Multiplicity Reactivation of 5-Iodouracil-Substituted, Nonviable Bacteriophage T4td8

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Nonviable, 5-iodouracil (IUra)-substituted bacteriophage T4td8 can be multiplicity reactivated. The data indicate that two nonviable, IUra-substituted T4td8 phage can complement each other intracellularly to produce viable progeny. Phage particles in lysates of T4td8-infected *Escherichia coli* BT⁻, prepared in the presence of varying mole fractions of IUra plus thymine, were examined by infecting with low and high dilutions of lysate. The yields of multiplicity reactivable particles were identical, regardless of the mole fractions of IUra present in the growth media. However, the yields of viable phage, measured at low multiplicities of infection, decreased with increasing mole fraction of IUra. The results are consistent with the hypothesis that the lethal effect of IUra is a consequence of its incorporation into DNA. Further, the IUra-induced lesion cannot involve genetic damage that shuts off expression at a single region of the genome.

5-Iodo-2'-deoxyuridine (IdUrd) currently is used as an antiviral agent for the treatment of herpes simplex keratitis (14). A number of steps in viral nucleic acid metabolism have been examined for susceptibility to metabolites of IdUrd (14). IdUrd accumulates as phosphorylated metabolites in the herpesvirus-infected cell preferentially (4), due to the induction of thymidine kinase by the herpesvirus in these cells. Although this "trapping" of IdUrd accounts for much of the drug selectivity for infected over uninfected cells, to date, inhibition of intermediary enzymatic processes by IdUrd metabolites does not provide an explanation for the ability of IdUrd to kill virus. Since 5-iodouracil (IUra) is incorporated into phage nucleic acid (5), and IdUrd into animal virus deoxyribonucleic acid (DNA) (13) or phage DNA (6), an alternative explanation exists. We have chosen bacteriophage T4 as a model system to examine the effects of IdUrd incorporation because of the ease with which T4 is manipulated experimentally. Also, since lethal effects of IdUrd incorporation into any viral genome likely involved a genetic mechanism, killing of herpes simplex virus and T4 by IdUrd probably involves a similar mechanism.

Growth of T4 on *Escherichia coli* is not impaired in the presence of IUra, and little incorporation of IUra into DNA occurs. However, with the thymine-requiring strains T4td8 and

E. coli BT⁻, IUra incorporation occurs and the burst size of viable progeny is reduced (D. M. Byrd and W. H. Prusoff, Chem. Biol. Interact., in press). (By viable, we mean the ability of a single phage to replicate such that a plaque will form under standard plating conditions.) When a single T4 growth cycle occurs, then monotonic relationships exist between the concentration of IUra in the growth medium, the degree of IUra substitution, and the extent of the lethal effect (8). In addition, substantial yields of IUra-substituted but nonviable T4 virions are produced (7). These nonviable particles can adsorb and inject DNA, but are defective in many ways, such as their inability to induce control levels of enzyme activity (7). The lethal effects of IUra and IdUrd are equivalent when a single growth cycle and synchrony prevail (Byrd and Prusoff, Chem. Biol. Interact., in press). Qualitatively different mechanisms can account for reductions in IUra-substituted T4 viability: sensitization to incident radiation, particularly near-visible light, or incorporation of IUra per se (2). In this work we have investigated further the mechanism of the lethal effect of IUra incorporation in the absence of light by examining whether IUra-substituted, nonviable T4 particles can be multiplicity reactivated. The results eliminate several possible explanations of the mechanism for IUra-induced lethality.

MATERIALS AND METHODS

Organisms. *E. coli* BT⁻ and *E. coli* BA were obtained from W. D. Rupp and E. B. Freese, respec-

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tively. Bacteriophage T4td8 was obtained from C. K. Mathews.

Methods. Preparation and purification of phage stocks, measurement of particle numbers by absorbance at 260 nm and viability (plaque-forming units/particle) by optical cross-section, tris(hydroxymethyl)aminomethane-salts medium, and determination of percentage of IUra substitution by CsCl isopycnic centrifugation have been described (2, 8; Byrd and Prusoff, in press). Standard plating techniques were used (1). Mouse antisera to T4 were prepared by the method of Sartorelli et al. (15). Growth of T4td8-infected *E. coli* BT⁻ in the presence of IUra has been described (Byrd and Prusoff, in press). All experiments were performed with cells and phage protected from near-visible light (2). IUra was recrystallized from hot ethanol. Multiplicity reactivation of purified phage stocks was carried out with *E. coli* BA grown in C medium (modified to contain 0.5% glucose instead of maltose [4]) to approximately 2×10^8 cells/ml, as estimated from turbidity at 660 nm. Cells were centrifuged ($2,000 \times g$ for 10 min), suspended in the adsorption buffer of Hershey and Chase (10) (modified to contain 0.025% gelatin and 100 μ M L-tryptophan), and mixed with phage stocks suspended in the same buffer, to give varying plaque-forming units (and particle) to cell ratios. After 10 min of incubation at 37 C, the incubate was centrifuged as above, resuspended, and appropriately diluted prior to plating with and without *E. coli* BA lawns to determine the numbers of infected centers and unkilld cells, respectively. The starting cell number was determined by plating appropriate dilutions of uninfected cells without a lawn. Colonies and plaques were counted after 24 h of incubation at 37 C. The fraction of unkilld cells was the ratio of unkilld colonies per milliliter to uninfected colonies per milliliter. The expected number of infected cells per milliliter was calculated as $(1 - e^{-x})$ (uninfected cells per milliliter), where X = plaque-forming units per unkilld cell at the end of the adsorption step. The found number of infected cells per milliliter was the concentration of infected centers per milliliter.

RESULTS

Multiplicity reactivation of nonviable, IUra-substituted T4 particles. IUra-substituted T4td8 with approximately 60% substitution and control, unsubstituted T4td8 were prepared by a single growth cycle of T4td8 on *E. coli* BT⁻ in the presence of IUra or thymine, respectively. Resuspended, log-phase *E. coli* BA were infected at different multiplicities of infection (MOI) (phage per cell) with IUra-substituted or unsubstituted phage, and, after a period of adsorption, appropriate dilutions were plated and the number of infected cells was determined (Table 1). In separate experiments, the unadsorbed phage in the supernatant fractions of infected cells, after centrifugation, were

TABLE 1. Multiplicity reactivation of IUra-substituted T4 particles

Cells		Unsubstituted		IUra substituted	
		Expt 1	Expt 2	Expt 1	Expt 2
Uninfected	cells/ml ($\times 10^{-8}$)	2.2	2.1	2.2	2.1
Particles/cell		3.9	3.3	5.4	4.6
MOI		3.9	3.3	0.27	0.23
Fraction of unkilld cells		0.20	0.02	0.11	0.005
Expected infected	cells/ml ($\times 10^{-8}$)	2.2	2.1	0.59	0.43
Found infected	cells/ml ($\times 10^{-8}$)	2.2	1.8	1.8	1.6
Found/expected		1.0	0.86	3.1	3.8

several orders of magnitude less than the concentrations of infected cells. Equivalent numbers of infected cells were found after dilution of infected cells in the presence or absence of anti-T4 antibody. Thus, correction of the results for unadsorbed phage was unnecessary. More infected cells were obtained with IUra-substituted phage at high MOI than were expected from the Poisson distribution and the number of viable progeny present at limiting dilutions. These results show that multiplicity reactivation of nonviable, IUra-substituted phage particles occurs. The fraction of unkilld cells varied for unknown reasons but was identical within an experiment for both unsubstituted and IUra-substituted T4, if the particle to cell ratios were equivalent.

Dimeric reactivation. We were interested to see if any two nonviable IUra-substituted phage particles could combine intracellularly to yield an infected cell (dimeric reactivation), or if some higher number of particles was required. Multiplicity reactivation of nonviable virions has been described in several systems (12, 16). In general, quantitative descriptions are carried out most easily by using highly inactivated virus preparations. In those cases where a high degree of inactivation either is not of interest or is not possible, then quantitative analysis is complicated by the presence of viable virus, since at high MOI most of the cells will be infected with viable virus.

We have found a graphical analysis of data, obtained by infecting cells at various high MOI, to be convenient in describing populations with low degrees of inactivation. For each sample the number (or fraction) of infected cells actually found is divided by the number (or

fraction) of infected cells expected, which is determined from the number of viable virions measured at limiting dilution, and the number of cells. The results for samples at different MOI are plotted as a function of either the MOI, or the number of particles per cell, if known. We calculated the hypothetical curves expected for dimeric and trimeric reactivation at experimentally determined degrees of viability and phage particles per cell (see Appendix) and compared the hypothetical curves with experimental results. Theoretical examples of dimeric and trimeric reactivation are readily distinguished (Fig. 1) with the simple assumptions, as for IURA-substituted T4, that (i) nonviable virus particles can enter eclipse (7), (ii) interference between virus particles does not occur, and (iii) the viable and nonviable populations are determinate. Results with several preparations of T4td8, having 65% or less replacement of DNA-thymine by IURA, were consistent with the hypothesis of dimeric reactivation of all nonviable, IURA-substituted phage particles (Fig. 2). At MOI much greater than 10, lysis from without became a problem.

Yields of reactivable T4 particles in lysates of T4td8-infected *E. coli* BT⁻ grown in the presence of different mole fractions of IURA and thymine. Previous data indicated that T4td8-infected *E. coli* BT⁻ produce equivalent yields of phage progeny particles when grown in the presence of IURA, regardless of the mole fraction of IURA present (Byrd and Prusoff, in press). However, we obtained these results by examining purified progeny particles physically and correcting for losses incurred in purification. Because of the efficiency of the reactivation process with purified IURA-substituted phage, we wondered not only if the yields of reactivable particles were equivalent when T4td8-infected *E. coli* BT⁻ were grown in the

FIG. 1. Hypothetical comparison of dimeric and trimeric reactivation. Curves (—, no multiplicity reactivation; ---, dimeric reactivation; ····, trimeric reactivation) were calculated from equations in the Appendix, with 0.10 as the fractional viability of the virus particles.

FIG. 2. Multiplicity reactivation of IURA-substituted T4td8 as a function of particles per cell. Experiments were carried out with *E. coli* BA as described. Each point represents a separate experiment (average of duplicate titrations). Symbols represent different control, unsubstituted (○, ▽, □) and IURA-substituted T4td8 preparations with 0.05 (●), 0.09 (■), and 0.11 (▼) fractional viabilities. The lines are theoretical and drawn according to the equation given in the Appendix to represent no multiplicity reactivation (—) and dimeric reactivation (---) for virus with 0.09 fractional viability, the median used.

presence of IURA, but also whether reactivable progeny particles were present which could have been lost during purification. Low and high dilutions of lysates from T4td8-infected *E. coli* BT⁻, grown in the presence of varying mole fractions of IURA and thymine, were used to infect *E. coli* BA cells (Fig. 3). The yields of progeny phage particles, determined by multiplicity reactivation, were identical, regardless of the amount of IURA in the medium, with consequent IURA incorporation and lethal effect.

DISCUSSION

The burst size of multiplicity reactivable phage progeny remains constant, as the mole fraction of IURA to thymine increases in the growth media of T4td8-infected *E. coli* BT⁻. However, the burst sizes of viable progeny decrease, and the extents of IURA-substitution increase at the same time (2). Progeny particle yields, measured after purification, are equivalent up to 65% IURA substitution (Byrd and Prusoff, in press). Together, these results indicate that progeny particles released after growth in the presence of IURA are identical to those obtained after purification. In addition to synthesis of normal yields of particles, with the exception of the presence of IURA in place of DNA-thymine, we have detected no physical

FIG. 3. Yields of multiplicity reactivable *T4td8* in lysates of infected *E. coli* *BT*⁻ cells grown in the presence of different mole fractions of IUra and thymine. *T4td8* was grown on *E. coli* *BT*⁻ in tris(hydroxymethyl)aminomethane-salts media containing the indicated mole fractions of IUra (calculated as moles of IUra/moles of IUra plus thymine), such that the total concentration of IUra plus thymine was 100 μ M. Burst sizes were determined as the ratios of plaque-forming units per milliliter (after growth at 37 C, lysis, dilution, and plating on *E. coli* BA) to infected centers (O). Infected centers were determined by mixing 0.5 ml of each lysate (6.8×10^9 plaque-forming units for the 0.0 mol of fraction lysate) with 1.8×10^8 *E. coli* BA in 2 ml of adsorption buffer (Δ). After 10 min at 37 C, unabsorbed phage were removed by centrifuging ($2,000 \times g$ for 10 min) the cells. Serial dilutions of the cells were plated on *E. coli* BA lawns and plaques were counted after 24 h of incubation at 37 C.

defect in IUra-substituted progeny. Functional virion proteins encapsulate and provide a normal apparatus for adsorption and injection (7). The efficient multiplicity reactivation of IUra-substituted progeny seen here is further evidence in this regard. Thus, IUra incorporation during a T4 growth cycle initiated by unsubstituted phage apparently has no significant effect on macromolecular synthesis or morphopoiesis. All of our data oppose inhibition of intermediary metabolism by some metabolite of IUra as a biochemical explanation for the lethal effect of IUra with, as far as we are aware, this single exception: if the fidelity of DNA replication is controlled by some apparatus separate from the replication machinery, and if this apparatus is altered or inhibited by the presence of a metabolite of IUra, then IUra could cause lesions in progeny DNA independently of incorporation into DNA. We know of no such hypothetical apparatus. In addition, the reduced viability of IUra-substituted phage and the biochemical

and genetic defects measured in IUra-substituted phage-infected cells are readily attributed to the presence of IUra in progeny DNA.

Some *amber* mutants of T4 are capable of genotypic and phenotypic rescue of most IUra-substituted, nonviable progeny phage, whereas *amber* mutants at other loci cannot rescue markers to any appreciable extent (8). From experiments with mutants at many locations, a genetic map of IUra-induced damage has been constructed (8). The extent of damage at some sites is extensive, relative to the viability of the IUra-substituted phage. Assuming that the lethal event is "inactivating," that is, that damaged genes either are absent or can make no products (6), then IUra-induced genetic damage apparently is nonrandom and extensive. Damage occurs at a large number of determinate sites in most nonviable particles. If the product of the fractional inactivation for even a few individual genes is calculated, the population is seen to be "over-killed" by several orders of magnitude relative to the fractional viability of the population which is found experimentally. From this description, multiplicity reactivation should not be possible with IUra-substituted phage. However, the present results clearly show that multiplicity reactivation does occur. Therefore, the assumption that the lethal event is an inactivating lesion must be questioned.

That dimeric reactivation occurs efficiently means that substantially greater than a half-genome-sized DNA molecule must be present. That any genes are missing from the IUra-substituted T4 particle, either as a result of a shortened DNA molecule or of a characteristic deletion, is unlikely. Measurement of ³²P content, and agreement of percentage of IUra substitution by two independent methods, isopycnic density centrifugation or chemical hydrolysis, indicate that the DNA content of IUra-substituted phage is the same as the DNA content of unsubstituted T4 ($\pm 7\%$) (Byrd and Prusoff, in press). Supporting evidence is presented above. Regardless of IUra-induced lethal effect, bacterial killing ability, which measures the number of T4 particles, was constant with particle absorbance at 260 nm, which mostly is a measure of DNA content. Since full-sized DNA molecules are present in each IUra-substituted T4 particle, then a genetic map of IUra-induced damage is incompatible with a circularly permuted population of DNA molecules (18), unless molecule size is reduced independently of DNA length by characteristic deletions. However, characteristic deletions would not permit efficient multiplicity reactivation.

The efficiency of multiplicity reactivation seen here means that a large portion of the

genome of each nonviable particle is genetically functional. A similar conclusion is obtained independently from genotypic and phenotypic reactivation results (8). A mechanism similar to that proposed for ultraviolet-induced damage, by which three or four critical sites, each controlling the function of a larger portion of the genome, are inactivated at random by IUra substitution, is consistent with multiplicity reactivation (9). However, this critical site mechanism is incompatible with the genetic map for phenotypic and genotypic reactivation of nonviable, IUra-substituted phage.

One simple model of the IUra-induced lesion, which is compatible both with the available marker rescue data and with efficient multiplicity reactivation, involves the assumption that T4 replication is not perfectly efficient with respect to gene products. For example, a certain number of gene products from a particular locus may be required intracellularly before the probability of assembling a viable progeny particle approaches one. In this model, different genes will vary in susceptibility to the presence of a certain degree of IUra substitution for DNA-thymine, both because specific susceptibility may vary (length of gene, base sequence, etc.) and because either catalytic or stoichiometric gene products may be involved (17). Further, susceptibility to IUra for each gene will depend on the relative impairment of gene product synthesis and on the relationship for that gene between gene product number and probability of assembling at least one viable progeny phage. A candidate explanation for the mechanism of impairment is mutagenesis. IUra is a mutagen for T4 (11). Mutagenesis has a probabilistic nature, and this explanation encompasses the additional possibility of phenotypic reactivation of inactive gene products, since mutagenesis will tend to the synthesis of impaired gene

products, as opposed to synthesis of fewer gene products.

APPENDIX

Let A , B , and C represent the concentrations or numbers of virus particles that individually are capable of forming infectious centers, virus particles capable of killing a cell that cannot individually form an infectious center, and cells, respectively. Also let $(A/C) = a$ and $(B/C) = b$. Then $a/(a + b) =$ viability of the virus preparation.

The virus population may be divided into hypothetical compartments (Fig. 4). From the Poisson distribution, the fraction of infected cells will be $1 - e^{-a}$, if all B virions also are incapable of combining to yield infectious centers in cells mixedly infected with more than one virion (and do not interfere with A virus infectivity). B virions will be maximally efficient in reactivation if any two B virions attaching to the same cell form an infectious center (and no B virions interfere with A virus infectivity). The fraction of cells receiving two or more B virions is $1 - (e^{-b} + be^{-b})$. The fraction of infected cells will increase by $(e^{-a})[1 - (e^{-b} + be^{-b})]$, and found/expected = $[1 - (e^{-a})(e^{-b} + be^{-b})]/(1 - e^{-a})$. For the requirement that three B virions are necessary for reactivation, found/expected = $\{1 - (e^{-a})(e^{-b} + be^{-b} + [b^2e^{-b}]/2)]/1 - e^{-a}$.

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FIG. 4. Compartments of infected cells for all multiplicities of infection and fractional viabilities. See Appendix for definitions of symbols. The total areas in the boxes represent the numbers of cells, whereas the shaded areas represent the fraction of cells infected.

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