Lethal and Mutagenic Effects of 5-Iodouracil on Bacteriophage T4td8rII

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Evidence was obtained which indicates that the lethal effect of 5-iodouracil (IUra) on bacteriophage T4 is not due to a mutagenic process. T4td8rII (thymine requiring, rapid lysis) double mutants were constructed. Reversion of T4td8rII to r+ was measured. First, reversion by growth in the presence of the structural analogues chlorouracil (CIUra) and bromouracil (BrUra) did not correlate with their relative lethal effects (for mutagenesis: IUra ≤ CIUra ≤ BrUra; for lethality: CIUra < BrUra < IUra). Second, reversion frequencies of T4td8rII in infected cells increased linearly with time of growth in the presence of IUra, whereas the frequency of lethality was constant with time. Third, reversion frequencies increased markedly at low levels of IUra substitution, whereas lethal effects were apparent only with extensive IUra substitution. Fourth, the reversion frequency of the nonviable fraction of IUra-substituted T4td8rII (as examined by multiplicity reactivation) did not differ significantly from that of the viable IUra-substituted T4td8 fraction. If mutagenesis caused lethality, then the nonviable T4td8rII fraction should accumulate mutations and have a higher reversion frequency.

Selective uptake of the antiviral agent 5-iodo-2'-deoxyuridine (IdUrd) explains much of its therapeutic index against herpes simplex virus. Phosphorylated metabolites of IdUrd accumulate in the virus-infected cell because of the induction of virus-specified thymidine kinase (17). However, inhibition of intermediary enzymatic process by IdUrd metabolites does not explain the ability of IdUrd to kill (20). IdUrd is incorporated into viral deoxyribonucleic acid (DNA) in place of thymidine, and malfunction of the resulting iodo-DNA provides an alternative explanation for the lethal effect of IdUrd (9).

We have chosen bacteriophage T4 as a model system to investigate the mechanism(s) of the lethal effect of IdUrd. For the thymine-requiring system of T4td8 grown on Escherichia coli BT, the lethal effect of 5-iodouracil (IUra) is equivalent to that of IdUrd (6). A monotonic, increasing relationship exists between the concentration of IUra in the medium, the percentage of IUra substitution in DNA, and the extent of the lethal effect (6). Two mechanisms that are qualitatively different account for the lethal effect: (i) sensitivity to incident radiation, particularly near-visible light, or (ii) incorporation of IUra per se (4). Inhibition of DNA replication does not account for the lethal effect in the absence of light (6). Normal yields of progeny particles occur, which absorb and inject competently, and which can be reactivated in several ways, although the particles are IUra substituted and nonviable (5, 13). (By nonviable, we mean that the particle is unable to replicate such that a plaque is formed under standard plating conditions.) IUra is a mutagen for T4 (19). Therefore, we reasoned that the IUra-substituted particles might be killed by accumulation of mutations formed during the DNA incorporation process.

In this paper we investigate the possibility of a mutagenic mechanism of lethality by examining reversion events with a well-characterized system, the rII locus of T4 (3). The results are not consistent with a mutagenic mechanism for the lethal effect of IUra.

MATERIALS AND METHODS


Methods. Preparation and purification of substituted T4 stocks, measurement of viability by optical cross section, tris(hydroxymethyl)aminomethane-salts medium, protection from near-visible light, and determination of percentage substitution by CsCl isopycnic centrifugation have been described.
Standard plating techniques were used for T4 and to detect the rII phenotype (2, 3). The td phenotype was determined by the white halo technique of Hall and co-workers (15). Mutation frequency (reversion frequency) was calculated as the ratio of plaque-forming units (PFU) on E. coli K(λ) to PFU on E. coli BA (K/B) (12). Multiplicity reactivation of IUra-substituted T4 has been described (5). T4d8d8rII is produced by regrowing E. coli BA with both phage types, each at input multiplicities of 4 phage per cell. After one round of growth, progeny were plated on E. coli OK 305, and plaques with td phenotypes (white halos) were picked. Plaques that did not lyse E. coli K(λ) lawns were regrown on E. coli BB as described by Fleming and Bessman (10). Stocks derived in this way were retested for td and rII phenotypes and were used only if recombinant viability, spontaneous reversion frequency, and frequency of white halo phenotype were consistent with those of the appropriate parental stocks. Functional survival of IUra-substituted T4d8d8 was examined with E. coli K(λ) grown to 2 × 10^8 cells per ml in C medium (modified to contain 0.5% glucose instead of maltose [8]) and resuspended in adsorption buffer (supplemented with 0.025% gelatin and 100 μM L-tryptophan [16]) at approximately 2 × 10^9 cells per ml. One-milliliter portions of cells were mixed with (i) 6 × 10^8 PFU of T4d8rII (AP72) (K/B ratio, 0.14 × 10^-6), (ii) 8 × 10^8 PFU of IUra-substituted T4d8 (optical cross section, 17 × 10^-11 cm^2/PFU), or (iii) (i) and (ii) together. After 10 min at 37°C, unadsorbed phage were removed by centrifuging infected cells, and appropriate dilutions of the pellet were plated on either E. coli BA or E. coli K(λ) lawns. The "found/expected" ratio is the PFU per milliliter obtained when both phage types are present divided by the sum of the PFU per milliliter obtained with each individual phage type.

RESULTS

Construction and characteristics of double mutants. We studied an rII mutant, AP72, that reverts strongly in the presence of bromouracil (BrUra) (12). T4rII(ΔP72) was recombined with T4d8d8 to produce a T4d8rII(ΔP72) genotype.

Three tests were performed to demonstrate that T4d8rII(ΔP72) did have the same properties attributed to T4rII(ΔP72) and that IdUrd did produce the same effect as bromodeoxyuridine (BrdUrd). First, the ability of both T4rII(ΔP72) and T4d8rII(ΔP72) to form plaques on E. coli K(λ) lawns increased after growth on either E. coli BB in the presence of BrdUrd, or on E. coli BT- in the presence of IdUrd. Second, IdUrd-induced reversion was genotypic instead of phenotypic. Stocks grown in the presence of IdUrd were plated, and individual plaques were picked from E. coli K(λ) lawns. Stocks were grown from each plaque on E. coli BB and tested for rII by plating on E. coli BA and E. coli K(λ). Nine out of nine plaques picked after IdUrd treatment of T4rII(ΔP72) plated equivalently on either E. coli BA or E. coli K(λ). Thirty out of thirty-one plaques picked after IdUrd treatment of T4d8rII(ΔP72) plated equivalently on E. coli BA and E. coli K(λ). The remaining plaque yielded progeny that plated less well on E. coli BA than on E. coli K(λ). All 31 plaques picked retained the td genotype. Third, progeny phage obtained after growth in the presence of IdUrd on E. coli BT- were regrown on the same host at a low multiplicity of infection in the absence of IdUrd and presence of deoxothymidine. The reversion frequencies of both T4rII(ΔP72) and T4d8rII(ΔP72) were not altered appreciably by this second round of growth, demonstrating that the rII- revertants resulted from errors of incorporation.

Structure-activity relationships. To examine structure-activity relationships, we studied the closely related halouracils, chlorouracil (ClUra), BrUra, and IUra. During a single, synchronous growth cycle in the absence of light, under conditions where approximately 70% substitution by halouracils occurs, full complements of physically intact phage particles were produced that were normal in their DNA content and ability to absorb to and kill bacteria, but were reduced in viability. Quantitatively, CIUra is less effective than BrUra, which is less effective than IUra with respect to lethality (6). Under the same conditions, the mutagenicity of the three analogues showed a different quantitative relationship (Table 1).

Kinetic relationships. The induction of lethality might increase with growth of intracellular progeny, if the intracellular thymine pool (created from host cell DNA breakdown) were preferentially incorporated into intracellular progeny early during the growth cycle. Previous experiments showed the transfer of E. coli BT- DNA-thymine to T4d8 progeny DNA to be equivalent with either thymine or IUra present in the growth media (6). Therefore, we examined the viability of intracellular T4d8 progeny accumulating in infected E. coli BT-cells at different times after growth in the presence of thymine or IUra. The appearance of
viable intracellular progeny is impaired by IUra to the same extent at earlier times as at the time of spontaneous cell lysis (Fig. 1).

Another possibility was that IUra impaired the synthesis of full complements of virus particles but gave rise to fully viable progeny at early times in the synthesis of intracellular progeny. We eliminated this possibility from consideration in a companion experiment. Infected cells were lysed after 15 min of growth in the presence of IUra, and the optical cross section of the progeny particles obtained after purification was compared with the optical cross section obtained after a complete growth cycle. They were approximately the same (19 \times 10^{-11} \text{ cm}^2/\text{PFU} \text{ and } 14 \times 10^{-11} \text{ cm}^2/\text{PFU}, \text{ respectively}).

Since a linear increase with respect to time in the reversion frequency of T4 intracellular progeny in the presence of BrdUrd was obtained by Susman and co-workers, we studied the kinetic correlation of IUra-induced reversion frequency to IUra-induced lethality in T4td8rII-infected E. coli BT\(^-\) (22). The reversion frequency increased linearly with time from the value of the control to the value found for the progeny after lysis, whereas, as stated previously, lethality is constant at any time during the growth cycle (Fig. 2).

**Fig. 2.** Kinetics of lethality and mutagenicity of IUra for T4td8rII(AP72). T4td8rII(AP72) was grown on E. coli BT\(^-\) in tris(hydroxymethyl)aminomethane-salts medium with 250 \(\mu\text{M} \text{ thymine or IUra. The cells were infected at a multiplicity of 2 phage per cell, and the density of infected cells in the phage growth flasks was } 6 \times 10^8 \text{ cells per ml. At the indicated times, 2-ml portions from each of the flasks were mixed with 8 drops of CHCl}_3 \text{ and chilled on ice. Each portion was diluted in adsorption buffer and plated on E. coli BA or E. coli K}(\lambda). The solid lines indicate the K/B ratios that were obtained at different times in the presence of IUra or thymine. The dashed line shows the ratio of PFU on E. coli BA in the presence of IUra to PFU on E. coli BA in the presence of thymine.

Dose-response relationships. A correlation between IUra-induced mutagenesis and lethal effects was obtained in dose-response studies. As the percentage of substitution of IUra for DNA-thymine increased, both reversion frequency and lethality of T4td8rII increased (Fig. 3). Percentage substitution varies with the ratio of IUra to thymine in the growth medium (6). However, reversion frequency increased most markedly at lower ratios of IUra to thymine in the medium, whereas the lethal effect is apparent only at higher ratios.

**Marker rescue of rII.** Since IUra substitution impairs the function of some T4 genes (14), we examined the effect of IUra substitution on rII\(^+\). T4td8 has equal viability when plated on either E. coli BA or E. coli K(\lambda). We infected E. coli K(\lambda) with IUra-substituted T4td8 and T4td8rII together, to see whether IUra-substituted T4td8 would supply the rII function not expressed by T4td8rII. As described by Krieg (18), when the mixedly infected E. coli K(\lambda) was plated on E. coli K(\lambda) lawns, where rII

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**Fig. 1.** Intracellular synthesis of progeny in the presence of IUra. T4td8-infected E. coli BT\(^-\) were grown in tris(hydroxymethyl)aminomethane-salts medium in the presence of 100 \(\mu\text{M} \text{ thymine (\(\bigcirc\)) or 100 } \mu\text{M IUra (\(\blacksquare\)). At the indicated times, 1-ml samples were mixed with 3 drops of CHCl\(_3\) and chilled on ice. Portions of each sample were diluted and plated on E. coli BA. The concentration of infected cells in each flask was } 4 \times 10^8 \text{ infected cells per ml, and the burst size was approximately 110 phage per cell.}
phage do not survive, genotypic rescue occurs. When plated on E. coli BA lawns, where rII phage form plaques, phenotypic rescue occurs. IUra-substituted T4d8 can both phenotypically and genotypically rescue T4d8rII (Table 2).

Mutation frequency of nonviable IUra-substituted T4d8rII. Since T4d8rII extensively substituted with IUra differs either from T4d8rII substituted with other halouracils or from T4d8rII slightly substituted with IUra, in a high degree of lethality relative to mutation frequency, we reasoned that mutations might accumulate in the nonviable fraction of the IUra-substituted T4+IId8 population. Since nonviable, IUra-substituted T4 reactivates itself at high multiplicities of infection, we could test this hypothesis (5).

Spontaneously arising revertants cannot be detected when highly mutagenized phage stocks are plated, because the number of newly arising revertants is small compared with the number of revertants already present. The burst size of IUra-substituted, multiplicity-reactivated T4d8rII (AP72) is not known. If it is small, then the reversion frequency of progeny will be about the same as that of the viable parental phage, because the number of r+ phage reactivated will be small. For this reason, we plated infected cells before lysis. Under these conditions, each cell in which multiplicity reactivation of nonviable phage takes place counts the same as each cell infected with a viable phage. The reversion frequency of multiplicity-reactivated phage in cells infected with substituted T4d8rII does not differ significantly from the reversion frequency of nonreactivated T4d8rII surviving the lethal effects of IUra substitution (Table 3).

**DISCUSSION**

We undertook these experiments to define the lethal effect of IUra in the absence of light. IUra, apparently, does not kill bacteriophage T4 by a mutagenic effect. The absence of a correlation between the structure-activity relationship for lethal effects compared with the

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**TABLE 2. Functional survival of IUra-substituted T4d8**

<table>
<thead>
<tr>
<th>Phage prepn</th>
<th>Phenotypic rescue (E. coli BA)</th>
<th>Genotypic rescue (E. coli K+K-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4d8rII (PFU/ml)</td>
<td>1.63 x 10^8</td>
<td>&lt;2.5 x 10^8</td>
</tr>
<tr>
<td>IUra-substituted T4d8 (PFU/ml)</td>
<td>0.91 x 10^8</td>
<td>0.76 x 10^8</td>
</tr>
<tr>
<td>IUra-substituted T4d8 plus T4d8rII (PFU/ml)</td>
<td>5.3 x 10^7</td>
<td>1.3 x 10^7</td>
</tr>
</tbody>
</table>

**TABLE 3. Multiplicity reactivation of IUra-substituted T4d8rII (AP72)**

<table>
<thead>
<tr>
<th>Determination</th>
<th>Unsubstituted</th>
<th>IUra-substituted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiplicity of infection (particles/cell)</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Multiplicity of infection (PFU/cell)</td>
<td>0.7</td>
<td>0.06</td>
</tr>
<tr>
<td>Infected cells expected (PFU/ml x 10^-9)</td>
<td>3.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Infected cells found (PFU/ml x 10^-9)</td>
<td>3.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Mutation frequency of progeny (K/B x 10^9)</td>
<td>0.004</td>
<td>1.3</td>
</tr>
<tr>
<td>Mutation frequency of infected cells (K/B x 10^9)</td>
<td>0.04</td>
<td>0.7</td>
</tr>
</tbody>
</table>
relationship for mutagenesis indicates that the lethality and mutagenicity of IUra are unrelated. Further, no kinetic correlation exists between the mutagenicity and the lethality of IUra for T4. Since the reversion frequency of survivors is the same as the reversion frequency of the nonviable fraction of the IUra-substituted population, the relationship between mutagenicity and lethality with respect to percentage of substitution (dose of IUra), if any, cannot be one to one. Finally, reversions do not accumulate in the nonviable fraction, as might be expected if the nonviable fraction experienced more mutational hits.

That extensive mutagenesis does not causally result in extensive lethality is not surprising. For example, Tessman discovered conditions under which hydroxylamine rarely produces lethal hits but is a potent mutagen (24). IdUrd-substituted T4d8 induces lower levels of several phage-coded enzymes, including deoxyuridylate deaminase (13). Goz and Aamodt showed IdUrd-substituted T4d8-induced deoxyuridylate deaminase to be immunologically equivalent to unsubstituted T4d8-induced deoxyuridylate deaminase (1), whereas, if IdUrd-induced mutagenesis caused the lower level of deoxyuridylate deaminase, cross-reacting but enzymatically inactive material would be expected.

In this paper, comparisons of reversion at a single genetic site induced by IUra with the lethal effect of IUra indicate that the mutagenic and lethal effects of IUra are unrelated. This finding applies to reversion at several other sites within the rII gene. Studies with T4d8rII recombinants at other rII sites (AP129, DAP-56, AP6-181, and AP275) do not contradict the results with T4d8rII(AP72) (D. M. Byrd and W. H. Prusoff, unpublished data). All of the mutants listed revert while incorporating BrdUrd (7, 12). The possibility exists that IUra induces other mutagenic processes within the rII gene. However, the predominant evidence indicates that BrUra-induced mutations are errors of incorporation (11). Errors of replication, postulated by Terzaghi and coworkers (23), would not explain the inability of IUra-substituted T4 to induce normal enzyme levels.

Generalization of the present results to all T4 genes warrants some caution. The best evidence that rII behaves like other T4 genes with respect to IUra substitution is that rII resembles some other T4 genes in undergoing marker rescue. Because AP72 is a point mutant of rII, the gene product of AP72 can interfere with the rII+ phenotypic function. Therefore, the present rescue studies yield a minimal estimate of marker rescue and are subject to an ambiguity not found in previous marker rescue studies of IUra-substituted T4, which utilized amber mutants (14).

Impairment of different T4 genes by IdUrd substitution is not constant; some genes are relatively sensitive, whereas others are relatively resistant (14). The present results argue that this distribution is not caused by different "hot spots" for IUra-induced mutagenesis within the genes. Comparison of marker rescue studies with multiplicity reactivation data for IUra-substituted, nonviable T4, led us to reject mechanisms that involve either a few "critical sites" in the genome for lethal damage or characteristic deletions of T4 (5). Instead, we postulated a model in which T4 replication is not perfectly efficient with respect to gene products. In this model, different T4 genes vary in susceptibility to IUra substitution due to variance in either specific susceptibility (length of gene, base sequence, etc.) or the catastrophic versus stoichiometric nature of the gene product (21). Although the present results reject mutagenesis as an explanation for impairment of gene product synthesis, we believe that the model remains valid as a working hypothesis for the mechanism of IUra-induced lethal effects.

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LITERATURE CITED


