CURING OF R-PLASMIDS WITH RIFAMPICIN

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SUMMARY

The in vitro curing (elimination) of R–plasmids in E. coli K-12 by subinhibitory concentrations of rifampicin is presented. The frequency of curing was found to be higher in exponential phase than lag or stationary phases of growth. Combination of a curing dose of rifampicin and non-inhibitory concentrations of trimethoprim or trimethoprim and ampicillin resistant E. coli K–12 strains. Rifampicin induced the loss of the R–plasmid in these strains thereby enabling the cured cells to be susceptible to the second antibacterial agent.

INTRODUCTION

Bacteria resistant to multiple unrelated antibacterial agents are often found to harbour their resistance genes on extra-chromosomal DNA elements (R–plasmids). In several instances these extra-chromosomal DNA elements can be transferred en bloc to sensitive bacteria of the same or different species (1, 2). This infective nature of R–plasmids has been shown to account for the resistance of some bacterial species to several antibacterial agents (3, 4, 5, 6) in clinical infections. It is remarkable however that R–plasmid mediated resistance to rifampicin has not been reported. The conversion of a resistant strain to a sensitive phenotype could probably overcome the increasing problem of resistance to antibacterial chemotherapy. However, this phenomenon has to be subjected to detailed clinical investigation before its value in antibiotic therapy can be assessed. The basis of this idea hinges on the observation that this method is not entirely new since some R–plasmids of *E. coli* and *Shigella flexner* have been spontaneously lost in situ and that the frequency of such loss could be increased by acridine dyes (7, 1, 3). Other factors which have been shown to cause the elimination of R–plasmids and sex-factors are treatment of *E. coli* with sodium dodecyl sulphate (8) A similar reaction was observed when *Klebsiella aerogenes* was treated with inhibitors of dihydrofolate (9) and thymidylate synthetase (10).

Rifampicin has earlier been reported to cure penicillinase producing plasmid from *Staphylococcus aureus* (11) and F'lac-plasmid from *E. coli* (12). This study scales to confirm the in vitro curing ability of R–plasmids conferring antibiotic resistance in *E. coli* with rifampicin.

MATERIALS AND METHODS

Bacterial strains: The bacterial strains used in this work were strains of E. coli k–12 and a clinical *E. coli* isolate. (Table 1).

Media: Diagnostic sensivity test agar, DST (Oxoid), nutrient broth, No. 2 (Oxoid), Davis & Mingioli (13) minimal medium, Eosin methylene blue (EMB) sugar agar., L. broth:

Difco Tryptone, 10 G; Difco Yeast Extract, 5G; Nacl, 10G; distilled water to 1 litre, pH 7.2.

Antibacterial agents: Rifampicin (Rif.), trimethoprim (Tp), nalidixic acid (Nal), and nitrofurantoin (NT) were purchased from Sigma Chemical Co. England. Ampicillin (Ap) and streptomycin (Sm) were of Glaxo Laboratories, England.

Determination of antibiotic minimum inhibitory concentration (M.I.C.)

About 10^5 – 10^6 colony forming units/ml of normal saline dilutions of overnight test cultures were spotted onto series of overdried DST agar plates containing progressively increasing concentrations of the test antibacterial agent. The plates were incubated at 37°C for about 24h and observed for growth. The lowest concentration of the antibacterial agent preventing visible growth was regarded as the M.I.C.

Curing of R–plasmids with rifampicin: 1 ml overnight culture of R^+ cells were subcultured in 10 ml nutrient broth containing the appropriate antibacterial agent to which the bacteria were resistant and grown to early exponential phase. The culture was decimally diluted to 10^{-5} in broth containing varying concentrations of rifampicin and incubated at 37°C. At time intervals, suitably diluted aliquots were plated on Lactose Eosin methylene blue agar plates. All the colonies on the plates were tested for their antibiotic resistance characters, by replica-plating on DST agar plates containing streptomycin (20 ug/ml); nitrofurantoin (30 ug/ml), gentamicin (10 ug/ml), ampicillin (20 ug/ml) and trimethoprim (10 ug/ml) respectively. Davis & Mingioli (13) minimal agar supplemented with trimethoprim (10 ug/ml) was also employed.

Infection with R–plasmids: The method of (6) was used.

Environmental effects on the curing of R–plasmids with refampicin.

Effect of pH of the growth medium was determined by innoculating 10^3 cfu/ml of R^+ cells onto nutrient broth at different pH values, Effect of growth medium on the efficiency of curing was determined using Davis & Mingioli (13) minimal medium, L–broth and nutrient broth.

Reversion of cured cells: Cured cells were tested for reversion to resistance by inoculating 10^3 onto plates with 10 ug and 20 ug nitrofurantoin/ml or 10 ug and 20 ug gentamicin/ml respectively.

Growth determination: Relative growth rates of R^+ and R– cells in the presence and absence of subinhibitory concentration of rifampicin (2.5 ug/ml) were determined by diluting overnight cultures in nutrient broth to approximately 10^5 cells/ml. Aliquots from each culture were taken, suitably diluted and viable counts determined in triplicate for each determination by the "spread plate" method.

Effects of combination of subinhibitory concentrations of rifampicin and ampicillin or trimethoprim on ampicillin or trimethoprim resistant strains.

Overnight test cultures were respectively diluted to about 10^3 c.f.u./ml in nutrient broth containing subinhibitory concentrations of rifampicin and varying concentrations of ampicillin. Supplemented glucose minimal media was used for the growth of trimethoprim treated cells. The antibiotic mixtures were also added to the growing cultures at log phase of growth. The cultures were incubated at 37°C and the increase in optical density at 600 nm using a Bausch and Lamb spectronic 20 spectrophotometer.

RESULTS

M.I.C. determinations: The M.I.C. of rifampicin on J62-1, J62-1(EO^{10}) and J62-1(JR225) was 3.75 ug/ml after three consecutive determinations. Ampicillin, nitrofurantoin, trimethoprim and nalidixic acid had MIC values of 5 ug/ml on the appropriate sensitive strain. Gentamicin and streptomycin had an M.I.C. value of 1 ug/ml on the appropriate sensitive strain. Resistance of J62-1(EO_{10}) to nitrofurantoin, trimethoprim and streptomycin was, respectively, to more than 60, 1000 and 50 ug/ml. Resistance of J62-1(JR225) to gentamicin and ampicillin was, to more than 80 and 1000 ug/ml respectively.

Curing of R–plasmids with rifampicin: Depending on the concentration of rifampicin, culture inoculum and the growth phase of the R^+ cells, loss of drug resistance in the test R^+ cells was observed at high frequency (Table II). Curing was observed between 4 to 6 h of treatment and was completed in mid-log phase of growth of the cells. As the incubation time increased after 6h the frequency of R–plasmid curing was observed to decrease. A subinhibitory concentration of rifampicin (2.5 ug/ml) cured the R–plasmids but higher frequencies of curing were observed as the concentration of rifampicin was increased (Table II). There was no appreciable killing of the cells by 2.5 ug rifampicin/ml (the used experimental curing concentration) within 8h. (Fig. 1).

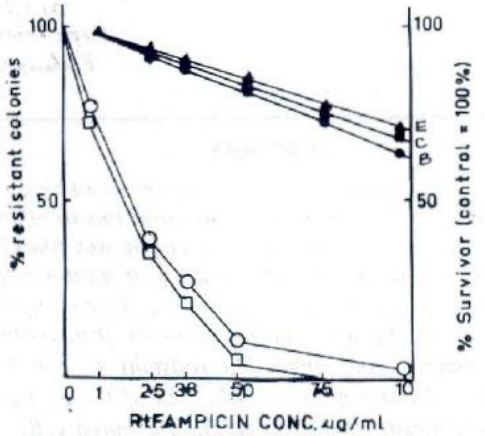

Fig 1. Rifampicin curing of J62–1(EO 10) and J62–1 (JR225) after 6 h treatment.

E. J62–1(EO_{10}); *, J62–1; *, J62–1(JR225);
F. J62–1(EO_{10}); *, J62–1(JR225)

Formation fo auxotrophic and/or rifampicin resistant mutants were not observed. Antibacterial susceptibility patterns of the parent host susceptible cell thus obtained after the curing of the resident R–plasmid was stable after many generations. No R+ revertant was obtained from the cured cells after several subculturing. The cured cells completely lost their R–plasmid determined resistance characters. The cured cells could be reinfected by R–plasmid, e.g. EO_{10} with the same frequency of about 68–72° as obtained with J62-1 recipient.

There was no difference in the frequencies of curing at pH 6.5 to pH 8.0 (Table III). Curing of R–plasmid in minimal medium and L–broth was not significantly different from those obtained from nutrient broth (Table IV).

Effect of combination fo R^+ resistant antibiotics with curing concentrations of rifampicin.

Figs. 2 & 3 show the combined action of 2.5 ug rifampicin/ml plus varying concentrations of ampicillin compared to the effect of 2.5 ug rifampicin/ml on an R–plasmid mediated ampicillin resistant strain. The results show that the combination of rifampicin and ampicillin inhibited ampicillin resistant strain and there was no appreciable inhibition of the growing cells by 2.5 ug rifampicin/ml. Inhibition of the ampicillin resistant strain was more remarkable when the antibiotics combination was added at exponential phase than at lag phase of growth. Aliquots of cultures taken after 2h of growth indicated that the cells have lost their R–plasmid mediated resistance spectra. The percentage frequency of the formation of antibiotic-susceptiole colonies was on the average 73% per hour of rifampicin contact on initiation of curing.

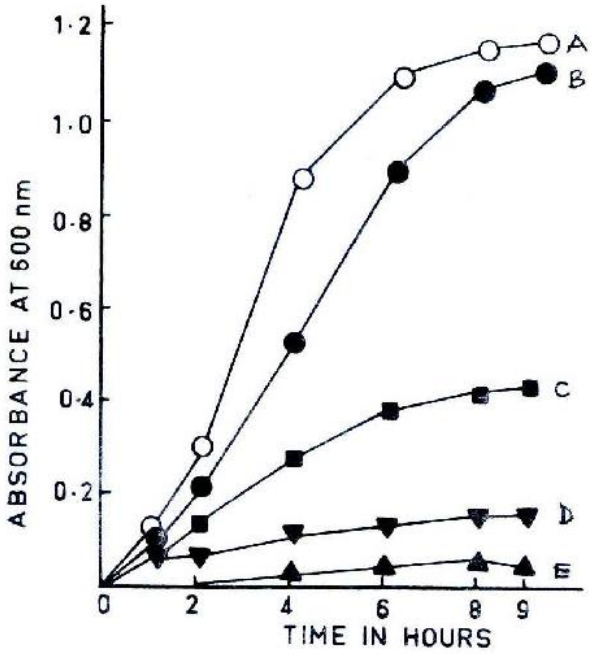

Fig. 2. Effect of rifampicin and the combined effects of rifampicin plus varying concentrations of ampicillin at lag phase of growth of ampicillin resistant E.coli K–12 J62–1(JR225).

A. culture (ca.2.5×10^3 cells ml) without antibiotics,
B, culture plus 2.5 ug Rif.ml/ml
C. culture plus 2.5 ug Rif.ml/ml and 5 ug Amp. ml/ml.
D. culture plus 2.5 ug Rif,ml/ml and 10 ug amp. ml/ml.
E. culture plus 2.5 ug Rif. ml/ml and 20 ug Amp. ml/ml.

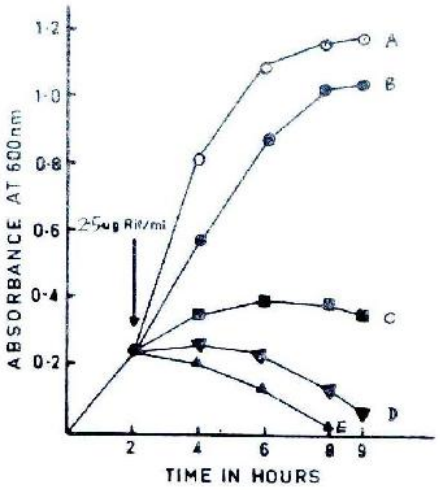

Fig. 3. Effect of rifampicin and the combined effects of rifampicin plus varying concentrations of ampicillin at exponential phase of groth of ampicillin resistant E. coli K–12 J62–1(HW15)

A. culture without antibiotic.
B. cutlure plus 2.5 ug Rig.ml/ml
C. culture plus 2.5 ug Rif. ml/ml and 5 ug Amp. ml/ml
D. culture plus 2.5 ug Rif, ml/ml and 10 ug Amp. ml/ml
E. culture plus 2.5 ug Rif. ml/ml and 20 ug Amp. ml/ml

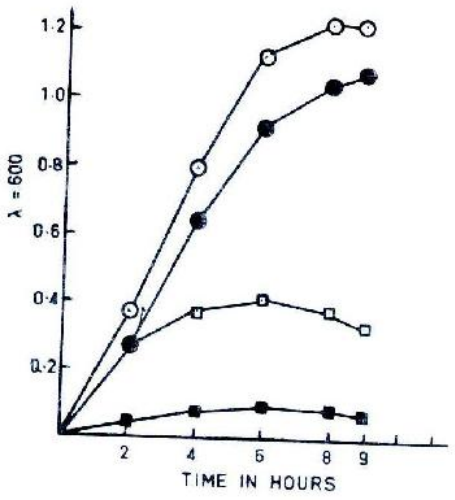

Table 4. Effect of Rifampicin and the combined effect of rifampicin plus verying concentrations of trimethoprim at lag phase of grwoth of trimethoprim resistance E. coli K–12 J62–1(EO_{10})

A. culture (ca 2.5×10^3 cells ml) without anitbiotic
B. culture plus 2.5 ug Rif.ml/ml
F. culture plus 2.5 ug Rif.ml/ml and 10 ug Tp. ml/ml.
C. culture plus 2.5 ug Rif. ml and 20 ug Tp. ml/ml.

Figs. 4 and 5 indicate that although J62-1(EO_{10}) was initially resistant to trimethoprim, the presence of the curing concentration of rifampicin enabled trimethoprim to inhibit the cells. Inhibition at exponential phase of growth was more remarkable than at lag phase. The percentage frequency of the formation of antibiotic susceptible colonies was about 71% per hour of rifampicin contact on initiation of curing.

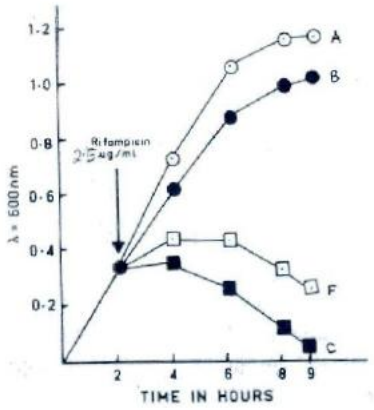

Fig. 5. Effect of Rifampicin and the combined effect of rifampicin plus varying concentrations of trimethoprim at exponential phase of growth of trimethoprim resistance E. coli K–12 J62–1(J62–1(EO10).

A. culture without antibiotic.
B. culture plus 2.5 ug Rif.ml/ml
F. culture plus 2.5 ug Rif.ml/ml and 10 ug Tp. ml/ml.
C. cutlure plus 2.5 ug Rif. ml/ml and 20 ug Tp. ml/ml.

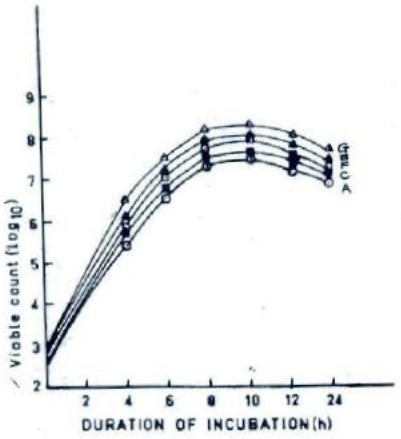

Fig 6. Treatment of E.coli K–12 J62–1 and J62–1 (EO_{10}), with 2.5 ug Rif.ml–1.

G. viable count of J62–1 without rifampicin.
E. viable count of J62–1 with rifampicin.
F. viable count of J62–1(EO_{10}) without rifampicin.
C. viable count of J62–1(EO_{10}) with rifampicin.
A. viable count of cured strain of J62–1(EO_{10}).

Growth of cultures:

Fig. 6 shows the viable counts of J62-1(EO_{10}) and J62 on treatment with 2.5 ug rifampicin/ml in nutrient broth 37°C. The curing concentration of 2.5 ug rifampicin/ml ha no appreciable effect on the survival of the organisms.

DISCUSSION

Rifampicin is a drug that has been principally used for tl treatment of mycobacterial infections (14). However, this pr dominant use is gradually eroding (15) and its value as a antistaphylococcal agent is being highly appreciated (16 Rifampicin (14) in combination with other agents such erythromycin, gentamicin, lincomycin, have been reported effective antistaphylococcal agents (17, 18). The curing R–plasmids by rifampicin requires actively growing cel hence from the results obtained, decreasing numbers of cure cells were found as the culture was approaching stationary an declining phases of growth. Results of reversion experiment with the cured cells and of reinfection of the cured cells wit R–plasmids showed that the cured cells obtained by th rifampicin treatment have totally lost their R–plasmid mediated resistance determinants but were still able to mat with R+ cells. Recently, rifampicin was suggested to mediat the segregation of an R–plasmid in chromosomally mediate rifampicin resistant mutant recipient often transferred as unit when isogenic recipients with chromosomal nalidixi acid or streptomycin resistance markers were used (19). I this work, rifampicin was found to completely cure the tes R–plasmids. The use of a combination of sublethal concen tration of rifampincin with antibiotics whose resistance ar R–plasmid mediated could require further investigation i *in vivo* survey. The results so far obtained in this study indicate that a minimum curing dose of rifampicin is firstly required to eliminate R–plasmids and make the host cell susceptible to antibiotics to which they are hither-to resis tant. This inhibition does not seem to be due to synergism between rifampicin and the second antibacterial agents in th combination in this study. The results indate that the suscepti bility could be due to the elimination of the plasmids b rifampicin at a high rate of about 70% per hour after curin has started.

The mode of action of rifampicin curing of R–plasmids i not yet understood. Rifampicin is an inhibitor of RNA poly merase (19, 12), it could be suggested that a minimal curin does of rifampicin inhibits R–plamid synthesis of RNA with out affecting that of the host cell with a consequent loss o the R–plasmid. This seems to be so because the rifampici curing was most appreciated when the cells were activel growing, and rifampicin does not interfere with bacteria cellwall or protein synthesis.

ACKNOWLEDGEMENT

The receipt of University of Benin staff research gran is acknowledged.

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