CHAPTER - 6

Vibriocidal action of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and mitomycin C (MC) on V. cholerae
INTRODUCTION

There are number of documented reports about the bactericidal action of N-methyl-N' -nitro-N-nitrosoguanidine (NTG) and mitomycin C on some micro-organisms (Adelberg et al., 1965; Megnet, 1965; Yanagisawa et al., 1967; Sinha, 1970; Metzer et al., 1974; Shiba et al., 1959; Sekiguchi and Takagi, 1960; Iijima and Hagiwara, 1960; Reich et al., 1961; Tsukamura and Tsukamura, 1962). However, relatively little is known about the vibriocidal activity of these two mutagens on V. cholerae.

In view of the above the present study was undertaken to determine the vibriocidal action of NTG and mitomycin C on V. cholerae on a comparative basis.
MATERIALS AND METHODS

Strain: A rabbit passaged *V. cholerae* Inaba strain 569B, obtained from Dr. N.K. Datta of Haffkine Institute, Bombay, was used throughout this study.

Media: Dehydrated Difco Bacto-nutrient broth and nutrient agar medium were used. Minimal medium used was as described by Braun (1965). Nutrient and minimal agar medium were prepared by adding 2% agar in liquid medium, pH adjusted to 7.6.

Mutagens: N-methyl-N’-nitro-N-nitrosoguanidine (NTG) of Aldrich Chemical Co. Inc. Milwaukee, Wisconsin, U.S.A. and Mitomycin C (MC) of Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan, were used. Fresh solutions in sterile distilled water were made for each use. The solutions were checked for sterility by streaking samples on nutrient agar.
Determination of vibriocidal effect of NTG on *V. cholerae* in nutrient broth.

A nutrient broth culture containing an average number of $6.4 \times 10^7$ cells / ml. (counted by means of viable count) was taken. The bacterial culture was divided in equal proportions in 10 ml. aliquots in 125 ml. Erlenmeyer flasks. Freshly prepared solutions of NTG were added to the culture samples to give the desired concentrations. Concentrations of NTG was different in different sets of experiment. Each experiment was done with an appropriate control. The treated samples were uniformly shaken with the Vortex mixer and were incubated at 37°C. Viable counts from treated and control samples were made at intervals (shown in fig. 6.1), according to the method described by Cruickshank (1965).

Determination of vibriocidal effect of NTG on *V. cholerae* in minimal medium (Braun, 1965).

A sample (0.5 ml) of an eighteen hour nutrient broth culture was added to 20 ml. of freshly prepared nutrient broth in a 125 ml. Erlenmeyer flask and incubated at 37°C with shaking until the culture attained 5 to $8 \times 10^9$
cells / ml. The culture was washed by passing through millipore filter (pore size 0.22 μ) and rewashed with sufficient quantity of minimal medium. Millipore filter membrane which retained the bacterial culture on its surface was suspended in 20 ml. of liquid minimal medium in a 125 ml. Erlenmeyer flask and diluted with fresh liquid minimal medium to give an average final concentration of 7.5 x 10^6 cells / ml. Vibriocidal activity in minimal medium was determined according to method, described previously in this chapter.

**Determination of vibriocidal effect of Mitomycin C on V. cholerae in nutrient broth.**

A nutrient broth culture containing an average number of 4.5 x 10^9 cells / ml. was taken. Higher number of cells were taken as because our trial experiments revealed that an average number of 10^6 cells / ml. present in a culture, were killed by mitomycin C at a concentration of 0.5 μg/ml. in about 2-3 minutes. Vibriocidal activity of mitomycin C was determined according to the same method described earlier in this chapter.
RESULTS

Vibriocidal effect of NTG on V. cholerae in nutrient broth.

Vibriocidal effect of NTG on V. cholerae in nutrient broth is shown in Figure 6.1. It can be seen from the graph that NTG even at concentrations of 5 and 15 /µg/ml. exerted significant killing effect on V. cholerae. NTG at a concentration of 25 /µg/ml. killed about 100% of the vibrios within 60 minutes of treatment. Complete killing of the vibrios in nutrient broth was also observed within 10 minutes of treatment at a concentration of 50 /µg/ml of NTG.

Vibriocidal effect of NTG on V. cholerae in minimal medium.

Vibriocidal effect of NTG on V. cholerae in minimal medium of Braun (1965) at pH. 7.6 is shown in Figures 6.2 and 6.3. It was observed that the V. cholerae strain 569B grew relatively less appreciably in minimal medium at least for the first 60 minutes than that in nutrient broth. NTG at a concentration of 25 /µg/ml. killed about 16% of vibrios in 10 minutes. 50% survival of vibrios was affected in about 40 minutes of treatment.
NTG at a concentration of 40 \(\mu\)g/ml. killed 100% of the organisms within 60 minutes of incubation. NTG at a concentration of 50 \(\mu\)g/ml. within 20 minutes caused 100% killing.

Vibriocidal effect of mitomycin C on *V. cholerae* in nutrient broth.

Vibriocidal effect of mitomycin C on *V. cholerae* in nutrient broth is shown in Figures 6.4 and 6.5. It can be seen from the graphs that mitomycin C exerted a drastic effect on the viability of *V. cholerae* in nutrient broth culture. 50% survival of vibrios was affected in about 60 minutes of treatment at a concentration of 0.1 \(\mu\)g/ml. Mitomycin C at a concentration of 0.5 \(\mu\)g/ml. killed about 100% of the vibrios within 60 minutes of treatment. Complete killing of the vibrios in nutrient broth was also observed within 10 minutes of treatment at a concentration of 1 \(\mu\)g/ml. of mitomycin C.

Vibriocidal effect of mitomycin C on *V. cholerae* in minimal medium.

Vibriocidal effect of mitomycin C on *V. cholerae* in minimal medium of Braun (1965) at pH 7.6 is shown in Figures 6.6 and 6.7. It was observed that mitomycin C also exerted pronounced killing effect on *V. cholerae* in minimal
Mitomycin C killed about 50% of the vibrios in about 60 minutes of treatment at a concentration of 0.1 μg/ml. A concentration of 0.5 μg/ml caused 100% loss of viability within 80 minutes of treatment. Mitomycin C at a concentration of 1 μg/ml killed the total bacterial population in minimal medium culture within 10 minutes of treatment.

DISCUSSION

The mutagens NTG and mitomycin C have been reported to be lethal as well as mutagenic for a variety of microorganisms (Adelberg et al, 1965; Megnet, 1965; Yanagisawa et al, 1967; Sekiguchi and Takagi, 1960; Iijima and Hagiwara, 1960). However, not much is known about their bactericidal action on *V. cholerae*. The results of the present study show that NTG even at concentrations of 5 and 15 μg/ml have exerted marked killing effect on *V. cholerae* in nutrient broth. Total elimination of the vibrio population in broth was observed after treatment with NTG at the concentrations of 25 and 50 μg/ml within sixty and ten minutes respectively. Our previous experience had shown that NTG at
concentrations of 5 and 15 \( \mu \text{g/ml} \) had no significant effect on the viability of \textit{V. cholerae} in minimal medium (unpublished data). However, it appears from the present study that NTG at a concentration of 25 \( \mu \text{g/ml} \) killed about 83\% of vibrio cells in minimal medium culture with in 60 minutes of treatment. 50\% survival of vibrios in minimal medium was affected with a treatment of NTG at a concentration of 25 \( \mu \text{g/ml} \) in round about 40 minutes time. In minimal medium a treatment with 50 \( \mu \text{g/ml} \) of NTG totally wiped out the vibrio population with in 20 minutes. However, from the data recorded in this present study it also appears that the vibriocidal activity of NTG was comparatively more drastic in nutrient broth than that in minimal medium, possibly the vibrio cells grew more appreciably in the former medium. Recently, Finkelstein \textit{et al} (1974) observed 97\% killing of vibrio cells with a treatment of 100 \( \mu \text{g/ml} \) of NTG for 30 minutes in Tris-maleic buffer. High and variable lethal activity of NTG has also been recorded by several other workers in other micro-organisms too. Adelberg \textit{et al} (1965) observed 95\% killing in \textit{Escherichia coli} after a treatment with 1 mg. NTG/ml. for 30 minutes in Tris-maleic buffer. In \textit{Schizosaccharomyces pombe}, there was only 20\%
survival after an exposure to 2 mg. NTG/ml. for 30 minutes (Magnet, 1965). Clutterbuck and Sinha (1966) found that the viability in Aspergillus nidulans was 1.3% after the treatment of the conidia with 0.5 mg. NTG/ml. for 30 minutes. Moore (1969) found that Coprinus lagopus was highly sensitive to NTG and after treatment with only 5 μg. NTG/ml. for 30 minutes the survival was 53%. Yanagisawa et al (1967) observed that 1.0 mg. NTG/ml. killed 80-90% of the Myxamoebae of Dictyostelium discoideum in 20-30 minutes in presence of 0.05 M potassium phosphate buffer. Sinha (1970) recorded 12.3% survival in Dictyostelium discoideum spores after an exposure to 1.0 mg. NTG/ml. for 30 minutes. Metzer et al (1974) reported that a growing culture of the group H challis strain of Streptococcus when exposed to 0.1 μg. NTG/ml. for 30 minutes at 37°C, caused about 99% killing.

In this present investigation mitomycin C (MC) has been found to exert more pronounced vibriocidal effect on V. cholerae than that of NTG. It was observed with interest that mitomycin C, even at very low concentrations had rapid and extensive killing effect on V. cholerae either in nutrient broth or in minimal medium. In nutrient
Mitomycin C at a concentration of 0.5 μg/ml resulted in the loss of 100% viability within 60 minutes of treatment. About 50% survival of vibrios was affected in about 60 minutes of treatment at a concentration of 0.1 μg/ml of mitomycin C. In minimal medium 100% of the vibrios were also killed by mitomycin C at a concentration of 0.5 μg/ml within 80 minutes of incubation. Mitomycin C killed about 50% of the vibrios in minimal medium culture in about 60 minutes of treatment at a concentration of 0.1 μg/ml. Mitomycin C at a concentration of 1 μg/ml completely wiped out the vibrio population within 10 minutes in both nutrient broth and minimal medium. An analysis of the above results indicate that the lethal activity of mitomycin C either in nutrient broth or in minimal medium was not much significantly different. Mitomycin C also been found to be bactericidal to mycobacteria (Tsukamura and Tsukamura, 1962) and E. coli (Shiba et al, 1959; Sekiguchi and Takagi, 1960; Iijima and Hagiwara, 1960; Reich et al, 1961). Studies of Suit et al (1967) showed that incubation with mitomycin C (5 μg/ml.) brought about rapid and extensive killing of strain, E. coli C thy-321 after about 20 minutes. Mitomycin C at a concentration of 20 μg/ml, has been found to exert lethal effect.
even on *Micrococcus radiodurans*, a highly ionizing radiation resistant, pigmented non-sporing bacterium (Moseley, 1967).

The comparative data recorded in the present study indicates that both the mutagens N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and mitomycin C (MC) under the conditions of experiment tried have exerted extensive vibriocidal effect on *V. cholerae*. It is also of worth mentioning that the vibriocidal action of mitomycin C on the *V. cholerae* strain tested was comparatively more drastic than that of NTG. In other words sensitivity of *V. cholerae* strain to mitomycin C was found to be greater that that towards NTG.
Fig. 6.1.

Vibriocidal effect of NTG on V. cholerae in nutrient broth.

- Untreated culture, Conc. of NTG: ●● = 5 μg/ml,
- ▲▲ = 15 μg/ml, ○○ = 25 μg/ml, ■■ = 50 μg/ml.
Fig. 6.2 shows vibriocidal effect of NTG on *V. cholerae* in minimal medium.

- ×× = Untreated culture,
- ●● = 25 μg/ml,
- ▲▲ = 40 μg/ml,
- ○○ = 50 μg/ml.

Fig. 6.3 shows vibriocidal effect of NTG on *V. cholerae* in minimal medium and percentage of killing.

- ×× = Untreated culture,
- ●● = 25 μg/ml of NTG.
Fig. 6.4 shows vibriocidal effect of mitomycin C on *V. cholerae* in nutrient broth.

- $\times\times$ = Untreated culture,

Conc. of mitomycin C:
- $\bullet\bullet$ = 0.1 $\mu$g/ml.,
- $\bigcirc\bigcirc$ = 0.5 $\mu$g/ml.,
- $\Delta\Delta$ = 1 $\mu$g/ml.

Fig. 6.5 shows vibriocidal effect of mitomycin C on *V. cholerae* in nutrient broth and percentage of killing.

- $\times\times$ = Untreated culture,
- $\bullet\bullet$ = 0.1 $\mu$g/ml. of mitomycin C.
Fig. 6.6 shows vibriocidal effect of mitomycin C on *V. cholerae* in minimal medium.

\[ \times \times \text{ = Untreated culture,} \]

Conc. of mitomycin C:
- \( \bullet \bullet = 0.1 \text{ ug/ml.} \)
- \( \Delta \Delta = 0.5 \text{ ug/ml.} \)
- \( \circ \circ = 1 \text{ ug/ml.} \)

Fig. 6.7 shows vibriocidal effect of mitomycin C on *V. cholerae* in minimal medium and percentage of killing.

\[ \times \times \text{ = Untreated culture,} \]

\[ \bullet \bullet = 0.1 \text{ ug/ml. of mitomycin C.} \]