Lethal Effect of Rifampicin in *Bacillus subtilis* as a Complicating Factor in the Assessment of the Lifetime of Messenger Ribonucleic Acid

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The bactericidal action of rifampicin was compared with that of chloramphenicol in growing and in sporulating cultures of *Bacillus subtilis* 168. Chloramphenicol kills cells only very slowly, but exposure to rifampicin kills over 95% of cells in a few minutes, causing gross physical damage, which is visible in both phase-contrast and electron microscopy. This is accompanied by a fall in O₂ consumption and by lysis. Experiments with synchronized cultures showed that susceptibility to the lethal effect of rifampicin is greater when the cells are dividing. The results suggest that the synthesis of some species of RNA other than mRNA may be necessary for the maintenance of cell integrity, although experiments with actinomycin D do not altogether fit this interpretation. However, we conclude that rifampicin is too toxic to use as an antibiotic for assessing the lifetime of mRNA.

The degree of stability of the mRNA produced in bacterial sporulation will obviously affect the importance attributed to translational control of the process. When actinomycin D was used as an inhibitor of RNA synthesis conflicting results were obtained. Balassa (1963) and Szulmajster *et al.* (1963) reported that the drug inhibited spore development in *Bacillus subtilis*, whereas Aronson & del Valle (1964) working with *Bacillus cereus* and Sterlini & Mandelstam (1969) with *B. subtilis* found that spore development could continue for some time after addition of the drug. The two latter groups concluded that relatively long-lived messenger was involved in sporulation.

However, actinomycin D is known to have secondary toxic effects (see, e.g., Acs *et al.*, 1963; Korn *et al.*, 1965; Laszlo *et al.*, 1966). Because of this Leighton & Doi (1971) re-examined sporulation using rifampicin, which is generally considered to be a specific inhibitor of RNA polymerase in *Escherichia coli* (Hartmann *et al.*, 1967; Wehrli & Staehelin, 1971) and in *B. subtilis* (Geiduschek & Sklar, 1969). Leighton & Doi (1971) found that the drug rapidly inhibited sporulation events, including the production of alkaline phosphatase. Further, they isolated a rifampicin-resistant mutant containing an altered RNA polymerase that functions normally at 37°C but is inactivated at 50°C. They showed that transferring the mutant cells to the higher temperature and thereby stopping RNA synthesis had the same inhibitory

effect on sporulation events as rifampicin had in the wild type. They concluded that continuous RNA synthesis was necessary during sporulation and that there was no reason to suppose that the mRNA produced during sporulation was any more stable than that found during vegetative growth.

In the course of attempting to repeat the work of Leighton & Doi (1971) we noticed that, after treatment with rifampicin for about an hour, the cells when viewed by phase-contrast microscopy were obviously damaged and much cell debris was evident. This indicated that rifampicin might be having deleterious side-effects of an unexpected sort and we therefore decided to investigate the effects of this drug on viability.

If rifampicin specifically inhibits the DNA-directed RNA polymerase it would stop protein synthesis very quickly by inhibiting the formation of new mRNA. (This is, of course, on the assumption that most of the mRNA in the cell is short-lived). The drug would at the same time stop the formation of ribosomal and transfer RNA, but cells already inhibited through lack of protein synthesis should have no immediate need of these. One would therefore expect that rifampicin would, at least in short time-exposures, be no more toxic than any inhibitor of protein synthesis. For these reasons we compared its effect on cell viability with that of chloramphenicol which is predominantly bacteriostatic rather than bactericidal (Hahn, 1967). We found very marked differences in cell survival after treatment with these two antibiotics, and the results suggest that continuing RNA synthesis is necessary for cell survival in some

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way not connected with the formation of mRNA. It is also apparent that cells in which RNA synthesis is prevented are so damaged that no reliable conclusion can be drawn about their biochemical potentialities.

Experimental

Materials

Chemicals. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Chloramphenicol and actinomycin D were from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Rifampicin was a gift from Dr. R. J. White, Lepetit, Milan, Italy.

Organisms. *Bacillus subtilis* 168 (trpC2) was used, which grows and sporulates normally in media supplemented with indole or tryptophan. *B. subtilis* ts-134 was also used. This has a requirement for thymine and is temperature-sensitive for initiation of chromosome replication. At 45°C any chromosome replication that has begun will be completed, but no fresh replication is initiated. At the permissive temperature (37°C) replication and growth are normal (Mendelson & Gross, 1967). We are indebted to Dr. R. Doi for sending his strain of *B. subtilis* (Marburg) and a temperature-sensitive rifampicin-resistant strain (ts-3).

Media. Glutamate minimal medium was the 'resuspension' medium described by Sterlini & Mandelstam (1969) and used by them to induce sporulation. CH medium was a 'rich' medium containing hydrolysed casein and inorganic ions (Sterlini & Mandelstam, 1969). All media were supplemented with L-tryptophan (20 µg/ml) and, when mutant ts-134 was used, with thymidine (20 µg/ml).

Methods

Growth and sporulation conditions. Cells were cultivated with shaking at 37°C. Growth was measured spectrophotometrically by using a calibration curve relating E_{600} to bacterial dry weight. For viability experiments involving vegetative cells of *B. subtilis* 168, cultures growing exponentially in CH medium were treated with the antibiotics when the bacterial concentration had reached 0.25 mg dry wt./ml. For experiments requiring cells initiated to sporulation, a culture at 0.25 mg dry wt./ml in CH medium was centrifuged and the cells were resuspended at the same density in resuspension medium containing L-glutamate and inorganic ions (Sterlini & Mandelstam, 1969). Cells shaking in this medium at 37°C yield about 80% refractile spores in 7–8 h.

For cell viability counts 0.1 ml of a culture was serially diluted in glutamate minimal medium and plated in duplicate on nutrient agar (Oxoid Ltd., London E.C.4, U.K.).

Synchronous growth of *B. subtilis* ts-134. A culture

(100 ml) was grown at 37°C in CH medium supplemented with thymidine. When the culture density had reached 0.1 mg dry wt./ml the flask was transferred to a water bath at 45°C and incubation with shaking was continued for 1 h. The culture was then diluted with fresh CH medium plus thymidine at 37°C. Total cell counts were determined at intervals by using a Petroff-Hauser counting chamber and phase-contrast microscopy (Dworkin *et al.*, 1972). The cells were first plasmolysed by treatment with 10M-CsCl to make cell septa visible. This eliminated any uncertainty resulting from chains of cells. At least 200 cells were counted for each point.

Assay of RNA and protein synthesis. Both RNA and protein synthesis were measured in exponentially growing cells in CH medium at a cell density of 0.18 mg dry wt./ml. To follow RNA synthesis the medium was supplemented with [5-³H]uridine (0.5 µCi/ml) and unlabelled carrier uridine (final concentration of 25 µg/ml). Duplicate samples (2 ml) were removed at intervals and mixed rapidly with 2 ml of ice-cold trichloroacetic acid (10%, w/v) containing uridine (500 µg/ml). The precipitates were collected on glass-fibre filters (Whatman, GF/C grade) and washed with 5 ml of ice-cold trichloroacetic acid (5%, w/v) containing uridine (500 µg/ml). The filters were dried in a vacuum at 80°C for 1 h and the radioactivity was determined by liquid-scintillation counting. Protein synthesis was followed in a similar manner, except that the medium was supplemented with L-[³⁵S]methionine (0.5 µCi/ml) and carrier L-methionine (20 µg/ml). Cell samples were treated with trichloroacetic acid (10%, w/v) containing L-methionine (500 µg/ml), collected on filters and washed with trichloroacetic acid (5%, w/v) containing L-methionine (500 µg/ml).

Electron microscopy. Samples of cultures were fixed and thin sections prepared as described by Kay & Warren (1968). Morphological assessment was made only on complete cells in longitudinal section.

Results

Inhibition of RNA and protein synthesis and bactericidal action of rifampicin and of chloramphenicol

The incorporation of [³H]uridine into the acid-insoluble fraction of growing cells of *B. subtilis* 168 was inhibited more than 95% by 100 µg of rifampicin/ml. The corresponding values at concentrations of 1 and 30 µg/ml were 86 and 93% inhibition respectively (Fig. 1a). Inhibition was maximal at 1–1.5 min after addition of the antibiotic. With rifampicin at 30 µg/ml the incorporation of [³⁵S]methionine continued at a gradually decreasing rate. The maximum inhibition was only reached 5–6 min after addition of the antibiotic (Fig. 1a), whereas incorporation of [³⁵S]methionine was almost completely inhibited by chloramphenicol (100 µg/ml) after the first minute.

Fig. 1. Inhibition of RNA and protein synthesis and the corresponding bactericidal effects of rifampicin and chloramphenicol

(a) Effect of rifampicin (30 $\mu\text{g/ml}$) on the incorporation of $[^3\text{H}]$ uridine and $[^{35}\text{S}]$ methionine into the acid-insoluble material of growing cells of *B. subtilis* 168. The antibiotic was added at zero time. $[^3\text{H}]$ uridine: $\circ$, control; $\bullet$, rifampicin. $[^{35}\text{S}]$ methionine: Δ , control; $\blacktriangle$, rifampicin. Chloramphenicol (100 $\mu\text{g/ml}$) completely inhibited $[^{35}\text{S}]$ methionine uptake within the first minute and is not shown in the Figure. (b) The corresponding bactericidal effects of rifampicin and chloramphenicol on growing cells. The antibiotics were added at zero time. $\circ$, Control; $\square$, chloramphenicol (100 $\mu\text{g/ml}$); $\blacktriangle$, rifampicin (1 $\mu\text{g/ml}$); $\bullet$, rifampicin (30 $\mu\text{g/ml}$).

Fig. 1(b) shows the corresponding effects on cell viability of the antibiotics used at the same concentrations. Addition of chloramphenicol (100 $\mu\text{g/ml}$) to exponentially growing cells stopped protein synthesis and growth almost immediately and produced a slow decline in cell viability. In contrast, rifampicin at both 1 and 30 $\mu\text{g/ml}$ killed over 95% of the cells within the first 2 min and caused a further slow loss of viability over the next 2 h. The rate of cell death was slightly greater with the higher rifampicin concentration. After 2 h of exposure to the antibiotic at 30 $\mu\text{g/ml}$ less than 1% of the original cell population remained viable.

The bactericidal effect of rifampicin was decreased if the cells were first washed twice in glutamate minimal medium before dilution and plating, but even so over 90% of the cells failed to form colonies. This partial reversal of the effect when the cells were washed has been reported also with *E. coli* K12 (Lancini *et al.*, 1969; R. J. White, personal communication).

The bactericidal effects of the antibiotics on cells initiated to the sporulation process were also in-

vestigated (Fig. 2). A culture growing in CH medium was transferred to a resuspension medium to initiate sporulation (see the Experimental section). Portions of the resuspended culture were treated with the antibiotics immediately after transfer and the remainder 3 h later when the sporulation process was well advanced. Chloramphenicol was again only slowly bactericidal at either time of addition (Fig. 2). Addition of rifampicin to cells that had just been resuspended caused very rapid cell death, which was even more severe than that found with cells exposed to the antibiotic during exponential growth (see Fig. 1b). However, cells that had been undergoing sporulation for 3 h were somewhat more resistant to rifampicin and even after 2 h of exposure to the antibiotic at 30 $\mu\text{g/ml}$ between 1 and 5% of the cells remained viable.

To ensure that the lethal effect we were observing was not peculiar to rifampicin, four other rifampicin derivatives (kindly supplied by Dr. R. J. White, Lepetit, Italy) were tested. These compounds (rifamycins S, SV, DMAO and X/P) retain the ability to inhibit RNA polymerase. (For the relationship

Fig. 2. Bactericidal effects of rifampicin and chloramphenicol on sporulating cells of *B. subtilis* 168

The antibiotics were added to portions of the culture at zero time (a) and after 3 h (b). Samples were removed at the times indicated and the viable-cell counts were determined. ○, Control; □, chloramphenicol (100 µg/ml); ▲, rifampicin (1 µg/ml); ●, rifampicin (30 µg/ml).

between structure and function of the rifamycins see Wehrli & Staehelin, 1971). Their effect on growing cells was essentially similar to that of rifampicin.

The experiments were repeated with the strains of *B. subtilis* provided by Dr. R. Doi. We confirmed his finding that the rifampicin-resistant mutant (ts-3) was not significantly affected by the antibiotic. However, in our conditions the wild-type strain behaved similarly to our own, i.e. at the end of 2 h over 99% of the cells were killed by the drug at 30 µg/ml. When we used a medium similar to that of Leighton & Doi (1971) to obtain cells that had been undergoing sporulation for about 3 h the survival rate after 2 h of exposure to the antibiotic was still less than 5%. The use of tryptose blood-agar plates to test viability produced no significant difference in the results.

Susceptibility to rifampicin during the growth cycle

The lethal effect of rifampicin on growing cells was initially severe, but it then declined leaving a significant fraction of viable cells even after 2 h of exposure

to the antibiotic (Fig. 1b). This resistant fraction may have represented cells that were at a particular stage in the growth cycle which made them less sensitive to the lethal action of rifampicin. This possibility was investigated by using a synchronized culture of mutant ts-134 (see the Experimental section) and determining the effects on viability of exposure to rifampicin (30 µg/ml) at different stages of the division cycle. Fig. 3 shows that after 1 h at the restrictive temperature (45°C) transferring the culture to 37°C results in a stepwise increase in cell numbers, each step representing roughly a twofold increase in cell number. After treatment with rifampicin the number of viable cells, expressed as a percentage of the total cell count, increased up to the point at which the first cell division took place, decreased during the subsequent period of cell division and then increased again while the cells were not dividing. During the second cell division the percentage of viable cells again declined steeply, but remained roughly constant during the subsequent plateau, only to decrease again during the third cell division. This increased sensitivity of the cells to rifampicin during the cell-division period was reproducible, but the increase in percentage viability shown in Fig. 3 after the first cell division was often replaced by a plateau similar to that shown in Fig. 3 after the second cell division.

Comparative morphology of cells exposed to rifampicin and chloramphenicol

The bactericidal effect of rifampicin suggested that it might be causing structural damage to the cells, and evidence of this was looked for by phase-contrast and electron microscopy. In the phase-contrast microscope many cells, after 1 h of exposure to rifampicin, seemed to have partially lysed, i.e. the perimeter of the cell appeared to be intact, but the interior was pale and granular. Electron micrographs confirmed this impression. They were prepared from samples removed at intervals after treatment of growing cells of *B. subtilis* 168 with rifampicin or chloramphenicol. After 1 h of exposure to rifampicin (at 1 or 30 µg/ml) the majority of cells appeared quite normal. However, the higher antibiotic concentration produced in about 20% of the cells the appearance shown in Plate 1(b). In these cells the cytoplasmic membrane, except at the poles of the cells, had retracted from the cell wall. The nuclear material was more dispersed and less distinct than it was in control cultures (Plate 1a). Prolonged exposure to rifampicin caused exudation of the cell contents into the medium (Plate 1c) and after 4 h most of the cells had lysed. In contrast, cells treated with chloramphenicol (100 µg/ml) under identical conditions all had an appearance after 1 h similar to that of cells from the control culture. Longer exposure produced only a low incidence of lysis in the cell population.

EXPLANATION OF PLATE I

Effect of rifampicin on appearance of cells

(a) A typical cell of *B. subtilis* 168 undergoing vegetative growth. The nuclear material appears as two compact light areas. (b) Characteristic appearance of a cell that has been exposed to rifampicin ($30\mu\text{g/ml}$) for 1 h. The cell membrane, except at the poles of the cells, has retracted from the cell wall. The nuclear material is dispersed and less distinct than in the control cell. (c) Cell beginning to lyse after exposure to rifampicin ($30\mu\text{g/ml}$) for 4 h. Each bar represents $1\mu\text{m}$.

Fig. 3. Susceptibility of cells to the lethal action of rifampicin during different stages of the division cycle

A synchronized culture of *B. subtilis* ts-134 was obtained by the procedure described in the Experimental section. Zero time was the point at which the culture was transferred to 37°C after a period of 1 h at 45°C. Periodically the culture was sampled for total-cell counts (○). At the same time 5 ml samples were withdrawn, incubated with rifampicin (30 µg/ml) with shaking at 37°C for 5 min and the viable-cell counts then determined (●).

Cells initiated to sporulation were also examined after treatment with the antibiotics immediately after transfer to the spore-inducing medium. Cells treated with rifampicin exhibited, after 1 h, cytological damage similar to that found in growing cells and in a roughly equivalent percentage of the population.

O₂ uptake after treatment of cells with rifampicin and chloramphenicol

The suggestion from the electron micrographs that rifampicin had a generally damaging effect was supported by a comparison of the rates of O₂ uptake

by growing cells of *B. subtilis* 168 after treatment with rifampicin (30 µg/ml) or chloramphenicol (100 µg/ml). Cells growing exponentially in CH medium at 37°C were treated with the antibiotics. Cell respiration was noted at 10 min intervals by using conventional Warburg manometry with untreated cells as a control. The control cells continued to grow and for this reason only the O₂-uptake rates between the cells treated with the antibiotics could reasonably be compared. For 30 min after exposure the rates of O₂ uptake with the two antibiotics were similar, but after 60 min the rate of uptake with rifampicin-treated cells was only 89% that of chloramphenicol-treated cells and during the next 30 min this rate decreased by a

Fig. 4. Bactericidal effects of rifampicin and actinomycin D on cells of *B. subtilis* 168 initiated to sporulation

Antibiotics were added to portions of the culture at zero time. Samples were removed at the times indicated and the viable-cell counts were determined. ○, Control; ▲, rifampicin (1 µg/ml); △, actinomycin D (1 µg/ml).

further 9%. The values are the averages taken from three separate experiments, but in all instances it was clear that rifampicin was having a more adverse effect on cell respiration than chloramphenicol.

Comparison of the bactericidal action of rifampicin and actinomycin D

We also compared the lethal action of actinomycin D with that of rifampicin. Cells initiated to sporulation in resuspension medium were treated with both antibiotics at 1 µg/ml. Actinomycin D killed about 40% in the first 30 min, with a slow decline in viability thereafter, so that even after 2 h of exposure to the drug between 10 and 20% of the cells were still viable (Fig. 4). With rifampicin the effect was both more rapid and more severe. When actinomycin D was tested on cells growing in CH medium the bactericidal effect was more marked and between 90 and 95% of the cells were killed in 30 min.

Sporulation of the wild-type and of the rifampicin-resistant mutant at 50°C

The rifampicin-resistant mutant ts-3 described by Leighton & Doi (1971) contains an RNA polymerase which is rapidly inactivated at 50°C. These authors reported that if the mutant is transferred to 50°C during sporulation further spore development ceased. This was taken as a further indication that the cells at the time of transfer did not contain stable mRNA.

We tested this mutant under our own conditions as follows. Cells were incubated in resuspension medium in the usual way at 37°C. At the time of resuspension and at further intervals up to 3 h portions of the culture were transferred to a bath at 50°C and incubated further. Measurements were made of alkaline phosphatase activity, refractility and the number of heat-resistant spores by the methods described by Sterlini & Mandelstam (1969). Each of these three indicators of spore development showed virtually no further increase after transfer of the cells to 50°C, although the cultures shifted at 3 h did develop a small number of heat-resistant spores (1–2%). This confirms the finding of Leighton & Doi (1971). However, in parallel experiments done at the same time with the wild-type organisms (our own strain and also that provided by Dr. Doi) we found that spore development was stopped equally abruptly at 50°C. Accordingly, we repeated all the experiments using the growth medium of Leighton & Doi (1971) and found again that spore development was halted both in the wild-type and in mutant ts-3.

We therefore have to conclude that, at least under our conditions, the cells were too damaged by the shift to 50°C to proceed with sporulation and that no conclusion could be drawn from the experiments.

Discussion

Reports differ on the bactericidal action of rifampicin. In *Escherichia coli* K12 there is a severe and rapid killing effect on growing cells (Lancini *et al.*, 1969) whereas in *Mycobacterium smegmatis* there is only a slow bactericidal action (White *et al.*, 1971). In *B. subtilis*, Leighton & Doi (1971) reported that 50% of the cells were killed in 2 h by rifampicin (30 µg/ml) and that a similar rate was observed with chloramphenicol (100 µg/ml). In our own experiments, we found a comparable slow cell-death rate with chloramphenicol, but rifampicin, also used at 30 µg/ml, was enormously more toxic both to growing and to sporulating cells. Since similar effects were obtained with other rifamycin compounds, it seems improbable that the discrepancy between our results and those of Leighton & Doi (1971) was due to chance contamination of our batch of antibiotic with something a great

deal more toxic. This was in any case unlikely, because the lethal effect of rifampicin was very obvious even when the drug was used at 1 µg/ml. If there had been an impurity it must have been producing an almost instantaneously lethal effect at some even lower concentration. Since we obtained similar results using both the same medium and the same strain of *B. subtilis* as Leighton & Doi (1971) we can only assume that the difference in results is due to some minor undefined difference in experimental procedure.

On the basis of the results illustrated in Figs. 1(b) and 2 we have to conclude that rifampicin is, at least in our conditions, more toxic than chloramphenicol, so that most of the cells are killed in a few minutes, except for a small but significant fraction which retain viability even after prolonged exposure. A synchronous population of *B. subtilis* (ts-134) was used to investigate the possibility that this small proportion of survivors represented cells at some relatively insensitive state produced during the cell-division cycle. Cells that have been incubated at 45°C for 1 h to induce synchronized growth emerge from the treatment relatively resistant to the drug, and the incidence of resistant cells doubles during the latent period preceding the first cell division (Fig. 3). Then, as the cells enter the first division the resistance falls sharply, recovers somewhat during the second latent period and then declines again with the next division. There is thus a very clear relationship between the cell-division cycle and the susceptibility of the bacteria to the lethal action of rifampicin, but it has to be borne in mind that we are considering only a small and atypical fraction of the population. The vast majority of the cells are sensitive at all stages of the division cycle and the survivors never exceed 4% of the total.

The lethal effect of rifampicin in *B. subtilis* could in principle be the result of its action in inhibiting RNA polymerase or it could be due to an effect on some entirely different process or component in the cell. The latter interpretation is supported by the fact that actinomycin D, at a concentration producing a comparable effect on RNA synthesis, has a much slighter effect on viability and allows a limited amount of spore development to proceed (see Sterlini & Mandelstam, 1969). However, all the other available evidence suggests that the action of rifampicin is, in fact, confined to the RNA polymerase. Thus, rifampicin-resistant mutants have an altered RNA polymerase (Heil & Zillig, 1970; Wehrli & Staehelin, 1971). Further, a single mutation appears to confer rifampicin resistance (Sonenshein & Losick, 1970) and to produce a temperature-sensitive RNA polymerase (Leighton & Doi, 1971). If these facts are correct, it seems necessary to conclude that the lethal effect is produced solely by inhibition of RNA synthesis. If the lethal action was due to an effect on synthesis of mRNA and therefore of protein, the

viable-cell count should be affected in a similar way by chloramphenicol. Since it is not, one has to conclude that continuous synthesis of some other form of RNA is necessary for cell survival. We have no idea what this RNA may be or why inhibiting its synthesis should affect the ability of the cells to form colonies. An alternative explanation is suggested by the fact that actinomycin D promotes a depolymerization of newly formed RNA in *B. subtilis* which is not simply the result of inhibiting RNA synthesis (Acs *et al.*, 1963). If rifampicin did the same and if this affected 'old' RNA and not merely newly synthesized material a quite short period of exposure to the drug might suffice to cause irreversible damage. Whatever the mechanism may be it is apparent that rifampicin produces physical damage that is visible both in phase-contrast and in electron microscopy and that it affects even such a crude indicator of biochemical capacity as O₂ uptake. It is not surprising to find that cells that are rapidly lysing fail to sporulate, and it is apparent that even if these cells possessed long-lived mRNA they would almost certainly be incapable of expressing it in terms of protein synthesis. We conclude that no reliable conclusions about the lifetime of mRNA can be drawn from experiments with so toxic a drug.

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