNA has been found in which single-strand breaks are lethal: *Bacillus megaterium* phage α (7). Single-stranded phages ϕ X174 and S13 are inactivated by single-strand breaks (19, 20).

Alterations in the DNA other than strand breaks caused by ionizing irradiations are to be expected. Some of these alterations are being quantified by P. A. Cerutti at the University of Florida. However, techniques for identification and measurement of base and sugar damage were beyond the scope of our investigation.

Transfection was not considered to be a fruitful system for our studies due to the extensive breakdown of the transfecting DNA during the normal process of transfection (12).

It was shown previously (14) that UV in the presence of H_2O_2 causes true strand breaks rather than alkali-labile sites in DNA. In our studies, this method for producing single-strand breaks was compared with their production by X rays.

MATERIALS AND METHODS

Survival of X-irradiated phage. Cells of *Haemophilus influenzae* (strain Rd), originally obtained from R. M. Herriott, were the indicator cells used for lawns when phage was plated. Mutants of the Rd strain, which were isolated in the laboratory of Jane K. Setlow and are more sensitive to UV than is the wild type, have been characterized in detail and described previously. (15, 16; M. E. Boling, S. Modak, G. Price, A. Mattingly, M. Gordon, and J. K. Setlow, *Biophys. J.* 8:A54). Plating techniques were also described previously (8). All platings were done in duplicate, and some of the survival curves were repeated under varying conditions as described further in the text. The double-stranded DNA phage HP1c1 was originally obtained from W. Harm. The molecular weight of the double-stranded DNA of this phage was determined by velocity sedimentation to be 2.2×10^7 and by measurements of calibrated electron micrographs of the DNA to be 2×10^7 (1).

The phages were irradiated always as whole phage and were routinely suspended for storage in dilution medium containing brain heart infusion (BHI; Difco Laboratories) at a concentration of 3.3 g per 100 ml. The phages were purified, dialyzed against BHI, and diluted from the phage stock in BHI before X irradiation. Irradiation was accomplished with a General Electric Maxitron 250 X-ray machine at 250 peak kV and 30 mA by using a 1-mm-thick aluminum filter with a half-value layer thickness of roughly 0.2 mm of Cu. The sample was kept at approximately 4°C during the irradiation, and a typical exposure rate was 4,192 rads/min.

Phage preparations were obtained and labeled with [3H]Thd as described formerly (1).

X-ray-induced strand breaks. Samples were sedimented in alkaline and neutral sucrose gradients as described previously by McGrath and Williams (11) in a Spinco ultracentrifuge with an SW50.1 rotor at 20°C. Thirty fractions from each gradient were collected as they dripped from a puncture at the bottom of the centrifuge tube and counted by the procedure of Carrier and Setlow (4). For neutral sedimentation, the phage DNA was extracted from the phage as described previously (1) before addition of DNA (less than 0.1 μ g) to the gradients as follows: the phage in BHI was held in a freezer at -70°C for 10 min and then thawed and placed in a 70°C water bath for 5 min. This procedure was repeated three times, with an additional 10 min in the water bath the last time. Under these conditions whole phage was sometimes seen at the bottom of the gradients. No extraction was necessary for the alkaline sedimentation, since 10 min in 0.5 N sodium hydroxide on top of the gradients prior to sedimentation was

usually sufficient to lyse the phage and denature the DNA.

H_2O_2 -induced single-strand breaks. UV irradiation was at a fluence rate of 29.5 J/m² per min in a 9-cm-diameter glass petri dish. The phage in M9 medium was at a concentration of about 10^9 per ml in BHI. H_2O_2 was diluted into the phage suspension to a concentration of 0.3%. (A control sample was placed in a glass petri dish with H_2O_2 , and portions were taken at the usual times without turning on the UV lamp.) Absorption through the sample (depth, about 1.6 mm) at a wavelength of 254 nm was 47% per cm of path length. The phage was titered for infectivity and sedimented in alkaline sucrose to obtain number-average molecular weights (M_n 's) (13).

Attachment of X-irradiated phage to cells. *H. influenzae* cells that were actively growing were mixed with phage, either X-irradiated with an exposure of 482 kR or unirradiated. Cells and phage were at a final concentration of 5×10^8 per ml. After an attachment period of 15 min at 37°C in BHI growth medium, the cells and phage were chilled in ice and separated by centrifugation in a Sorvall Superspeed RC-2 automatic refrigerated centrifuge at 2°C at $7,710 \times g$ for 5 min in an SS-34 centrifuge head. Round-bottom Pyrex glass tubes were used. The pellet was suspended in 0.5 ml of BHI. Then the supernatant and suspended pellet samples were dissolved in glass scintillation vials in 3 ml of NCS Tissue Solubilizer (Amersham/Searle Corp.), and toluene counting fluid containing 12 g of Perma-blend 1 (Packard Instrument Co., Inc.) per gallon was added to fill the vial.

Removal of free DNA from phage suspensions. Enzymatic digestion of phage DNA was accomplished by incubation at 37°C for 30 min with 0.1 mg of pancreatic DNase per ml and 0.5 mg of snake venom phosphodiesterase per ml in the presence of 10^{-3} M $MgSO_4$. To test these conditions, *H. influenzae* DNA at 10^{-4} mg/ml and labeled with 3H at 1.2×10^5 cpm/ μ g was degraded from 900 to about 30 acid-insoluble cpm.

RESULTS

Phage survival. Figure 1A shows no significant variations among wild-type and four UV-sensitive mutants in their ability to repair X-ray damage in the whole phage. The survival curves are exponential throughout the range of exposures. The X-ray exposure (D_0) required to reduce the survival to 37% was approximately 90 kR. Plating these phages at 9 or 14 days after the irradiation gave the same results. Platings of phages at the time of irradiation gave on three occasions D_0 values of 128, 132, and 140 kR. Hence the measured sensitivity increases after irradiation, but does not change after reaching the level seen at 9 days. Lawn cells were doubling exponentially or were stationary cells that had been stored frozen in 18% glycerol and thawed just before plating.

FIG. 1. Survival curves for X-irradiated whole phage. (A) Plated with wild-type and mutant cells; wild type (●), DB117 (▲), DB115 (△), DB112 (■), and DB116 (○). (B) X-rayed in BHI broth (●), diluted 10^2 in BHI broth prior to irradiation (○), and diluted 10^2 in 0.067 M phosphate buffer, pH 7 (△). Plated on wild-type cells. (Note 4:1 difference in scales of abscissas.)

Figure 1B shows that diluting phage into phosphate buffer (pH 7) before X irradiation increases sensitivity considerably. The same dilution made into BHI has no effect on sensitivity, the phages obviously being protected by the BHI.

Strand breakage with X rays. The production of strand breaks was measured by sedimentation of the DNA from irradiated phages in alkaline and neutral sucrose gradients (Fig. 2 and 3). Figure 2 includes a number of alkaline sucrose gradients representing two separate experiments. The same purified phage preparation was used in both experiments, but the irradiation was given at different times. Incomplete lysis of the phage may account for the occasional counts at the bottom of the gradients, particularly for the neutral gradient profiles in Fig. 3. The freeze-thaw-heat method of DNA extraction used before neutral sedimentation did not lyse the phage as completely as did the alkali in the alkaline sucrose gradients. Neither method always completely lysed the phage. Figure 3 represents two separate neutral sucrose sedimentation experiments done with the same phage preparation, but sedimented at different times after the irradiation. Sedimentation was carried out at 41 days after irradiation for experiment A and at a few hours after irradiation for experiment B. There is reasonable agreement between results from the two experiments despite the difference in conditions caused by the time differential. This suggests that the change seen in the survival curves between very early times after

irradiation and later times is not due to an increase in postirradiation double-strand breaks.

In Fig. 4 the reciprocals of the M_n values for single strands are plotted against X-ray exposures. The D_0 value, which produced an average of one break per strand and thus halved the value of M_n , was estimated from this plot.

UV and H_2O_2 . Figure 5 shows alkaline sucrose profiles for the DNA from phages which were irradiated with UV either in the presence or absence (control) of H_2O_2 . There was no change in these profiles as a function of UV alone. However, in the presence of the peroxide, the molecular weights of the single strands decreased. The single-strand breaks produced in this way seem to have no effect on the survival of the phage. Figure 6 shows the change of $1/M_n$ for single-stranded DNA versus duration of irradiation with and without peroxide. The

FIG. 2. Alkaline sucrose gradients of DNA from X-irradiated phage. (A) and (B) represent separate experiments as described in the text. The total number of counts obtained varied no more than $\pm 10\%$. Sedimentation was at 40,000 rpm for 160 min. Sedimentation profiles are labeled with numbers representing exposures (in kR).

FIG. 3. Neutral sucrose gradients of DNA from X-irradiated phage. (A) and (B) represent two separate experiments as described in the text. The total number of counts obtained from these gradients varied no more than 10%. Sedimentation was at 40,000 rpm for 100 min. Sedimentation profiles are labeled with numbers representing exposures (in kR).

FIG. 4. Change in reciprocal of M_n for single strands after X irradiation. Values for $1/M_n$ were calculated from gradients shown in Fig. 2A (○) and 2B (●).

FIG. 5. Alkaline sucrose gradients of DNA from phages irradiated with UV, without (A) and with (B) H_2O_2 . Sedimentation was at 40,000 rpm for 160 min. Sedimentation profiles are labeled with numbers representing length of UV exposure (in minutes) at a fluence rate of $29.5 J/m^2$ per min.

M_n of single strands decreased by a factor of 3 as a result of a UV-irradiation dose of $295 J/m^2$ in the presence of H_2O_2 or by about 200 kR of X rays (Fig. 4). The corresponding decreases in infectivity of the phage at these doses was much greater than a factor of 3. An X-ray dose of 200 kR gave a survival of about 10% (Fig. 1A), whereas a UV dose of $180 J/m^2$, with or without peroxide, gave a survival of less than 0.1% (Fig. 7).

Attachment of phage to cells. Table 1 shows the distribution of radioactivity between the pellet and the supernatant medium after at-

tachment of the phage to cells and separation of the infected cells and unattached phage as described in Materials and Methods. The decrease in attachment seen at this exposure (482 kR) suggests that if the loss of attachment is expo-

FIG. 6. Change in the reciprocal of single-strand M_n as a function of UV dose with (○) and without (●) H_2O_2 . UV dose at 10 min was $29.5 J/m^2$.

FIG. 7. Survival curves for phage treated with H_2O_2 (○), UV (●), and both H_2O_2 and UV (△). The fluence rate of UV was $29.5 J/m^2$ per min.

nential, the exposure required for reducing attachment to 37% of the control value would be approximately 1,100 kR. This could account for only 8% of the inactivation of the phage, which reaches 37% survival after only 90 kR of X irradiation.

Phage lysis. Evidence for lysis of the phage by X irradiation is obtained by measuring the amount of DNase-sensitive DNA present as a function of X-ray exposure. Phages that were X-rayed and then treated with DNase had fewer acid-insoluble counts per minute as a function of X-ray exposure. Without irradiation there was a 37% decrease after DNase treatment in the number of acid-insoluble counts, indicating the presence of some DNase-sensitive DNA in the purified phage preparation (Fig. 8). At an exposure of 369 kR, 50% of the DNase-resistant, acid-insoluble DNA present before irradiation was lost by exposure to DNase. Figure 8 shows the percentage of acid-insoluble counts remaining after DNase treatment at the various X-ray exposures. The figure also shows separate data for DNase-treated and -untreated phage survival as a function of X-ray exposure. Treatment of the various irradiated samples with DNase did not affect the number of phages able to infect the lawn cells, suggesting that viable phages have no DNase-sensitive DNA.

DISCUSSION

The UV-sensitive mutants DB115, DB116, and DB117 are each defective in repairing single-strand breaks including those resulting from excision, and the mutant DB112 is deficient in excision of pyrimidine dimers caused by UV (15). Although DB117 is more sensitive to X rays than are the wild-type cells (14), generally the UV-sensitive mutants used are not defective as hosts for repair of X-ray damage in phage (15). Whatever the process for repair of single-strand breaks caused by X rays or for editing of nucleotide alterations, it must differ from steps in UV repair which excise pyrimidine dimers. If the slightly increased X-ray sensitivity seen in DB117 for colony formation is a defect in repair of single-strand breaks, the repair of strand breaks in the phage DNA is

FIG. 8. Percentage of acid-insoluble counts per minute in samples irradiated at 92, 184, and 369 kR X rays expressed as percentage of counts remaining after treatment with a mixture of pancreatic and snake venom DNases (Δ). Percentage of phage survival with ($\bullet$) and without ($\circ$) DNase treatment before plating of the phage with cells.

either carried out by a different repair system or such repair is not rate limiting for the production of phage. That single-strand breaks or alkali-labile sites are repaired by other means in this mutant has been demonstrated by Kimball et al. (9) for breaks produced by treatment with alkylating agents. Fangman and Russel (5) also showed that certain cells defective for normal DNA synthesis, but not defective for repair DNA synthesis, are sensitive to X rays but not to UV.

The sensitivity to X rays, as measured by whole phage survival, is increased on 100-fold dilution into phosphate buffer, as if the BHI medium acts as a protective compound suppressing the indirect effect of free radicals.

Direct calculation from the data shown in Fig. 4 yields a D_0 value of 105 kR. A more refined calculation suggests that the true value may be about 50 kR (M. L. Randolph, private communication). Thus the energy deposited directly in single strands per break is 40 to 80 eV, which is well within the range of values cited by Setlow and Setlow (18). Double-strand breaks were, as seen by comparison of the curves in Fig. 2 and 3, much less frequent than single-strand breaks, but the differences in sedimentation data for double strands at zero and

TABLE 1. Distribution of phage after attachment for 15 min at 37°C and separation by centrifugation

Exposure (kR)	Radioactive counts			Total in pellet (%)	
	In pel- let	Unat- tached	Sum	Input	
0	521	511	1032	1041	51
482	213	459	672	624	32

400 kR were too small to permit quantitative estimates of double-strand break frequencies.

From our data it appears that strand breaks cannot account for all the lethality of X rays, since in our phage the survival of intact strands is more resistant than survival of infectivity. The rate for strand breakage is less than for inactivation, particularly for double-strand breaks. Thus the contribution made by loss of injection due to such breaks would be small. Although the production of single-strand breaks approaches the rate of survival loss, a correlation can be made only if the lethality of such breaks has an efficiency of 1. The production of large numbers of single-strand breaks by the use of UV in the presence of H_2O_2 with no apparent loss in survival appears to cast serious doubt on a one-to-one relationship between single-strand breaks and phage inactivation. This, of course, presupposes that the breaks are equivalent whether caused by X rays or by UV with H_2O_2 .

Figure 8 shows that although lysis does occur to the extent that half the DNA present in a sample irradiated with an exposure of 370 kR is DNase-degradable after, but not before, irradiation, treatment of the sample with the DNase does not affect the infectivity of the phage in the sample. This suggests that if DNA is protruding from the phage particles as a result of irradiation, such particles are not infective; otherwise, DNase treatment would render them uninfected and would decrease the number of survivors in that sample.

Because of repair, rare occurrence, or innocuous effect, none of the specific forms of X-ray damage measured in this work, nor their sum, seems to account for the observed reduction in survival. X-ray-induced single-strand breaks may be completely ineffective in preventing infection, and although the inability of the phage to attach or to inject its DNA would always prevent infection, these defects occur more rarely. The portion of the X-ray-induced nucleotide damage that remains unrepaired is probably sufficient to cause most of the decrease in plaque formation. This conclusion is in accord with conclusions drawn from studies with other phage systems (22).

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