Effects of Furazolidone on the Infection of Vibrio cholerae by Bacteriophage φ149

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Received for publication 30 November 1972

Furazolidone in concentrations which had little effect on the growth of host organisms greatly reduced the yield of phage φ149 from the host Vibrio cholerae OGAWA 154. This phage was resistant to the in vitro action of the drug. The phage yield of infected bacteria depended significantly on the time of addition or withdrawal of the drug. The average burst size of the drug-treated and infected bacteria decreased exponentially with increase in drug concentration. The latent period of phage multiplication and also the eclipse period did not change significantly from the control values. A concentration of 0.05 μg of furazolidone per ml inhibited DNA synthesis by about 50% in phage-infected cells and only by about 18% in noninfected ones, relative to the respective controls. RNA and protein synthesis were affected by a much smaller degree both in infected and noninfected cells. Quantitative deduction of the length of furazolidone-treated cells from their phage adsorption characteristics and its agreement with previous electron microscopy data indicated that furazolidone did not affect the phage receptors.

The antibacterial properties of nitrofurans were first recognized by groups in the United States (8) and Germany (7) during the 1940's. Since that time, certain derivatives have been widely used in clinical and veterinary medicine. The chemotherapeutic properties of a number of nitrofurans have been reviewed by Paul and Paul (16, 17).

Furazolidone or N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone, a relatively new member of the synthetic group of nitroated compounds, also displays a wide spectrum of antibacterial activity (8). Our previous study revealed that this drug inhibited DNA synthesis in and caused filamentation of Vibrio cholerae cells (6, 18). The knowledge of the mode of action of a drug on noninfected bacterial cells cannot, a priori, be assumed to hold good in phage-infected ones. There are agents which affect DNA synthesis differently in noninfected and phage-infected cells (2, 4, 5, 10–12, 22, 23). Hence the action of furazolidone on the V. cholerae cells infected with cholera phage φ149 should be of interest. A preliminary account of this investigation will be presented elsewhere. (S. N. Chatterjee and M. Maiti, Indian J. Exp. Biol., in press).

MATERIALS AND METHODS

V. cholerae strain OGAWA 154 and cholera phage φ149, obtained through the courtesy of S. Mukerjee, Former Director, Cholera Research Centre, Calcutta, were used in these experiments.

Sodium [32P]orthophosphate was purchased from Bhabha Atomic Research Centre, Trombay, India. Chemically pure furazolidone was obtained as a gift sample from Smith, Kline, and French (India) Ltd., Bombay.

Bacterial growth and estimation. Nutrient broth (NB) containing 10 g of peptone (Difco), 5 g of NaCl, and 10 g of beef extract (ICAS, Italy) in 1 liter of distilled water (pH 7.4) was used as the general culture medium for the study of phage-infected or noninfected bacteria. Bacterial growth was estimated by measurement of the absorbance at 650 nm by a photoelectric colorimeter and viability (colony forming units per milliliters) by the conventional pour plate method. For study of the incorporation of radioactive phosphorus into different chemical fractions of phage-infected and noninfected V. cholerae cells, sodium [32P]orthophosphate was added to the peptone-water (PW) medium (18) which contained, in 1 liter, 30 g of peptone (Difco) and 5 g of NaCl (pH 8.0), and the activity was adjusted so that appreciable counts per minute were obtained in DNA after chemical fractionation of the cells.

Assay of phage yield. The log-phase (2.5 × 10^8 cells/ml) V. cholerae cells were infected with phages (2.5 × 10^6 PFU/ml) at 37 C in NB medium. After a 10-min adsorption period, the bacteria were spun down at 6,000 × g for 5 min and transferred in suitable portions to prewarmed NB containing no, or various concentrations, of furazolidone. The samples were incubated for 120 min at 37 C to allow intracellular
multiplication of the phages and bursting of the cells to take place, mixed with a few drops of chloroform, and then assayed for plaque-forming titer.

The double agar layer method (1) was routinely used for phage assay. The top and bottom agar layers in plates used for phage assay contained 0.7% and 1.1% agar respectively in NB medium.

Measurement of lysis of phage-infected cells. Phage was added, at a multiplicity of infection (MOI) of 6.0, to 10 ml of log-phase NB culture of V. cholerae in an Erlenmeyer flask (capacity 100 ml), and the mixture was incubated at 37 C. After allowing 15 min for the phage adsorption to take place, the mixture was diluted (1:5) into fresh NB medium (containing no or the desired amount of furazolidone) prewarmed to 37 C and incubated at 37 C with aeration. Optical density at 650 nm was recorded at desired time intervals during incubation by a photoelectric colorimeter.

Determination of burst size. The burst size was determined by one-step growth and by single-burst experiments. In the one-step growth experiment, the log-phase V. cholerae cells (2.5 x 10^8 cells/ml) were infected with phage φ149 (2.5 x 10^3 PFU/ml) in NB medium at 37 C. After 10 min, the mixture was centrifuged and the pellet was suspended in the original volume of fresh NB medium prewarmed to 37 C and containing zero or 0.05 μg of furazolidone per ml. Dilutions were then made in either drug-free or drug-containing media to prepare first and second growth tubes (1).

In a single-burst experiment, 0.9 ml of log-phase cells (2.5 x 10^9 cells/ml) was infected with phage φ149 at an MOI of 1. After 10 min, the mixture was centrifuged and the pellet was suspended in NB medium containing no or 0.05 μg of furazolidone per ml. Further dilutions were then made in either drug-free or drug-containing NB medium to result in about 1 infected bacterium per ml, and about 50 small tubes were prepared each containing 0.5 ml of this final dilution. After a 90-min incubation period at 37 C the content of each tube was assayed for phage.

Premature lysis. One-step growth experiments were arranged for phage-infected cells incubated in the presence or absence of furazolidone, and the first and second growth tubes were prepared as described above. Samples were then taken at intervals from the growth tubes and subjected to premature lysis by shaking with chloroform (21).

Methods of fractionation for isotopic studies. Usually, log-phase cells (5 x 10^9 cells/ml) were incubated for 10 min at 37 C (MOI of about 6) or without phage in PW medium. The mixture was then centrifuged, washed to remove unadsorbed phage, and suspended in the original volume of PW medium. A 1-ml amount of the above suspension was then mixed with 9 ml of fresh medium containing no, or the appropriate concentration of, furazolidone and also 32P and incubated at 37 C with aeration. The distribution of 32P activity into the DNA and RNA fractions of the cells was determined at desired intervals of time by using the membrane fractionation techniques as described by Britten et al. (3) and modified by Roodyn and Mandel (19). The membrane filters used were 27-mm in diameter and of 0.45-μm pore size as obtained from Schleicher and Schull Co., Keene, N. H. For measurement of radioactivity, the filters were fixed on aluminum planchets with a layer of rubber cement, dried at room temperature and measured by a thin-window counter (Geiger Müller). Counts were continued until a standard error of less than 1% was obtained. The counts were corrected for background and 32P decay.

Protein estimation. For protein estimation, suitable portions (9 ml) of infected or noninfected culture were removed at desired intervals of time and chilled rapidly. The cells were harvested by centrifugation and washed twice in physiological saline. The washed cells were suspended in 2.5 ml of 0.5M HClO4 at 80 C for at least 25 min. Acid-insoluble material was collected by centrifugation, digested overnight in 1 M NaOH at 37 C, and estimated for protein by the method of Lowry et al. (14) using crystalline bovine serum albumin as the standard.

Determination of the length of drug-treated cell from phage adsorption data. Log-phase cells were exposed to 0.5 μg of furazolidone per ml of NB medium for 3 h at 37 C. The cells were then washed with NB medium only and suspended in one-tenth of its original volume. Kinetics of adsorption of the phage φ149 to furazolidone-treated or untreated cells were determined as described previously (18). Since the multiplicity of infection was kept low, the kinetics of phage adsorption could be described as follows (13): 

\[ \frac{\Delta p}{\Delta t} = K_b p \]

where \( p \) is the number of phages adsorbed (PFU/ml), \( K_b \) is the adsorption rate constant, \( b \) the bacterial concentration, and \( p \) the phage concentration at time \( t \). The adsorption rate constant was related to the diffusion constant (D) of phage particles by Schlesinger (20) as \( K = 4\pi DR \) (ii), where \( R \) is the radius of a sphere having the same surface area as that of the bacterium. Using equation (ii) and assuming that the rod-shaped bacteria increased only in length after drug treatment (this was found true from electron microscopy) the length \( l_4 \), of the drug-treated cell could be obtained from the equation

\[ l_4 = K_a K_b l_3/K_a \]

(iii), where \( K_a \) and \( K_b \) are the adsorption rate constants for untreated and drug-treated cells, respectively, and \( l_3 \) is the length of untreated cell.

RESULTS

Effects on the phage yield of infected bacteria. When the free phages were suspended in NB medium containing different concentrations of furazolidone, no significant decrease in the number of plaque-forming units was noted in any of the cases even after 200 min of incubation at 37 C (Fig. 1). In the presence of furazolidone the intracellular synthesis of the cholera phage φ149 was significantly affected. With increasing concentrations of the drug, there was a progressively decreasing phage yield (Fig. 1). At the drug level of 0.2 μg/ml, the phage yield was reduced by 98% (control 7.90 x 10^7 PFU/ml; experiment 0.19 x 10^7 PFU/ml), whereas the bacterial growth diminished by only about 25% (control optical density [O.D.])
Effects on the lysis of phage-infected bacteria. After infection of the cells by phage φ149 at an MOI of 6.0, optical density of the culture continued to rise to about 45 min and then, after a short stationary phase, started decreasing (Fig. 2). The absence or presence of different amounts of furazolidone did not alter the rise period, but the degree of lysis attained subsequently was significantly affected. Increasing concentration of the drug caused increasing inhibition of lysis of the infected bacteria.

Effects on the phage multiplication characteristics. In the previous experiments relating the effect of the drug on the phage yield in bulk cultures, some of the newly released phage particles might get adsorbed to unlysed bacteria or to cell wall debris, and hence might not have contributed to the overall plaque-forming units assayed. In consequence, the total number of progeny phage particles produced in bulk cultures might have been underestimated. This difficulty could be avoided by using the one-step growth experiment of Ellis and Delbruck (9). Such experiments showed that the presence of 0.05 μg of furazolidone per ml made no significant difference in the length of the latent period (36 min), but the average burst size was reduced by 50% (Fig. 3). With increasing concentration of the drug, the burst size decreased exponentially (Fig. 4), and with 0.1 μg of furazolidone per ml, the burst size was reduced to about 18% of the control value. It was of interest to know whether the reduction in burst size in the presence of the drug was due to fewer phage progeny from all the productive bacteria, or whether phage production was normal in some bacteria but completely inhibited in the remainder. The single-burst technique was used to study the number of phage particles produced by individual bacteria. Furazolidone reduced the number of phage progeny of those bacteria which remained productive. At an MOI of 1, only 8 of the 40 plates showed plaques in control experiment (in the absence of drug). The totals for these plates were 5, 11, 211, 12, 150, 55, 12, and 5. The average burst size as calculated from the Poisson distribution formula was 51. In the presence of furazolidone (0.05 μg/ml) with the same MOI the value of burst size was 6 with only 7 out of the 45 plates presenting plaques. The totals for the individual plates were 1, 3, 3, 6, 22, 4, and 2.

The premature lysis technique was used to determine whether furazolidone delayed the appearance of the first infective phage particles. It was found that 0.05 μg of drug per ml did not significantly alter the normal eclipse period (Fig. 3). The rates of appearance of intracellular infection phages in the presence of 0.05 μg of furazolidone per ml were significantly less than in the control from about the 25th min onward (Fig. 3).
V. cholerae cells infected with phage φ149 and grown in the presence or absence of furazolidone are shown in Fig. 5, 6 and 7, respectively. In noninfected bacteria, DNA and protein synthesis in the presence of 0.05 μg of furazolidone per ml were always less than the respective control values. The RNA synthesis in the presence of the drug was initially somewhat higher, but after about 50 min it became less than in the

Effects on macromolecular synthesis in noninfected and phage-infected cells. Time courses of synthesis of DNA and RNA, as measured by the incorporation of $^{32}$P into the respective chemical fractions, and of protein in
control. In phage-infected cells, DNA, RNA, and protein synthesis in the presence of the drug were always less than the respective control values, but, comparatively, DNA synthesis was markedly reduced. In phage-infected cells, DNA synthesis in presence of 0.05 μg of furazolidone per ml was reduced by 48 to 58% of the respective control (drug-free system) value, whereas in noninfected cells it was reduced by about 18% only. RNA and protein synthesis in the presence of the drug were affected by a comparatively smaller degree both in phage-infected and noninfected cells.

Effects of the time of addition or withdrawal of the drug on phage yield. Two groups of experiments with conditions similar to those of the one-step growth technique were done to determine when furazolidone evoked its inhibitory action on the phage production by infected bacteria. Fig. 8 shows that if the drug was added any time within the first 25 min of the latent period of infection, inhibition in phage production was maximal and consistently the same, irrespective of the time of addition. If, however, the drug was added at later stages of infection, its effect on the inhibition of phage production by the infected bacteria was significantly reduced. The greater the delay in the addition of the drug, the greater the phage yield.

The results of the converse set of experiments (Fig. 9) revealed that if the drug was withdrawn any time within the first 25 min of phage infection, the effect on the inhibition of phage production was least and the phage yield was the same as in the control. But if the drug was withdrawn at later stages of infection, its effect on the inhibition of phage production by infected bacteria became significant. The greater the delay in withdrawal of the drug, the less the phage yield. It could therefore be concluded that furazolidone was active on the phage-producing machinery of the infected bacteria from about the 25th min of infection onwards, and before this time the furazolidone-sensitive system was either not present or not operative.

Phage adsorption on furazolidone-treated cells. When the cells were pretreated for 3 h at 37 C with 0.5 μg of furazolidone per ml of NB medium and then exposed to phages, faster and greater adsorption of the phages relative to the control (untreated cells) was obtained (Fig. 10). However, in both experimental and control conditions, adsorption was biphasic in nature. Adsorption rate constants (from the first and major phase of the biphasic data) for the untreated (K_a) and for the drug-treated (a) cells were determined as 1.026 x 10^-9 ml/min and 2.7 x 10^-8 ml/min, respectively, using equation (i). Now with the help of equation (iii) we found that the length of the furazolidone-treated cells became 21 μm, i.e., seven times the length of untreated cells.

DISCUSSION

Studies on the adsorption kinetics of phage φ149 on the untreated and furazolidone-treated cells revealed that the lengths of the treated

Fig. 7. Protein synthesis in noninfected and phage-infected V. cholerae cells in presence and absence of 0.05 μg of furazolidone per ml of PW medium at 37 C. Symbols: O, noninfected cells; ●, noninfected cells + furazolidone; Δ, phage-infected cells; ▲, phage-infected cells + furazolidone.

Fig. 8. Effects of time of addition of furazolidone on the phage yield of infected bacteria. The first growth tubes of the one-step growth experiment were prepared as described in Materials and Methods. For addition of furazolidone within 36 min of infection (latent period), samples were diluted at the required time into second growth tubes containing 0.05 μg of furazolidone per ml. For exposure at later periods of time, several second growth tubes were prepared (before onset of lysis) and furazolidone was added directly at the appropriate times. Samples (0.1 ml) from all the second growth tubes were taken after 100 min at 37 C for assay of PFU. Phage yield is expressed as PFU/ml of second growth tube.
ones increased about seven times that of the untreated ones. Direct electron microscope examination revealed that the lengths of the treated cells ranged between 6 and 11 times the length of the untreated ones (6, 18) and is thus in reasonable agreement with the lengths deduced presently from adsorption data. This implies that the phage receptors present on the surface of furazolidone-treated or untreated cells were at least functionally identical.

The reproduction of phage φ149 was more sensitive to inhibition by furazolidone than growth of the host V. cholerae cells. Concentrations of furazolidone which had a small effect on growth of host cells markedly reduced phage reproduction. This is in conformity with the finding of Bailey et al. (2) and Iosson and Fry (12) for a temperate phage λ–host Escherichia coli strain K-112 system subjected to treatment with 5-aminoacridine, another inhibitor of DNA synthesis.

If furazolidone affected the synthesis of RNA, DNA and/or protein, then this might be the reason why phage production was less in the furazolidone-treated bacteria. A 0.05 μg/ml concentration of furazolidone caused an interesting change in the pattern of macromolecular synthesis in phage-infected cells as compared to that in noninfected ones. In phage-infected cells, DNA synthesis was reduced by 48 to 58% of the respective control value (drug-free system), whereas in noninfected cells it was reduced by about 18% only by the same amount of the drug. RNA and protein were affected by a comparatively smaller degree both in infected and noninfected cells. It is thus apparent that DNA synthesis in phage-infected cells is preferentially more affected by furazolidone. The 50% reduction in DNA synthesis by 0.05 μg of furazolidone per ml of medium agrees reasonably with the 50% reduction in burst size observed.

A comparison of the action of furazolidone with that of two other nitrofurans already studied would be of interest. 3-Aminot-6 [2-(5-nitro-2-furyl) vinyl]-1,2,4-triazine hydrochloride was reported to inhibit markedly DNA synthesis in noninfected E. coli B (10). However, the same drug did not inhibit the DNA synthesis in T2-infected cells. Similarly, (5-nitro-2-furyl)-vinylquinoline also did not affect the DNA synthesis in phage-infected cells at concentrations which markedly affected the phage reproduction and also the DNA synthesis in noninfected bacteria (23). Nalidixic acid had little inhibitory effect on SPOI phage DNA synthesis at concentrations that drastically inhibited B. subtilis DNA synthesis (11) and also caused a delay in eclipse and latent periods. 6-(p-hydroxyphenylazo)-uracil (HPUra) is a more specific inhibitor of the host Bacillus subtilis DNA synthesis (4, 5). In contrast with the action of these drugs, furazolidone has been found to affect DNA synthesis more in phage-infected cells than in noninfected ones. In this...
respect it is similar, however, to 5-aminocacidine acting on phage-infected *E. coli* K-112 cells (12).

It is interesting to note that the inhibitory action of furazolidone became more and more prominent when withdrawals of the drug were made at increasing times after the 25th min. Similarly, the inhibitory action of the drug became less and less prominent when the drug additions were made at increasing times after the 25th min. Thus furazolidone was active from about the 25th min onwards, and before this time the furazolidone-sensitive system was either not present or not operative, or it was operative but the damage was recoverable during the subsequent drug-free period. Biochemical analysis revealed that DNA synthesis in phage-infected cells was most vulnerable to furazolidone treatment. It could therefore be that withdrawal of the drug after the 25th min of infection could lead to significant recovery of DNA synthesis, and the earlier it was withdrawn, the greater the recovery. It may be noted in this respect that the recovery of DNA synthesis in noninfected cells of *V. cholerae* subsequent to the withdrawal of furazolidone was very fast (18). Thus the patterns of phage yield may be explicable in terms of drug action of DNA synthesis. Furthermore, the time at which it became effective could imply that it was also interfering with a late stage in the reproduction of infective phage particles (i.e., phage assembly), since it was at about this time that the complete phage particles first appeared within the cells. So far it has not been possible to decide whether furazolidone did or did not have any action on the process of phage assembly.

ACKNOWLEDGMENTS

We are indebted to A. B. Chowdhury, Director, School of Tropical Medicine, for his kind interest in this work. This work was financially supported by a research grant from the Indian Council of Medical Research, New Delhi. Grateful thanks are due to Smith, Kline, and French (India) Ltd., Bombay, for the gift of a sample of furazolidone.

LITERATURE CITED


