Dye mediated photoinactivation of bacteriophages by nitrogen laser

RUCHI SACHDEVA, N S BHAGWANANI and D S CHITNIS*,†

*Department of Microbiology and Immunology, *Department of Pathology, Choithram Hospital and Research Centre, Indore 452 001, India*

†*Corresponding author (Fax, 91-731-470068; Email, medicine@bom4.vsnl.net.in).*

The nitrogen laser ($\lambda = 337{\cdot}1$ nm) was documented to have photosensitized inactivation of bacteriophages P1 and phage A having *Escherichia coli* and *Salmonella typhi* as their respective hosts. Methylene blue and crystal violet had a direct virucidal effect whereas toluidine blue revealed accentuated lethal effect on photosensitization with N_2 laser for both of the bacteriophages taken in the study. The other dyes such as congo red, neutral red, auramin O and safranine showed differences in their virucidal activity among the two bacteriophages. However, malachite green did not show any change for the two viruses both by itself and on irradiation with the laser. A possibility of photosensitizing effect of N_2 laser for the therapy of viral infections needs to be explored.

1. Introduction

The photodynamic effect of certain compounds on viruses has been documented (Hebeda *et al* 1995). The photodynamic inactivation of viruses such as HSV and SV40 with heterocyclic dyes such as neutral red (Rapp *et al* 1973), methylene blue (Chang and Weinstein 1975) and toluidine blue (Seemayer *et al* 1973) has been reported. The inactivation of viruses in fresh human plasma by visible light in the presence of phenothiazine dyes such as methylene blue and toluidine blue has also been documented in the last few years (Mohr *et al* 1992). There are also reports on the photosensitized killing of *Streptococcus sanguis* on irradiation with He Ne laser in the presence of toluidine blue (Soukos *et al* 1996).

The photodynamic action of dyes such as methylene blue and toluidine blue on bacteriophages was reported earlier (Perdrau and Todd 1933; Clifton and Lawler 1930; Yamamoto 1958). However, there is scarce data on the effect of laser radiations on bacteriophages (Tiphlova and Karu 1989). Our preliminary work on the irradiation of bacteriophage suspensions with N_2 laser did not reveal any virucidal effect of this near UV wavelength laser (personal observation).

The N_2 laser ($\lambda = 337{\cdot}1$ nm) is being used successfully for the treatment of tuberculous pulmonary cavities (Bhagwanani *et al* 1996). We have also reported the *in vitro* bactericidal and fungicidal effects of near UV N_2 laser in our laboratory (Sachdeva *et al* 1995). There being paucity of data on the effect of N_2 laser on viruses, we planned to study the effect of N_2 laser on viruses. The bacterial viruses being easier to handle were selected for the study. The photodynamic action of N_2 laser over bacteriophages was carried out to study the inactivation of viruses on photosensitization using a wide variety of microbiological dyes such as crystal violet, methylene blue, neutral red, toluidine blue, malachite green, auramin O, congo red and safranine.

2. Materials and methods

The N_2 laser with a power output of 5·5 mW was used for the study. The energy density of the laser for irradiation was 5·4 J/cm^2. The N_2 laser used for the study was fabricated by the Centre for Advanced Technology, Indore.

Escherichia coli AB1157 bacteria and its bacteriophage P1 stocks were maintained on Lauria Bertani (LB)

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agar and LB broth respectively. The *E. coli* was grown overnight in LB broth to the late logarithmic phase (5×10^8 cells/ml). Bacteriophage P1 (titre of stock $1\text{–}5 \times 10^9$ PFU/ml) was diluted in phosphate buffered saline (PBS) having pH 7·4 to a titre of 5×10^3 PFU/ml. The phage density was 5×10^3 PFU/ml to allow the penetration of the laser beam through the phage suspension. This was done on the basis of our earlier studies wherein the penetration of 337 nm wavelength light was confirmed to be poor in high density bacterial suspensions (Sachdeva *et al* 1997).

Salmonella typhi bacterial culture and its bacteriophage phage A were maintained on nutrient agar and nutrient broth respectively. The densities of *S. typhi* bacterial culture grown overnight in nutrient broth and phage A diluted in PBS were the same as for *E. coli* and its bacteriophage P1.

One mg/ml stock solutions of the dyes were used for both the bacteriophage P1 and the phage A and the final concentrations of toluidine blue, neutral red, methylene blue, safranine, crystal violet, malachite green, auramin O and congo red were 100 μg/ml for experimentation in all cases. The stock solutions of crystal violet for P1 bacteriophage and malachite green for phage A were 100 μg/ml and 200 μg/ml respectively. The final concentrations of crystal violet for P1 bacteriophage and malachite green for phage A during irradiation were 10 μg/ml and 20 μg/ml, respectively. It needs to be mentioned here that the final concentrations of the dyes in bacteriophage suspensions selected were above their minimum inhibitory concentrations for the respective bacterial hosts.

2.1 *Laser-dye-phage interaction*

The size of the N_2 laser beam was 1 cm^2. Therefore, to ensure the irradiation of the phage suspension, polysterene microwells (diameter = 60 mm) were used. The phage suspensions (120 μl) were irradiated with N_2 laser in the presence of the dye for 20 min. Another microwell at least 3 cm apart from the irradiated well contained the same but non irradiated phage suspension. Another phage suspension diluted identically with PBS instead of the dye was used to study the effect of the dye alone on the bacteriophages. One hundred μl of the irradiated and the control bacteriophage suspensions were transferred to sterile test tubes immediately after 20 min. The tubes were incubated for 10 min at 37°C to allow the bacteriophages to infect the bacteria. Three ml of molten top agar (LB/nutrient soft agar for bacteriophage P1 and phage A respectively) at 56°C was added to each tube, vortexed and plated on the respective agar plates. The number of plaques were counted after incubation at 37°C for 24 h.

2.2 *Statistical analysis*

The significance of the microbiological dyes on either of the bacteriophages was calculated by the paired *t* test using the GB stat programme from Dynamic Systems Inc., Silver Spring, USA. The *p* value for the effect of dye alone on the bacteriophages was calculated by taking the pairs of the two non-irradiated suspensions. The photosensitized inactivation of the bacteriophages was analysed by comparing the phage suspension with dye (dye only) against the irradiated suspension in the presence of the dye (dye + laser).

3. Results

3.1 *Laser-dye-phage interaction*

The effect of various dyes on the two bacteriophages is shown in table 1. Methylene blue itself completely inhibited the bacteriophage P1 at the final concentration of 100 μg/ml ($p < 0.0001$). An inhibitory effect of methylene blue on *S. typhi*-phage A was also observed ($p = 0.0012$). However, the virucidal effect of methylene blue dye on phage A was less pronounced than that observed in case of bacteriophage P1. The N_2 laser irradiation did not accentuate the killing of phage A in the presence of methylene blue ($p = 0.0695$). The number of plaques obtained on the treatment of bacteriophage P1 with toluidine blue was observed to be reduced in the control with the dye. Further, the irradiated dye containing either of the viral suspensions revealed a reduction in plaque counts. The chemically similar neutral red dye had no virucidal activity by itself and even on photosensitization with N_2 laser.

The effect of safranine alone and on photosensitization with N_2 laser on the two bacteriophages was different. The dye by itself had no virucidal effect on P1 bacteriophage but the direct effect was pronounced on phage A ($p = 0.0097$) while on photosensitization with the laser the virucidal effect was significant on the bacteriophage P1 and not on phage A.

The virucidal effect of malachite green and congo red both alone and on photosensitization with N_2 laser was not seen for either of the two phages.

Crystal violet had a direct lethal effect on bacteriophage P1 at a concentration of 10 μg/ml. The effect of crystal violet for phage A was observed at a concentration of 100 μg/ml and its direct effect, though significant, was not as pronounced as that observed with P1 bacteriophage. Further, the photoinactivation of phage A by crystal violet was not significant.

Auramin O had a significant direct virucidal effect only on phage A ($p = 0.0067$), while no significant change in plaque counts of P1 bacteriophage was observed.

4. Discussion

The different dyes exhibited varied effects on the two bacteriophages. The studies on HSV, SV40 and other viruses have revealed that neutral red can photoinactivate viruses on treatment with the dye (Wallis and Melnick 1965) or by growing the viruses in the cells containing dyes (Rapp *et al* 1973). Toluidine blue has been found to produce lesions at the molecular level of the viral RNA resulting in the complete inhibition of MM virus infectivity (Smelt *et al* 1976). The mutagenic effect of toluidine blue (Dunipace *et al* 1992) and increase in the chromosome damage by it (Au and Hsu 1979) has been reported. However, there are also reports which suggest that toluidine blue has no carcinogenic effect on the hamster cheek pouch (Redman *et al* 1992). Further, oral rinsing or direct topical use of 1% toluidine blue solution in humans has no toxic effects (Mashberg 1983). Photooxidation has been proposed to be the mechanism of this inactivation (Spikes and Livingston 1969). The involvement of singlet oxygen in the inactivation of viruses by methylene blue/light has also been documented (MAuller-Breitkreutz *et al* 1995).

The appropriate concentration of the dye is also required to cause the inhibition of viruses with lasers. Crystal violet by itself was inhibitory to the bacteriophage P1 at lower concentrations than those for phage A. The dependence of loss of the biological activity of phages on the chemical dose has been suggested by studies of phage nucleoprotein-psoralen interaction (Ronto *et al* 1992). The psoralen photoreaction with bacteriophages induced by light has been documented (Toth *et al* 1988).

The DNA has also been regarded to be the target for the dye-light inactivation (Simon and Van Vunakis 1962). The photooxidation of guanosine in the DNA occurs in the presence of methylene blue (Saito *et al* 1975). It is possible that the DNA-dye mediated complex is formed by the interaction of the dye with nucleotide bases

Table 1. Effect of different dyes on the viability of bacteriophages.

	Bacteriophage P1 (mean plaque count)				Phage A (mean plaque count)			
Dye	Number of sets	Control (PBS)	Control (dye)	N_2 laser (dye)	Number of sets	Control (PBS)	Control (dye)	N_2 laser (dye)
Methylene blue	8	174·75 ± 39·87 p1 < 0·0001	0·125 ± 0·35	–	5	481·6 ± 112·37 p1 = 0·0012	47·6 ± 29·56	22·4 ± 12·97 p2 = 0·0695
Toluidine blue	8	801·25 ± 116·44 p1 = 0·0098	619·5 ± 183·11	412·63 ± 172·87 p2 = 0·0005	8	240·25 ± 57·29 p1 = 0·9152	237·75 ± 45·63	93·63 ± 44·41 p2 < 0·0001
Neutral red	5	540·8 ± 72·42 p1 = 0·0546	460·2 ± 63·38	424·2 ± 38·53 p2 = 0·0985	4	295·5 ± 32·71 p1 = 0·2516	310·25 ± 26·47	268·5 ± 22·37 p2 = 0·1748
Auramin O	4	579·25 ± 31·5 p1 = 0·312	532·75 ± 75·35	523·75 ± 51·996 p2 = 0·8016	5	205 ± 32·95 p1 = 0·0067	144·8 ± 24·79	178·6 ± 44·56 p2 = 0·1258
Congo red	7	939 ± 137·88 p1 = 0·0644	886 ± 118·58	1026 ± 244·09 p2 = 0·0965	5	141·6 ± 17·69 p1 = 0·2665	132 ± 4·90	131·2 ± 19·99 p2 = 0·9361
Crystal violet	7	178·71 ± 58·1 p1 = 0·0002	1·14 ± 2·04	–	4	239 ± 63·07 p1 = 0·0228	174·75 ± 33·77	191·5 ± 30·74 p2 = 0·2173
Malachite green	5	340·2 ± 61·29 p1 = 0·2143	242 ± 195·48	200·2 ± 175·55 p2 = 0·4721	5	215·4 23·77 p1 = 0·7464	209·4 ± 49·02	187·8 ± 43·83 p2 = 0·4085
Safranine	6	581·67 ± 58·1 p1 = 0·1827	517·17 ± 70·86	203·17 ± 33·42 p2 < 0·0001	6	178·67 ± 21·63 p1 = 0·0097	115·5 ± 33·23	105·5 ± 17·33 p2 = 0·4115

p1 and p2 are the probability values by paired *t* test for control (PBS) compared with control (with dye) and the non-irradiated control (with dye) compared to N_2 laser irradiated suspension of phages in the presence of dye respectively.

resulting in the production of mutagenesis of base substitution type (Gutter *et al* 1977). In case of methylene blue, the photooxidation of gaunosine residues may lead to an excision of base pairs resulting in a single strand break in the virus genome. Methylene blue has been documented to inhibit HIV virus after photosensitization (Bachmann *et al* 1995). This effect is possibly due to its action on the reverse transcriptase and alteration in the size of virus associated proteins and destruction of the viral RNA (Bachmann *et al* 1995). The incorporation of neutral red within the virus structure during replication of the virus has also been proposed to make the virus photosensitive (Crowther and Melnick 1961). However, in our study, neutral red had neither a direct nor a photoinactivation effect against both the bacteriophages taken in the study. The negative effect of the photodynamic inactivation with neutral red on recurrent infections with HSV has been reported (Myers *et al* 1975).

There also lies a possibility of the lysis of bacteriophages on photodynamic treatment with N_2 laser. The photoinactivation of bacteriophage T4 by methylene blue resulting in the damage to both the injection apparatus and the viral DNA has been reported (Kadish *et al* 1967).

The photobiological nature of low level laser on bacteriophage T4-*E. coli* interaction has been reported to require primary photoacceptors in bacterial cells since the bacteriophages lack the specific chromophores for absorption of light (Tiphlova and Karu 1989). Therefore, the dye molecules used in the study may be acting as the photoacceptors to trap the energy of N_2 laser. McGuff and Bell (1966) had hypothesized that the bacterial preparation might absorb an increased amount of laser energy by the incorporation of dyes. The presence of dye such as methylene blue in the broth was suggested to increase the sensitivity of micro-organisms to the effects of laser radiations using ruby laser (Klein *et al* 1965). It was reported by Herczegh *et al* (1971) that the ruby laser light was absorbed by the dye molecule attached to the protein coat of the phage and the release of energy to the protein coat of the phage caused the protein damage.

The present study suggests the direct inactivating effect for both the phenothiazine dyes (viz., methylene blue, toluidine blue) on the bacteriophages used in the study. However, the photosensitization with N_2 laser did not further increase the virucidal effect of methylene blue on phage A. Possibly the chemically similar phenothiazine and phenozine dyes and also the triphenyl methane dyes differ in their virucidal activity against *E. coli*-P1 and *S. typhi*-phage A interactions. Among the other dyes only safranine was documented to have a photosensitized virucidal effect on bacteriophage P1. The molecular mechanism of photosensitization was not studied presently and it needs to be carried out. There exists a possibility that the penetration of the dye into the virus increases on photosensitization, resulting in the accentuation of inactivation of the bacteriophages on irradiation with N_2 laser. The photosensitization observed in some cases could also be due to the interaction leading to protein coat modifications or by the action of nucleic acids.

Therefore, to conclude, the extent of the virucidal activity of the different dyes varies among the species of the bacterial viruses. The preliminary work on the bacteriophage and the UVA wavelength N_2 laser interaction suggests the virucidal effect of certain dyes. Further, the absorption of the dyes at 337 nm may not be the only deciding factor for the interaction of the dye with the phage. The work needs to be extended further as this approach could open new avenues for the use of the modality in therapeutics for the photodynamic inactivation of viral infections.

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