INHIBITION OF COLIPHAGE MULTIPLICATION AND R PLASMID TRANSFER BY DESDANINE

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Desdanine inhibited the plaque formation of male-specific coliphages but not that of other coliphages tested. Desdanine also suppressed the multiplication of both RNA phage Qβ and filamentous DNA phage f1 at the concentration of 3.13~6.25 μg/ml which had no influence on the growth of their host cells. However, the inhibitory effect on the phage multiplication was not due to the inactivation of phage particles nor the prevention of phage adsorption and penetration into the host cells. Desdanine also inhibited the transfer of R plasmid, R 100-1, in *E. coli* at 6.25~12.5 μg/ml without affecting the viability of donor and recipient cells.

Infectious process of male-specific phages are similar to the transfer of conjugative plasmids,[1] and several compounds,[3,4,6,8,10] which inhibited the multiplication of RNA coliphages have been examined for prevention of the transfer of R plasmids which carry the drug-resistant determinants and often cause difficulties providing effective chemotherapy against infectious diseases.

During the course of our screening for antibiotics which inhibit the plaque formation of male-specific coliphage Qβ, desdanine,[7] which is a known antibiotic and has been reported to inhibit the synthesis of nucleic acid in *E. coli*,[9] was isolated from the culture broth of *Streptomyces* sp. strain No. B-96936. Further studies showed that the antibiotic possessed inhibitory activity against the transfer of R plasmid, R 100-1 in *E. coli*.

The present paper deals with the effect of desdanine on the multiplication of coliphages and the transfer of R plasmid, R 100-1.

Materials and Methods

Bacterial strains and phages. RNA phage Qβ and filamentous DNA phage f1 and their host *E. coli* W 3110 F^+ were mainly used for phage experiments. *Escherichia coli* JE 2100 carrying R plasmid, R 100-1 which is a derepressed mutant of the R plasmid, R 100 and determines the resistance to tetracycline, chloramphenicol, streptomycin and sulfonamide was used as R donor, and *E. coli* NA 15 which is a nalidixic acid resistant mutant derived from *E. coli* W 3110 and requires aspartic acid, methionine and proline, as R^- recipient.

Media. LENNOX broth (L broth)[5] containing 2 mM $CaCl_2$ with or without agar was used as the medium for phage experiments. Trypticase-soy broth (BBL) was used for R matings and L broth at pH 7.6 for curing experiments. Drug resistance markers were selected on the plates of trypticase-soy agar (BBL) containing 100 μg/ml of chloramphenicol, 50 μg/ml of tetracycline and/or 50 μg/ml of nalidixic acid.

Antiphage activity. A 0.2 ml portion of exponentially growing culture of host cells, having reached an O.D. of 0.25 at 600 nm, was inoculated into 5 ml of soft agar (the medium containing 0.7% agar), and 0.1 ml of diluted phage lysate was added and gently mixed. The mixture was poured on the base agar (the medium containing 1.2% agar) in a plastic dish.

Phage titer of the lysates used was as follows; male-specific phages, T_1, T_2 and T_4, 2×10^5 plaque forming units (PFU)/ml; phages T_7 and ϕX 174, 1×10^4 PFU/ml. Paper discs previously immersed in the solutions of drugs tested were dried in air and placed on the plate which was then incubated at 37°C for 18 hours.

Phage infection. Exponentially growing culture of host cells at a density of $2\sim4\times10^8$/ml was infected with Qβ or f1 at the multiplicity of infection (MOI) of 0.05 at 37°C. After 5-minute standing for phage adsorption, the culture was quickly diluted 10^4-fold into prewarmed medium to avoid subsequent adsorption and was incubated at 37°C. At 80 minutes after infection, the culture was treated with chloroform and diluted appropriately. Phage titer was determined by standard agar overlay technique.

Assay of ribonuclease sensitive step of phage Qβ. The assay or ribonuclease (RNase) sensitive step was carried out by the method of DANZIGER and PARANCHYCH[2] with minor modification. Fifty micrograms of desdanine which was dissolved in 50 μl of methanol was added to 4.9 ml of the exponentially growing culture of host cells, and kept for 5 minutes at 37°C. Then 250 μg of pancreatic RNase (Miles) which was dissolved in 50 μl of sterile distilled water was added to the culture, and further stood for 5 minutes at 37°C. After this treatment, phage Qβ was added to the culture at MOI of 0.001 and incubated for 70 minutes at 37°C. Then the culture was treated with chloroform, diluted and assayed for PFU.

R mating. Overnight cultures of R donor and recipient were mixed in a ratio of 1:9 and incubated at 37°C for 2 hours without shaking. Then the mixture was diluted and spread on selective plates. Colonies resistant to chloramphenicol, nalidixic acid, and both were counted as R^+, recipient phenotype and R^+ conjugants, respectively.

Curing of R 100-1. Overnight culture of *E. coli* JE 2100 in the medium containing 25 μg/ml of chloramphenicol was diluted 10^4-fold with the medium and a 0.1 ml portion of the diluent was inoculated into 5 ml of the medium containing 5 μg/ml of desdanine and shaken at 37°C. Colonies susceptible both to chloramphenicol and to tetracycline in the survivors were counted as cured cells.

Chemicals. Desdanine was purified from the culture broth of *Streptomyces* sp. strain No. B-96936 isolated in our laboratory. The antibiotic was dissolved in 80% methanol and used. Rifampicin, chloramphenicol, nalidixic acid and tetracycline were obtained from Takeda Chemical Industries, Ltd., Sankyo Co. Inc., Daiichi Seiyaku Co. Ltd. and Lederle (Japan) Ltd., respectively.

Results

Effect of Desdanine on the Multiplication of Male-specific Coliphages

In the preliminary experiment, it was found that desdanine inhibited the plaque formation of phage Qβ. Based on the result, antiphage activity of desdanine against other coliphages was examined. As shown in Table 1, desdanine inhibited the plaque formation of male-specific phages, Qβ, f2, MS2 and f1, but not that of ϕX 174, T_1, T_2, T_4, and T_7. This spectrum of antiphage activity of desdanine was similar to that of rifampicin which was used as the control substance. Inhibition of the plaque formation of phages Qβ and f1 is shown in Fig. 1. Inner zone around paper discs represents inhibition of the growth of host cells and outer whitish zone inhibition of the plaque

Table 1. Antiphage spectrum of desdanine against various coliphages.

Inhibitory activity against the plaque forming ability of phages was expressed as the symbol of plus (positive) or minus (negative).

Antibiotic	RNA phage			DNA phage					
	Qβ	f2	MS2	f1	ϕX 174	T_1	T_2	T_4	T_7
Desdanine	+	+	+	+	−	−	−	−	−
Rifampicin	+	+	+	+	−	−	−	−	−

Fig. 1. Effect of desdanine on the plaque formation of coliphages. Upper disc, desdanine; lower disc, rifampicin.

Details are described in the text.

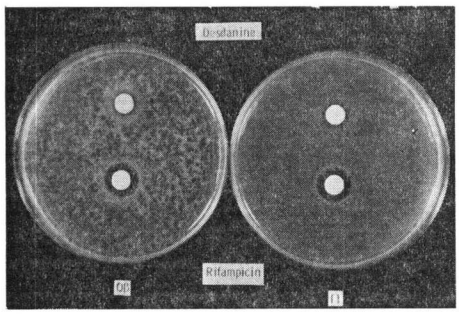


Fig. 3. Effect of addition time of desdanine on the multiplication of phages Qβ and f1.

The infected culture described in Fig. 2 was diluted with prewarmed medium and incubated at 37°C. Desdanine was added at appropriate intervals to give a final concentration of 10 μg/ml.

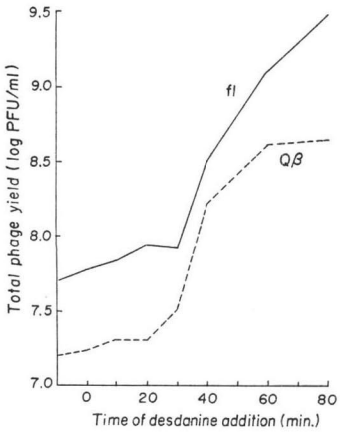


Fig. 2. Effect of various concentrations of desdanine on the multiplication of phages Qβ and f1.

The phages were added at MOI of 0.05 and allowed to adsorb for 5 minutes at 37°C. Then the culture was diluted with prewarmed medium containing a suitable amount of desdanine and incubated at 37°C.

Total phage was assayed as described in Materials and Methods.

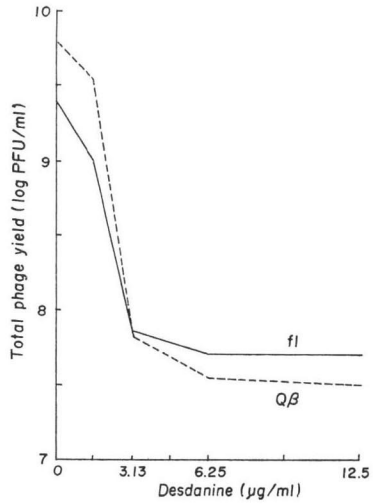


Table 2. Effect of desdanine on the infectivity of phage Qβ in the presence of RNase.

Complete condition consisted of 2×10^8/ml of host cells, 1×10^5 PFU/ml of phage Qβ and 50 μg/ml of RNase in the medium.

Details are described in Materials and Methods.

Condition	PFU/ml ($\times10^3$)	Percent PFU remaining
Complete	4.8	3.69
+Desdanine	5.4	4.15
+Desdanine−Host	130.0	99.85
−RNase−Host	130.2	100.00

formation of the phages.

Desdanine also inhibited the multiplication of Qβ and f1 in liquid medium. Relationship between phage yield and concentration of desdanine is shown in Fig. 2. Total phage yields of Qβ and f1 at 80 minutes after infection were drastically reduced in the presence of 3.13 μg/ml or higher concentration of desdanine. To further examine the effect of desdanine, it was added at varying periods after phage infection. As shown in Fig. 3, inhibitory effect of desdanine on the phage multiplication began to reduce at 30 minutes after infection.

It is known that RNase prevents the penetration of infectious RNA phages into the host cells and gives rise to an eclipse of the viral infectivity.[2,11] Based on this notion, assay of the RNase sensitive step was carried out in the presence of desdanine. Table 2 shows that the eclipse of Qβ infectivity was normally observed whether desdanine was present or not. It appears that desdanine did not prevent the adsorption of the phage particles and penetration

of the phage RNA. Furthermore, PFU of phage Qβ was not reduced when desdanine was added to the medium without host cells. This result also indicates that desdanine did not inactivate the phage particles. In addition, it was confirmed that the inhibitory effect of desdanine on the multiplication of phage f1 was not due to the inactivation of phage particles nor the prevention of phage adsorption (data not shown).

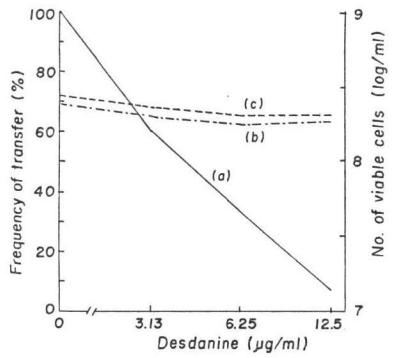
Effect of Desdanine on the Transfer of R Plasmid, R 100-1

In view of the similarity of the infection of male-specific coliphages and the transfer of conjugative plasmids, the effect of desdanine on the transfer of R plasmid, R 100-1 was studied by means of the mating system between *E. coli* JE 2100 and NA 15. In the experiment of Fig. 4, frequency of the transfer of R 100-1 indicated the value of 33.5% at 6.25 μg/ml of desdanine and that of 0.76% at 12.5 μg/ml of it. However, viability of donor and recipient cells was not affected at any concentration tested.

Fig. 4. Inhibition of R 100-1 transfer by desdanine.

Twenty microliters of desdanine solution were added to 0.9 ml of the donor culture in a test tube. Then 0.1 ml of the recipient culture was added to mix.

Frequency of the transfer (*a*) is indicated as percent of R^+ conjugants among chloramphenicol resistant cells (*b*). (*c*) indicates the number of nalidixic acid resistant colonies.



Curing Activity of Desdanine

In order to clarify whether desdanine possessed curing activity of R plasmid, R 100-1 or not, following experiments were performed. As shown in Table 3, cured cells in the survivors of *E. coli* JE 2100 were not increased in the presence of desdanine. In the control experiment, 57.4% of survivors were cured in the presence of 25 μg/ml of ethidium bromide. The result indicated that desdanine did not cure the plasmid at the concentration of 5 μg/ml. However, it was not possible to test at 10 μg/ml or higher concentration of desdanine since *E. coli* JE 2100 did not grow at more than 10 μg/ml of the antibiotic in this experiment.

Table 3. Curing activity of desdanine to R 100-1.

Susceptibility of 100 colonies picked at random was tested on the plate containing tetracycline or chloramphenicol.

Numbers of colonies susceptible to both of them were indicated as the values of percent cured.

Conc. (μg/ml)	Inoculum size	Time of incubation	No. of survivors	Percent cured
0	2.3×10^8/ml	0 hour		0
		24	6.3×10^8/ml	1
		72	1.2×10^8/ml	4
5	2.3×10^8/ml	0		0
		24	6.8×10^6/ml	1
		72	3.4×10^8/ml	0

Since differential inhibitory effect of desdanine on the growth of *E. coli* JE 2100, JE 2100 R^-, W 3110 F^+ and W 3110 F^- was not observed, desdanine might not act by selectively inhibiting the plasmid-bearing cells (data not shown).

Discussion

The results in this paper showed that desdanine possessed inhibitory activity against both the multiplication of male-specific coliphages and the transfer of R plasmid, R 100-1, in addition to the antibacterial activity.

Several compounds which inhibit the multiplication of RNA coliphages and also interfere with the transfer of R plasmids have been reported. Distamycin A,[10] levallorphan[6,8] and requinomycin[3,4] inhibit both phage adsorption and mating pair formation resulting from conformational changes of the cell surface. Unlike these compounds, desdanine did not inhibit both adsorption of phage Qβ and penetration of the phage RNA. Therefore the result suggests that antiphage activity and the inhibition of R 100-1 transfer of desdanine may be different from the effects with these other compounds.

SAEKI *et al.*[9] have recently shown that desdanine inhibited nucleic acid synthesis resulting from the inhibition of nucleoside diphosphokinase in *E. coli*. This finding suggests that desdanine may also interfere with the availability of substrates for nucleic acid syntheses in the infection of male-specific coliphages and in the conjugal transfer of the R plasmid. However, the possibility that desdanine might act at another specific site in the multiplication of coliphages and/or in the conjugal transfer of the R plasmid cannot be ruled out since inhibitory action of desdanine in this report was observed when the growth of *E. coli* was not affected at any concentration of the antibiotic tested.

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