Inhibitory Effect of Hydroquinone and Related Agents on Multiplicity Reactivation and Genetic Recombination of Bacteriophage T4

Hiroshi MAEZAWA, Kenshi SUZUKI and Yukio KIHO*

Department of Molecular Biology, School of Medicine, Tokai University
*Institute for Plant Virus Research, Tsukuba Science City
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The multiplicity reactivation of T4D phage irradiated with UV was inhibited by treatment of infected cells with 0.5 mM hydroquinone (HQ). HQ also inhibited genetic recombination of T4B rII mutants. The recombination frequency was reduced by treatment of infected cells with HQ in a buffer when compared with an untreated control. In addition to HQ, the effects of quinone, an oxide form of HQ, mercaptoethanol and α-tocopherol, antioxidants, were also investigated. Quinone and mercaptoethanol inhibited recombinations but α-tocopherol did not.

(Key Words: Hydroquinone, Multiplicity Reactivation, Recombination, Bacteriophage)

INTRODUCTION

Many of the damages produced in bacteriophages by ultraviolet light (UV), ionizing radiation and chemicals are repaired by various repair systems coded for by the genes of host bacteria and phages. It is well known that UV-, radiation-, and chemical-damages produced in T2, T4, T5 and T6 phages are repaired by the multiplicity reactivation (MR) on host bacteria (15, 13, 21). It has been proposed that the phenomenon of MR is related to the production of a normal chromosome set from two or more damaged phage chromosomes by recombination (11, 3) and it is assumed that the same mechanism must be involved in both R and genetic recombination of phages. This concept was supported by the fact that the T4 phage deficient in the gene x or y lacks both MR and recombination (3).

It is also known that various radicals, such as OH*, e\text{aq} and O₂⁻ are involved in photo-and radiation-biological actions. This notion is supported by the fact that radical scavengers reduce the radiation effects on biological systems. Hydroquinone (HQ) acts as a scavenger of oxygen (22) and its biological effects have been investigated with some systems. In E. coli, the derivatives of HQ (or quinone) act on the electron transfer system of respiration and of oxidative phosphorylation (7). The growth of Chlorella vulgaris is inhibited with 1 mM HQ (10). It has also been shown that HQ inhibits the (Na⁺-K⁺)-ATPase activity of microsomes from the rat intestine (4) and the indolylacetic acid oxidase activity in maize roots.

Hiroshi MAEZAWA, Department of Radiology, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259-11, Japan
In this report, it will be shown that HQ inhibits the MR of T4 irradiated with UV and that HQ also reduces the frequency of genetic recombination between rII mutants. In addition, p-benzoquinone, an oxide of HQ and mercaptoethanol, an antioxidant, also reduced the recombination frequency. However, \( \alpha \)-tocopherol, an antioxidant, had no effect on recombination.

MATERIALS AND METHODS

Bacteriophage and bacterial strains.

T4D wild-type phage was used for experiments of multiplicity reactivation (MR). Mutants of T4B phage (N55, N21, r221 and A105) were used for phage crosses. These markers of mutant phages were mapped by Benzer (2) on the rII cistron. \( E. \) coli B was used as the host bacterium for MR and crosses. It was also used as the plating indicator for mutant and wild-type phages. \( E. \) coli K12 (\( \lambda \)) AB1157 was used as the indicator for selecting wild-type recombinants (1).

Media and reagents.

The 3XD medium (pH 6.9), used for the preparation of T4 phage stocks, contained 4.5g of \( \text{KH}_2\text{PO}_4 \), 26.5g of \( \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \), 3g of \( \text{NH}_4\text{Cl} \), 15g of Difco Casamino acids, 13g of glycerine, 0.3g of \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 1.67mg of \( \text{CaCl}_2 \) and 0.03g of gelatin per liter of distilled water. Bacteria were grown in nutrient broth (pH 7.2) containing 8g of Difco nutrient broth, 5g of NaCl and 0.4g of glucose per liter of distilled water. M9 buffer (pH 6.9) contained 15g of \( \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \), 3g of \( \text{KH}_2\text{PO}_4 \), 0.2g of \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 4.5g of NaCl, 1g of \( \text{NH}_4\text{Cl} \) and 0.02g of gelatin per liter of distilled water. Adsorption of T4B rII mutants on bacteria was carried out in M9 buffer supplemented with 50mg/l of L-tryptophan (adsorption buffer) (24). Salt-poor nutrient broth (0.5g of NaCl per liter) was used for incubating infected bacteria to obtain free phage particles. The bottom- and top-agar media used for plaque assay contained 1.2g and 0.65g of agar per 100ml of nutrient broth (no supplemented glucose), respectively. Hydroquinone (HQ), p-benzoquinone (Q), \( \alpha \)-tocopherol and mercaptoethanol were purchased from Wako Pure Chemical.

Preparation of T4 phage stocks.

Phage stocks were prepared in the following way. \( E. \) coli B cells were propagated to \( 3 \times 5 \times 10^8 \) cells/ml in 3XD medium at 37\(^{\circ}\)C with aeration. T4D wild-type or T4B rII mutant phage was infected in to \( E. \) coli B cells at a multiplicity of 0.001 and 0.01, respectively. The infected cells were incubated for 4hr at 37\(^{\circ}\)C and lysed with chloroform. The lysate was centrifuged and the supernatant was treated with pancreatic deoxyribonuclease (10\( \mu \)g/ml, Sigma) for 40 min at 37\(^{\circ}\)C. The phage particles were collected by ultra-centrifugation (18,000\( \times \)g, 60 min) and resuspended in M9 buffer (T4D) and in adsorption buffer (T4B) and used as a stock suspension. The titer of stock suspensions was determined to be \( 1 \times 5 \times 10^{11} \) particles/ml.
Multiplicity reactivation of phage irradiated with UV.

*E. coli* B was grown up to about $2 \times 10^8$ cells/ml in nutrient broth with shaking at $37^\circ$C. The cells were centrifuged (3,500 rpm, 15 min) and resuspended in M9 buffer at a concentration of $2 \times 10^8$ cells/ml. T4D phage was irradiated in M9 buffer with UV emitted from a low-pressure mercury lamp (Toshiba, 15 watts) at a dose rate of $1 \text{ J/m}^2\cdot\text{sec}^{-1}$. Unirradiated- and irradiated-phages were adsorbed onto cells with a given multiplicity in M9 buffer (supplemented with 2 mM KCN), and unadsorbed phage particles were removed by centrifugation for 15 min at 3,000 rpm. Infected cells were incubated without shaking in nutrient broth with or without HQ. After 15 min, the culture was diluted and the number of infective centers was measured by the agar-layer method.

Phage crosses

*E. coli* B cells propagated to about $3 \times 10^8$ cells/ml in nutrient broth were collected by centrifugation and resuspended in adsorption buffer. The cells were starved for 1 hr at $37^\circ$C, sedimented and resuspended in the adsorption buffer at a concentration of $3 \times 10^8$ cells/ml. The cells were infected with phage particles at a multiplicity of infection (m.o.i.) of five for each of two rII mutants with or without HQ. Twenty min after infection, more than 97 per cent of phage particles were adsorbed onto cells irrespective of the presence of HQ. The infected cells were filtered onto a membrane filter (pore size of 0.45 \(\mu\)m), washed with M9 buffer and resuspended in the same buffer. Aliquots of the suspension were immediately plated on *E. coli* B to measure the number of total infective centers and on K12 (\(\lambda\)) to score bacteria bearing wild-type recombinants. The rest of the suspension was diluted to 50-fold in salt poor nutrient broth and cells were incubated at $37^\circ$C for 70 min without HQ or other materials to be tested. After the incubation, chloroform was added to the suspension (24) and the progeny particles were plated both on *E. coli* B and K12 (\(\lambda\)) cells to count the total number of progeny particles and the number of wild-type recombinant particles, respectively.

RESULTS

Inhibition of multiplicity reactivation by HQ

The wild-type T4D as well as *E. coli* B cells were inactivated very little, if any, when they were exposed to 1 mM HQ in nutrient broth for 15 min at $37^\circ$C (data not shown). Also, there was very little inactivation of T4D-infected *E. coli* cells (m.o.i.: 12) as revealed by the number of infective centers. However, when T4D was irradiated prior to infection (m.o.i.: 12) there was a remarkable inactivation under the same conditions. The results are shown in Fig. 1. It should be noted that UV-survival of free phage particles was not influenced by incubation in the presence of HQ before plating (see Table 1). Therefore, it is assumed that the inhibitory effect of HQ is exerted on a certain mechanism(s) in host cells.

The resistance to UV and the shape of survival curves are different depending on m.o.i., showing a typical MR. The results are shown in Fig. 2. It can be seen that HQ reduces survivals at m.o.i. 12, while the effect of HQ
is very small at m.o.i. 0.45. In addition, HQ reduces the big shoulder at m.o.i. 12, but it does not alter the slope of exponential inactivation. These results strongly suggest that HQ inhibits the MR of UV-irradiated T4. Similar tendencies were also observed at m.o.i. 6 (data not shown). The fluence of UV to T4 necessary to inactivate the phage-host complex to 1 per cent (D₀.₀₁) is summarized in Table 1. It is evident that larger effects of HQ are observed for larger m.o.i.

![Fig. 1 Effect of hydroquinone on T4-host cell complex.](image)

*Fig. 1* Effect of hydroquinone on T4-host cell complex. E. coli B cells were infected with unirradiated- or UV-irradiated (40J/m²)-T4D phage at a m.o.i. of 12 in nutrient broth containing various concentrations of hydroquinone and incubated for 15 min. The number of infective centers were assayed after the incubation. Each circle represents the mean of four independent experiments and bars represent the deviation.

<table>
<thead>
<tr>
<th>m.o.i.</th>
<th>D₀.₀₁ * (J/m²)</th>
<th>ratio (-HQ/ +HQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>free phage</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>0.45</td>
<td>61</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>102</td>
<td>77</td>
</tr>
<tr>
<td>10</td>
<td>127</td>
<td>85</td>
</tr>
</tbody>
</table>

* D₀.₀₁ represents the fluence required to reduce survivals to one per cent.
Effect of Hydroquinone on Multiplicity Reactivation and Recombination of T4

**Fig. 2** Multiplicity reactivation of UV-T4D and the effect of HQ. T4D was irradiated with UV and infected into *E. coli* B cells with a m.o.i. of 12 (○,●) or 0.45 (□,■). The infected cells were incubated in nutrient broth with (solid line) or without HQ (0.5 mM) (broken line). The number of infective centers was determined after incubation. The inactivation curve for free phage (without post-irradiation incubation) is also shown (dotted line).

**Effects of HQ and other agents on genetic recombination of rII mutants**

Since the mechanism of MR is believed to be closely related to recombination of the genetic material of T4 (3, 11) the effects of HQ were investigated for recombination systems of rII mutants. The formation of the wild-type recombinant phages was assayed by plating phage-host complexes or the progeny phages derived therefrom on both *E. coli* B and K12 (λ) strains (1, 24). In these experiments, phages were added to host bacteria, *E. coli* B, with indicated m.o.i. in adsorption buffer with or without agents. The mixture was incubated for 20 min at 37°C and assayed for infective centers using *E. coli* B and K12 (λ) as indicators. The results are shown in Table 2.

In a typical cross, N55 × N21, the frequency of the wild-type formed by a cross was $3.92 \times 10^{-1}$, i.e. about 40 per cent of the infective center produced the wild-type progeny, while infection of each rII mutant produced the wild-type at the order of $10^{-6}$ to $10^{-7}$. HQ at 0.5 mM and Q at 5 μM reduced the yield of the wild-type to about ten per cent of the
control. α-tocopherol had no effect at 10mM. Similar results were obtained for other recombination systems, r221×A105, N21×r221. In one cross, r221×A105, mercaptoethanol at 72mM revealed a major inhibitory effect.

Table 2 Effect of hydroquinone and related agents on recombination of T4 rII mutants.*

<table>
<thead>
<tr>
<th>phage</th>
<th>m.o.i.</th>
<th>Agent added</th>
<th>Indicator</th>
<th>Assayed for infective center</th>
<th>Frequency (K12(λ)/B)</th>
<th>Yield of** progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>N55</td>
<td>5 - 8</td>
<td>no</td>
<td>B</td>
<td>K12 (λ)</td>
<td>2.08 × 10^7</td>
<td>—</td>
</tr>
<tr>
<td>N21</td>
<td>5 - 8</td>
<td>no</td>
<td>B</td>
<td>K12 (λ)</td>
<td>4.4 × 10^-6</td>
<td>—</td>
</tr>
<tr>
<td>N55×N21</td>
<td>5:5</td>
<td>no</td>
<td>B</td>
<td>K12 (λ)</td>
<td>6.72 × 10^6</td>
<td>56</td>
</tr>
<tr>
<td>HQ 0.5 mM</td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>1.58 × 10^6</td>
<td>—</td>
</tr>
<tr>
<td>HQ 0.25 mM</td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>1.07 × 10^6</td>
<td>—</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>10 mM</td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>2.26 × 10^7</td>
<td>3.47 × 10^-1</td>
</tr>
<tr>
<td>r221×A105</td>
<td>3 : 3</td>
<td>no</td>
<td>B</td>
<td>K12 (λ)</td>
<td>4.56 × 10^7</td>
<td>—</td>
</tr>
<tr>
<td>HQ 0.5 mM</td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>6.05 × 10^5</td>
<td>1.53 × 10^-2</td>
</tr>
<tr>
<td>HQ 0.25 mM</td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>4.0 × 10^2</td>
<td>1.1 × 10^-4</td>
</tr>
<tr>
<td>mercaptoethanol</td>
<td>72 mM</td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>3.68 × 10^6</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>4.0 × 10^2</td>
<td>1.1 × 10^-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>3.46 × 10^7</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>2.54 × 10^5</td>
<td>7.61 × 10^-3</td>
</tr>
<tr>
<td>N21×r221</td>
<td>5 : 5</td>
<td>no</td>
<td>B</td>
<td>K12 (λ)</td>
<td>5.72 × 10^6</td>
<td>—</td>
</tr>
<tr>
<td>HQ 0.5 mM</td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>4.96 × 10^3</td>
<td>8.7 × 10^-4</td>
</tr>
<tr>
<td>HQ 0.25 mM</td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>2.23 × 10^7</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>2.32 × 10^5</td>
<td>1.04 × 10^-2</td>
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<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>5.84 × 10^6</td>
<td>2.89 × 10^-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>1.82 × 10^6</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>1.20 × 10^5</td>
<td>6.58 × 10^-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>1.02 × 10^7</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>K12 (λ)</td>
<td>8.47 × 10^5</td>
<td>8.67 × 10^-2</td>
</tr>
</tbody>
</table>

* The frequency of wild type phage in stocks of N55 and N21 was 7.4 × 10^-7 and 6.7 × 10^-8, respectively. For deletion mutants, r221 and A105, the frequency was much lower, less than 10^-9.

** Yield of progeny is defined as the ratio of the total number of progeny particles to the number of plaque forming infective centers.

In relation to the present experiments, the following points should be noted: (1) HQ, Q and mercaptoethanol killed E. coli cells and E. coli-T4 complexes significantly during incubation in adsorption buffer at 37°C for
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20 min. For example, survivals for HQ at 0.5 mM, Q at 5 μM and mercaptoethanol at 72 mM were determined to be 10, 60 and 13 per cent of the control for E. coli-T4 complex, respectively. However, these agents did not reveal serious lethal effect for free phage particles (survival was more than 80 per cent under the same conditions). (2) Irradiation with UV increased the frequency of recombinant formation. In this case HQ also reduced the frequency of recombinant formation. (3) The inhibitory effect of HQ and Q on recombinant formation was also observed when the free phage released by lysis with chloroform was assayed on two E. coli strains (date not shown). (4) The inhibitory effect of HQ and Q was small under certain conditions. For example, in a cross of N55 × N21 (total m.o.i. of 10), infected complexes were exposed to HQ at 0.5 mM in nutrient broth for 70 min at 37°C. In this case, the frequency of wild-type progeny phage released from cells was 76 per cent of the control although the yield of progeny was reduced to 50 per cent of the control. The reason remains unknown.

DISCUSSION

The present study demonstrated that HQ enhances the killing of UV-irradiated T4 phage when phage particles are multiply infected into host bacteria, while this effect of HQ was very small at a low multiplicity of infection. These results together with the fact that HQ reduced the big shoulder of the UV-survival curve of T4 typically observed in MR without changing the slope of exponential inactivation suggest that HQ inhibits MR of UV-irradiated T4. This idea was supported by the evidence that HQ reduces the frequency of genetic recombination of T4 rII mutants. It was also observed that Q is a much more potent inhibitor of genetic recombination and mercaptoethanol is also active in this sense, but not α-tocopherol.

The mechanism of action of these agents is still unknown. There is a possibility that some of the enzymes involved in genetic recombination (3) and in MR of UV-irradiated T4 (9, 11, 23) are inactivated by the agent. Inactivation of certain enzymes by HQ has been reported (4, 20). It should be noted here that HQ and Q also reduced the yield of T4 progeny as shown in Table 2, indicating that a certain step of phage reproduction must have been partially blocked. Under these circumstances, it appears that the target enzyme might be involved in phage growth as well as in genetic recombination.

There is another possibility that the effect of HQ is exerted by an indirect influence on the process of recombination, not by the direct action on recombination enzymes. In fact, the frequency of recombination is affected by physiological conditions. For example, Tomizawa and Anraku (24) reported that the frequency of recombinant progeny in crosses of T4 is increased when phage-host cell complexes are preincubated for a long time in buffer before the complexes are transferred to nutrient broth medium when compared with the case in which the complexes are retained for a limited period of time for adsorption in the buffer. In the present study it was found that HQ is less effective when phage-host cell complexes
are exposed to HQ in nutrient broth medium instead of adsorption buffer.

Biological effects of HQ should be due to the reduction of biological substrates involving the oxidation of HQ (18). There is also a possibility that effects of HQ involve products from the oxidation of HQ. HQ can be converted to a semiquinone form (8, 14) and quinone (6, 25) in an aerated solution and in cells. The semiquinone form and quinone can react with biological molecules in \textit{in vitro} and \textit{in vivo} (5, 8, 12, 16, 17, 19). Production of oxidizing products by incubation in adsorption buffer (20 min, 37°C) is not evident spectrally, but because the concentration required for Q to inhibit recombination was two orders lower than that for HQ, there is a possibility that part of the inhibitory effect of HQ is due to these oxidizing products.

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