Inhibition of T4 Bacteriophage Multiplication by Superinfecting Ghosts and the Development of Tolerance After Bacteriophage Infection

DONNA HARDY DUCKWORTH

Department of Microbiology, University of Virginia School of Medicine, Charlottesville Virginia 22903

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Simultaneous addition of T4 phage and ghosts to host cells prevents infective center formation. Cells which have been infected with phage for less than 2 min are also inhibited by superinfecting ghosts. After this time, a chloramphenicol-inhibitable reaction occurs which causes the phage-infected cells to become increasingly tolerant of added ghosts.

Although it has been known for some time that a primary infection by ghosts will inhibit the multiplication of superinfecting phage (2, 9, 13), it has been suggested that phage would prevent the effects of ghosts in a simultaneous infection (23).

During an investigation of the accumulation of various sugars by *Escherichia coli* B after phage and ghost infection (H. Winkler and D. Duckworth, *in preparation*), it was observed that simultaneous infection by phage and ghosts resulted in an inhibition of active transport of the same magnitude as that produced by infection with ghosts alone. As this was a rather unexpected observation, the phenomenon was investigated further and it was found that ghosts can inhibit a number of functions of phage-infected cells, including infective center formation. A chloramphenicol-inhibitable reaction which occurs starting at about 2 min after phage infection causes the phage-infected cells to become tolerant to the ghosts.

MATERIALS AND METHODS

Organisms and media. The organisms used in most of the experiments are the same as those used in a previous study (6). Their source and relevant characteristics are described in Table 1, along with organisms not previously used. The histidine auxotroph *E. coli* B(*his*⁻) was isolated by R. J. Kadner by using nitroguanidine-treated cells and penicillin selection (1, 12). B/4 is a spontaneous mutant of B which cannot adsorb T4. T*4 phage (10) were grown in a phosphoglucosyltransferase-negative mutant of *E. coli* K and titered on *Shigella sonnei*. The amber mutant of T4, T4 am E957, is a mutant in gene 1, the deoxynucleotide kinase gene, and cannot synthesize DNA, late proteins, or phage when allowed to infect *E. coli* B. It was

chosen for routine use (as ghosts) to avoid any problems of residual phage multiplication.

All media have been previously described. When M-9 was used, 200 µg of tryptophan per ml was added before the addition of phage or ghosts, except for the experiments with 5-methyl-tryptophan. Bacterial cell titers were determined from turbidity measurements or by colony counts, and phage titers were determined by the standard agar-layer technique.

Preparation and assay of ghosts. T4 am E957 to be used in the preparation of ghosts were grown on *E. coli* CR63, purified by sucrose gradient centrifugation (6), and then dialyzed and concentrated by centrifugation. Ghosts were prepared by the addition of 2 volumes of 3 M sodium acetate to 1 volume of T4 am E957 phage which titered between 2×10^{12} and 5×10^{12} phage/ml. Equilibration of the phage in the salt solution for 15 min at 0°C was followed by rapid dilution into 100 volumes of cold distilled water. Inactivation of plaque-forming ability was usually greater than 99.5%. The shockate material was treated with deoxyribonuclease and then centrifuged at $100,000 \times g$ for 3 hr. The pellets containing the ghosts were suspended in a small volume of M-9 salt solution and used without further treatment. Radioactively labeled ghosts were made from phage grown in the presence of a ¹⁴C-amino acid algal hydrolysate (1 µCi/ml in the presence of 0.5% Casamino Acids) purchased from Nuclear-Chicago Corp.

The ghosts were titered by a modification (6) of the method of Duckworth and Bessman (8). Details of this assay and characteristic data are given in the legend to Fig. 2.

ONPG transport. The cells for the orthonitrophenyl-β-D-galactopyranoside (ONPG) transport assay were grown in the presence of 5×10^{-4} M isopropyl-β-D-thiogalactopyranoside (IPTG, Calbiochem) for at least two generations. The cells were then centrifuged and resuspended in the absence of IPTG in the medium in which they were grown. ONPG hydrolysis by whole cells was measured in a Gilford recording spectrophotometer as an increase in optical density at 420 nm.

TABLE 1. *Organisms used*

Organism	Relevant characteristics	Source
Bacteria		
<i>Escherchia coli</i> B	Wild type, nonpermissive for amber mutants, restrictive for non-glucosylated T4 (T*4)	M. J. Bessman
<i>E. coli</i> B(<i>his</i> ⁻ ₁)	Histidine auxotroph	See text
<i>E. coli</i> B/4	Resistant to T4	See text
<i>E. coli</i> CR63	Permissive for amber mutants	M. L. Dirksen
<i>E. coli</i> DF2000	Phosphoglucosyltransferase-negative	D. Fraenkel
<i>Shigella sonnei</i>	Nonrestrictive for nonglucosylated T4 (T*4)	Department of Microbiology, University of Virginia School of Medicine
Phage		
T4 am E957	Amber mutation in gene 1	R. S. Edgar
T4 am ⁺	Wild type	Revertant of T4 am E957
T*4	T4 with nonglucosylated DNA	See text

Reaction mixtures contained 1 ml of 5×10^8 cells with phage or ghosts (where indicated) at a multiplicity of 5, 1.9 ml of 0.025 M sodium phosphate buffer (pH 7.2) containing 0.5% NaCl, and 0.1 ml of 0.03 M ONPG (Calbiochem). When the cells were infected with phage or ghosts, measurements were made from 1 min postinfection to 12 min postinfection. After this time, lysis may occur and greatly increase the rate of hydrolysis.

Infective center formation. Infective center formation was measured by incubation of phage with bacterial suspensions containing approximately 5×10^8 cells/ml for various times, followed by dilution into a sufficient concentration of antibody to T4 phage to inactivate 99% of the unadsorbed phage within 1 min. After further dilution, the suspensions were plated with an excess of host bacteria, and the infective centers were determined from the number of plaques.

Antibody preparation. Antibody to T4 phage was prepared by injecting rabbits subcutaneously at several sites with a total of 1 ml of T4 am E957 phage suspension containing 5×10^{12} phage/ml. After 2 weeks the process was repeated. Bleeding was 18 days after the second series of injections. For very high titer antiserum, the rabbits may be reinjected once after 2 months and bled 1 week later.

RESULTS

Transport of ONPG by infected cells. When ONPG is added to a culture of whole lactose operon-induced cells, it is transported into the cell by the lactose permease and broken down by β -galactosidase and the orthonitrophenol is released. As the rate-limiting step in this series of reactions is the entry of the ONPG into the cell, the hydrolysis of ONPG by intact cells may be used as a measure of the transport of this compound into the cells (14, 16). The addition of energy poisons partially inhibits the transport of ONPG by whole cells (16, 26). In *E. coli* B, the level of inhibition obtained with 0.03 M

sodium azide plus 0.1 M sodium fluoride (complete energy poisoning) depended, to some extent, on the state of the cell culture. Maximum inhibition ($\sim 70\%$) was obtained if the inoculum (grown from a slant in the medium to be used for the experiment) used was no more than 18 hr old.

Figure 1a (lower curve) shows the inhibition of ONPG transport by ghosts and phage. The level of inhibition obtained with a ghost multiplicity of 5 was very nearly equal to that obtained with azide plus fluoride. There was no inhibition when ghosts were added to B/4 cells. Figure 1a also shows that phage caused an inhibition which was very much less than the inhibition caused by ghosts, usually on the order of 5 to 10% with a multiplicity of 5. Whether this was due to some contaminating ghosts has not been determined, but as some phage preparations give almost no inhibition this is a possibility. T*4 phage, with a plating efficiency of 6% on *E. coli* B as compared to *S. sonnei*, caused only a 10% inhibition of ONPG transport when used at a multiplicity of 8 (as calculated from the titer on *Shigella*). Simultaneous addition of T4 am⁺ phage and ghosts was found to cause an inhibition of the same order of magnitude as that obtained with ghosts alone. In the next experiment, cells which had been infected with T4 am⁺ phage for a period of 10 min were used. When ghosts were added to these 10-min phage-infected cells, there was no inhibition of the ONPG transport. Therefore, some reaction had occurred that had made the cells "immune" to the effects of the ghost. Figure 1b (upper curve) shows the levels of inhibition obtained when ghosts were added to cells which had been infected with phage for various periods

FIG. 1. *In vivo* hydrolysis of ONPG by *Escherichia coli* B. Cells were grown in M-9 plus 1% Casamino Acids to a concentration of $\sim 5 \times 10^8$ cells/ml. IPTG (5×10^{-4} M) was present for at least two generations. The cells were then centrifuged and resuspended in M-9 salt solution containing 200 μ g of L-tryptophan per ml. Each cuvette contained 1 ml of cells, phage or ghosts (Γ) or both at a multiplicity of 5 (where indicated), 1.9 ml of 0.025 M sodium phosphate buffer (pH 7.2) containing 0.5% NaCl, and 0.1 ml of 0.03 M ONPG. The increase in optical density (OD) at 420 nm was measured in a Gilford recording spectrophotometer. Part a (lower curves): 1, uninfected cells; 2, cells infected with T4 am E957 ghosts; 3, cells infected with T4 am⁺ phage; 4, cells infected simultaneously with phage and ghosts. Part b (upper curves): 1, cells infected with phage for 1 min before the addition of ghosts; 2, cells infected with phage for 2 min before the addition of ghosts; 3, cells infected with phage for 5 min before the addition of ghosts; 4, cells infected with phage for 10 min before the addition of ghosts.

of time. From these data, it appears that the reaction which makes the phage-infected cells "immune" to the ghosts starts at about 2 min after infection and is complete by 10 min after infection. When T4 am E957 was used to infect its nonpermissive host for 10 min, there was no inhibition of ONPG transport by superinfecting ghosts, indicating that phage deoxyribonucleic acid (DNA) synthesis is not necessary for the reaction to take place.

Inhibition of phage multiplication by ghosts.

As the above data indicated that the ghosts could cause inhibition of transport in phage-

infected cells, I looked for an inhibition of phage multiplication by ghosts. Increasing amounts of ghosts were added to constant amounts of T4 am E957 phage. The mixed suspensions were titrated and used to infect *E. coli* CR63 grown in broth at a multiplicity of five phage per cell. After 2 hr, cell lysis was completed with chloroform. Table 2 shows that, although the presence of ghosts in the phage suspensions used for the infections did not decrease the plaque titer of these suspensions, increasing amounts of ghost/phage caused the yields of progeny phage from cells multiply infected with these phage suspensions to decrease.

Inhibition of infective center formation by ghosts. The decreased yields of progeny phage obtained when cells were multiply infected with phage suspensions which also contain ghosts could be caused by a decrease in the number of progeny phage from each infected cell or by an inhibition of infective center formation. The following experiments show that the inhibition of phage multiplication noted above was due to the inhibition of infective center formation when phage and ghosts were added simultaneously to cells. The data in Table 3 are taken from an experiment in which *E. coli* B was grown in broth to a concentration of 8×10^8 cells/ml and then infected with T4 am⁺ phage and phage ghosts for various times relative to each other. It can be seen that, as was previously known (2, 9, 13), phage can not form infective centers when they are added to cells previously infected with ghosts. The phage also could not form infective centers when they were added to the cells simultaneously with the ghosts. Note also that the number of infective centers did not change markedly if the cells were infected with phage for times varying between 1 and 10 min.

TABLE 2. Inhibition of phage multiplication by ghosts

Sample	Ghosts/ phage	Plaque titer ^a	Yield ^b
1	0	1.2×10^{11}	1.4×10^{11}
2	0.05	1.2×10^{11}	9.4×10^{10}
3	0.10	1.2×10^{11}	6.3×10^{10}
4	0.20	1.3×10^{11}	5.9×10^{10}
5	0.50	1.3×10^{11}	2.6×10^{10}
6 ^c	1,000	1.2×10^8	1.6×10^8

^a Titer of the phage-ghost suspensions used for the infections.

^b *E. coli* CR63 in broth at 5×10^8 cells/ml was infected with a multiplicity of 5 plaque-forming units/cell. After 2 hr, chloroform was added and the cultures were titrated for progeny phage.

^c Control with no added phage. It was used to infect the cells at a multiplicity of 5 ghosts/cell.

TABLE 3. Inhibition of infective center formation by ghosts

Cells	First incubation ^a	Second incubation ^a	Infective centers ^c
<i>E. coli</i> B in broth (8×10^8 cells/ml)	Ghosts for 5 min	Phage for 5 min	0.5
	Ghosts and phage (simultaneous) for 5 min	None	0.3
	Phage for 1 min	None	6.6
	Phage for 1 min	Ghosts for 5 min	1.5
	Phage for 2 min	None	6.0
	Phage for 2 min	Ghosts for 5 min	3.3
	Phage for 5 min	None	6.9
	Phage for 5 min	Ghosts for 5 min	4.5
	Phage for 10 min	None	6.8
	Phage for 10 min	Ghosts for 5 min	6.1
Chloramphenicol ^b -treated <i>E. coli</i> B	Phage for 10 min	None	6.9
	Phage for 10 min	Ghosts for 5 min	0.2
Puromycin ^d -treated <i>E. coli</i> B	Phage for 10 min	None	4.7
	Phage for 10 min	Ghosts for 5 min	1.8

^a Ghosts and phage (T4 am⁺) are used at a multiplicity of 4.

^b Cells grown in broth; incubated for 10 min with 100 μ g of chloramphenicol per ml.

^c Values are expressed $\times 10^{-8}$.

^d Cells grown in broth; incubated for 10 min with 2 mg of puromycin per ml.

This is consistent with the half-time of adsorption (~ 30 sec) of phage to these cells when these same conditions are used. If, however, the cells were infected with phage and then ghosts were added at increasingly later times after phage addition, the number of infective centers increased steadily. If ghosts were added to the cells after they had been infected with the phage for 10 min, there was almost no inhibition of infective center formation. If chloramphenicol was present during these 10 min, complete inhibition of infective center formation was obtained. Tests were conducted to determine whether ghosts would adsorb to cells infected with phage. Ghosts labeled with radioactive amino acids were adsorbed to either uninfected or phage-infected cells. The same amount of label was adsorbed in each case. Therefore, I shall refer to the reaction that the phage-infected cells undergo as the development of tolerance, in analogy to the term colicin tolerance (18, 19).

Table 4 shows the results of an experiment similar to the one in Table 3, except that the *E. coli* B was grown in M-9 plus 0.4% glucose to a concentration of 6.5×10^8 cells/ml. A synthetic media were used so that the effects of amino acid starvation and 5-methyl-tryptophan could be tested. For the histidine starvation experiment, the histidine auxotroph of *E. coli* B was used. Histidine starvation and 5-methyl-tryptophan treatment were shown to cause complete inhibition of β -galactosidase induction in unin-

fected cells, and 5-methyl-tryptophan is known to prevent early enzyme synthesis in T4-infected cells (17a). These treatments, however, did not prevent the development of tolerance by the phage-infected cells. Whether protein synthesis is necessary for the reaction is, therefore, an open question. Chloramphenicol was seen to be as effective in preventing the development of tolerance in synthetic media as it was in broth. Sodium azide at a concentration of 0.08 M also completely inhibited the development of tolerance, indicating that the reaction is energy-dependent.

Figure 2 shows the effect of ghost multiplicity on several reactions which ghosts are able to inhibit: β -galactosidase induction, colony formation, and infective center formation at various times relative to phage infection. Here again the development of tolerance was observed. The break observed in the curve for the 5-min phage-infected cells is mathematically predictable given a heterogeneous population of cells, all of which can adsorb the ghosts but only some of which can be inhibited.

The development of tolerance was also observed with an r_{II} mutant of the phage when it infected *E. coli* B.

DISCUSSION

It has been proposed that the attachment of the phage protein coat to the cell surface causes allosteric changes in the cell membrane which lead to the loss of many membrane-associated

TABLE 4. *Inhibition of infective center formation by ghosts*

Cells	First incubation ^a	Second incubation ^a	Infective centers ^b
<i>E. coli</i> B in M-9 + glucose (6.5×10^8 cells/ml)	Ghosts and phage (simultaneous) for 10 min	None	0.7
	Phage for 10 min	None	5.4
	Phage for 10 min	Ghosts for 5 min	5.1
Chloramphenicol ^c -treated <i>E. coli</i> B	Phage for 10 min	None	4.2
	Phage for 10 min	Ghosts for 5 min	<0.1
5-Methyl-tryptophan-treated ^d <i>E. coli</i> B	Ghosts and phage (simultaneous) for 10 min	None	0.7
	Phage for 10 min	None	6.5
	Phage for 10 min	Ghosts for 5 min	4.8
Histidine-starved <i>E. coli</i> B ^e	Ghosts and phage (simultaneous) for 10 min	None	0.5
	Phage for 10 min	None	4.0
	Phage for 10 min	Ghosts for 5 min	3.8

^a Ghosts and phage (T4 am⁺) used are at a multiplicity of 5.

^b Values are expressed $\times 10^{-8}$.

^c Cells grown in M-9 + glucose; incubated for 10 min with 100 μ g of chloramphenicol per ml.

^d Cells grown in M-9 + glucose; incubated for 10 min with 200 μ g of 5-methyl-tryptophan per ml.

^e *E. coli* B (*his*⁻) grown in M-9 + glucose + 100 μ g of histidine per ml; washed and incubated for 10 min in the absence of histidine.

functions and that the injection of the phage DNA or the internal protein, or both, and its attachment to the cell membrane allow the membrane to remain functional (6, 7). If this were the case and if the changes produced by attachment were changes in the entire cell membrane and not just local effects, it would not be unreasonable to expect that a simultaneous infection by phage and ghosts would produce the same effect as infection by phage alone. In fact, all of the available data indicate that this should be the case. Sechaud et al. (23) state that the injected germinal substance of the phage can counteract the effect of the ghost, but they did not present their data on this subject. Herriott and Barlow (13) found that there was less inhibition of infective center formation by ghosts when these were added simultaneously with phage to starved cells. This experiment is difficult to interpret, however, as the relative multiplicities of phage and ghosts are not given, and in the control the number of infective centers was low due to the starvation. Bourgaux (3), with ghosts of phage N, a phage which is similar to the T-even phages, found that these caused less superinfection inhibition if the phage were added with the ghosts rather than at a later time.

I do not, however, observe any protective effects by the phage when they are added to cells

simultaneously with ghosts. Cells infected simultaneously with both phage and ghosts are inhibited with respect to ONPG transport and infective center formation. Thiomethylgalactoside transport is also inhibited by a mixture of phage and ghosts (H. Winkler and D. Duckworth, *in preparation*). With a slightly different system, but one which can differentiate between the effects of phage and ghosts, Swift and Wiberg also observed that phage plus ghosts act like ghosts alone. These workers have found that both phage and ghosts can inhibit the colicin E2-induced breakdown of host DNA but that the effect of phage multiplicity is markedly different from the effect of ghost multiplicity. If phage and ghosts are added to their system simultaneously, they observe the effect of the ghosts not of the phage (R. Swift and J. Wiberg, *personal communication*). Therefore, it must be concluded that either the protein coat has entirely different properties when it is acting as a ghost than it does when it is acting as part of the phage or the phage has the ability to prevent or reverse the effect of only its own protein coat. The former possibility does not seem as evolutionarily reasonable as the latter.

One might ask if the injection of just the DNA of the phage could account for the apparent reversal of the effect of its protein coat. This is

FIG. 2. Effect of multiplicity of ghost infection on host functions. The volume indicated on the abscissa is the volume of ghost suspension added per milliliter of cells. *Escherichia coli* B was grown in broth to 5×10^8 cells/ml and then infected with various multiplicities of ghosts. For the β -galactosidase induction, infection with ghosts was for 2 min before the addition of 5×10^{-4} M IPTG. After 8 min at 37°C, the cells were chilled, centrifuged, resuspended in 0.025 M sodium phosphate buffer, and treated with toluene. The per cent survival is calculated from the extent of enzyme synthesis at 8 min in the infected cells as compared to the extent of the enzyme synthesis at 8 min in uninfected cells. A multiplicity of 1 is defined by the volume of ghosts which causes a 37% survival of enzyme-forming capacity; in this case, a multiplicity of 1 is given by 0.025 ml of ghost suspension. The ghost titer is calculated from this to be 2×10^{10} ghosts/ml. The per cent survival of colony formation was measured in the cultures used for the β -galactosidase synthesis after 10 min. For the infective center formation, cells were infected with T4 am^+ phage at a multiplicity of 5 before the addition of ghosts, and the assay for infective centers was performed as indicated in Materials and Methods. Symbols: $\circ$, β -galactosidase synthesis; $\square$, infective center formation, phage added 2 min before the addition of ghosts; $\blacktriangle$, infective center formation, phage added 5 min before the addition of ghosts; $\bullet$, infective center formation, phage added 10 min before the addition of ghosts. The closed circles ($\bullet$) represent surviving colony formers, and the open triangles ($\triangle$) represent infective center formation when ghosts and phage are added simultaneously; no lines have been drawn through these two sets of points.

not known, but it has been observed that the DNA may be extensively damaged before phage begin to take on the characteristics of ghosts. Extensively irradiated phage cause very little

inhibition of ONPG transport, and phage irradiated to a 10^{-8} survival cause only a small release of labeled uracil compounds (D. Duckworth, unpublished data). The latter leakage is extensive in ghost-infected cells (6). T*4 phage whose DNA is broken down upon infection (10) also has the properties of phage, not of ghosts, as far as transport of ONPG is concerned.

The development of ghost tolerance after phage infection is perhaps the most interesting result of this study. Damage by the phage which is later repaired by phage-induced enzymes has been the hypothesis most commonly put forth to account for the initial events in phage and ghost infection (see reference 7 for a more detailed discussion of this). Evidence of changes on the cell surface after phage infection which might be used to support this hypothesis has been presented by several authors (4, 5, 11, 17, 24, 25), but the major work upon which the damage and repair hypothesis is based (21, 22) has never been confirmed (7, 15). In addition, any initial membrane damage by the phage which even approaches the same magnitude as the changes produced by ghosts alone has not been observed. There is no loss from the phage pool of nucleotides (6) and no transient inhibition of thymidine transport (6) or of ONPG transport. All of these above-named reactions occur to what might be called a drastic extent after ghost infection. It is thus possible that the development of tolerance is just another manifestation of the reactions taking place on the surface of the infected cell, the purpose of which has yet to be determined.

Another fact which comes to light from this work is that in measuring the effects of phage on their host cells one must take care to use phage stocks which are not contaminated with ghosts. Whether observations of extensive leakage from phage-infected cells (21, 22) and work which shows an effect of phage multiplicity on inhibition of host functions (20) can be explained by the presence of ghosts is not known. But the use of an assay based on the ability of ghosts to inhibit ONPG transport should be very useful for checking the purity of phage stocks for ghost contamination.

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