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THE SEARCH FOR NEW STERILIZING ANTI-TUBERCULOSIS DRUGS

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1. ABSTRACT

To be of use in the control of tuberculosis, any new drug must be capable of shortening the duration of treatment by accelerating sterilizing activity, that is the rate at which *Mycobacterium tuberculosis* is killed in the lesions. The most difficult to kill are the extra-cellular bacilli in cavities. Persistence during therapy arises because there is a proportion of slowly metabolising bacilli (persisters) in the cavitary bacterial population at the start of treatment. Bacterial growth is slowed by low oxygen tension, quorum sensing and old age, but probably not by cellular immunity, since there are few professional phagocytic cells in cavities. The degree of phenotypic resistance to the bactericidal action of drugs can go through several stages: (i) the non-replicating stages 1 and 2 of micro-aerophilic adaptation, described by Wayne; (ii) a "tolerant" population that survives exposure to high rifampicin concentrations and is capable of growth in liquid medium but not on solid medium; and (iii) a population found in the sterile state of Cornell model mice which cannot grow initially in either liquid or solid medium but will eventually cause re-activation of tuberculosis. In all of these stages the bacilli are phenotypically resistant; there is no selection for genomic drug resistance. Rifampicin and pyrazinamide are the two drugs largely responsible for sterilizing activity during current treatment. Pyrazinamide is unique amongst anti-tuberculosis drugs in having no genomic site of action and having greater bactericidal activity as bacillary metabolism slows down; it is remarkably effective in human disease. The development of a new drug with a similar mode of activity might be very fruitful, especially if there were no need for an acid environment. Current methods advocated for drug development pass through a number of complex stages: choice of a genomic target, development of an in vitro assay, high throughput screening and identification of lead compounds, often with scaling up of synthesis of the molecule and preliminary studies of toxicity and animal pharmacology before tests are done for sterilizing activity. If the drug is not good at sterilizing, all of this initial work will be largely wasted as it would only have a very limited role in the treatment of MDR disease. One of the most important steps necessary is the development of rapid and simple tests to screen for sterilizing activity. Of tests currently available, none of those employing mice seem adequate, though a screen using a streptomycin dependent *Mycobacterium tuberculosis* seems the most hopeful. A set

of *in vitro* tests is described. There is an urgent need to develop these tests further since the factors slowing growth are closer to those in tuberculous cavities than in mouse models. They have the advantages of simplicity and require only small amounts of a new molecule.

2. THE AIM

The main reason for finding new anti-tuberculosis drugs is to shorten the period of treatment and so improve the control of tuberculosis (1). Assuring regularity in taking drugs throughout the current 6-8 months of treatment is often difficult or impossible. In many developing countries some supervision of dosage is given during the initial 2-month intensive phase of chemotherapy, but supervision of drug-taking throughout the subsequent continuation phase is rarely possible. Irregularity in drug taking leads to failure during treatment or relapse after it, and to the emergence of drug resistant tubercle bacilli. This article concentrates on the search for sterilizing drugs that have the potential for shortening treatment.

Drugs may also be useful for treating patients with multiple drug resistant (MDR) strains of *Mycobacterium tuberculosis*, but this need, though of importance on humanitarian grounds, is unlikely to help in the control of the disease.

2.1. Abbreviations

The following abbreviations are used, INH for isoniazid, RMP for rifampicin, PZA for pyrazinamide, EMB for ethambutol, SM for streptomycin, PAS for p-aminosalicylic acid and RCT for randomized clinical trial.

3. THE LESIONS OF PULMONARY TUBERCULOSIS

3.1. Histopathogy

The lesions of tuberculosis in human disease differ considerably from those found in the mouse and to a lesser extent from those found in the guinea-pig. The most thorough study of the histological appearances of human tuberculous organs, combined with approximate estimates of the numbers of visible (acid-fast) and culturable *Mycobacterium tuberculosis*, was carried out by Canetti on specimens obtained at resection operation and at post-mortem examination during a period from 1938 onwards (2,3). In brief, he first described the lesions with a good prognosis. They consisted of classical tubercles with occasional small areas of central solid caseum surrounded by macrophages, epithelioid and giant cells. These were surrounded in turn by zones containing lymphocytes and finally minimal attempts at fibrosis. The bacilli, usually present in only moderate numbers, grew initially within macrophages. These lesions tend to heal spontaneously. In contrast, lesions with a bad prognosis produced cavities. The first step was caseation in which all cellular elements in a solid block of tissue, including inflammatory cells, died at much the same time, presumably as a result of a delayed type hypersensitivity (DTH) reaction (the Koch phenomenon). The number of viable bacilli, now extra-cellular, also decreased sharply. Solid caseum could liquefy, and in the lungs, the liquid contents would then connect with a draining bronchus so that an air supply to the lesion was established. Liquefaction was accompanied by an enormous multiplication of bacilli. Bacilli grew so profusely that they formed solid clumps near the air-caseum interface and were also numerous throughout the liquid caseum. This multiplication was presumably due to the good oxygen supply and to the nutrients provided by the liquefied caseum. A typical cavity was surrounded by a collagenous capsule. From the air-caseum interface outwards there was first the zone of bacillary multiplication and then an area of capillaries and blood lakes next to the capsular wall. There were hardly any inflammatory cells within the capsule, but there was a large area of perifocal inflammation outside the capsule presumably arising from diffusion through it of bacillary cell wall components. Since there were few phagocytic cells near the bacilli, cellular immunity could play no part in limiting cavitary growth. It is as if a block of culture medium had been inserted into the lungs. The immune system appears to play a part in the early stages of infection and in the spread of the disease by bronchial transfer to fresh lung areas, but is has no effect on the extra-cellular bacilli in cavities once these have developed. It is these bacilli that are difficult to kill and prolong treatment. The speed with which drugs kill bacilli in sputum, that originate in cavities, is almost unaffected by the immuno-suppresion caused by HIV infection (4,5).

3.2. Extra- or intra-cellular site of bacilli

An important issue is that the action of drugs is almost entirely on the large numbers of extra-cellular bacilli which exist in cavities. Furthermore, those few bacilli that are intra-cellular are likely to become extra-cellular when caseation develops. Thus, in the initial phase of drug action, it may well be unnecessary for a drug to act within cells. In the later phases of treatment, we do not know whether persisting bacilli are located in the remnants of caseum or whether some extra-cellular bacilli might be phagocytosed. There is also evidence that residual bacilli might be intra-cellular but not within professional phagocytic cells (6). The eventual balance in bacillary numbers between the two possible sites is unknown, but the main thrust of chemotherapeutic action during the first 2 months of therapy is largely or entirely on extra-cellular bacilli.

3.3. Oxygen tension

A further point of great importance is the critical role of oxygen tension within the lesions. Rapid bacillary growth is only possible when cavities are “open” as a result of establishing an air-way through to bronchi. The air-way is often tortuous and can easily be blocked temporarily or permanently. Cavities without an air-way are termed “closed”. The numbers of bacilli within the caseum of a closed cavity is substantially lower than in open cavities, so further demonstrating the crucial role of an adequate oxygen supply for growth. Early experiments in housing guinea pigs infected with *M. tuberculosis* under different O_2 pressures also demonstrated the important role it played in the development of disease (7). We must assume that

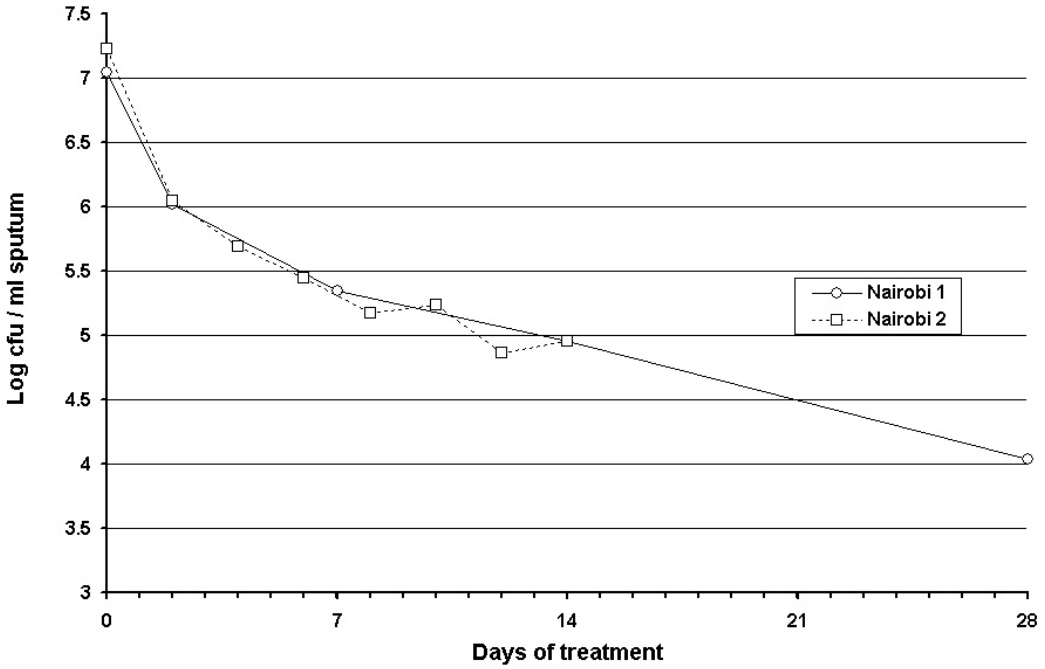

Figure 1. Viable counts of *M. tuberculosis* per ml sputum during the first month of treatment with regimens containing INH. Sputum was collected over-night at fixed intervals from the start of treatment, an aliquot was homogenized with dithiothreitol, which homogenizes by breaking -S-S- bonds but has not antibacterial activity, and plated on selective 7H11 plates. The results in two studies, both on patients in Nairobi, are shown. Data from references 5 and 10.

most of the adaptations to dormancy in the bacilli are triggered by changes accompanying ageing of the bacilli, including quorum sensing (8) and by limitations of the oxygen supply.

4. THE NATURE OF BACTERIAL PERSISTENCE

4.1. Speed of bactericidal drug action during chemotherapy

Pulmonary tuberculosis is almost unique in requiring chemotherapy for at least 6 months to prevent the likelihood of relapse afterwards. In studies of early bactericidal activity, the bacilli in cavitary lesions are sampled during the early part of treatment by counting colony-forming units (cfu) in sputum on selective medium (9,10). These studies have shown that there appear to be two rather distinct phases of bactericidal activity. In the first, "early bactericidal" phase, lasting only 2 days, there is rapid bactericidal action with a rate of kill of $\log_{10}$ 0.60 cfu/ml sputum / day. In the second, "sterilizing" phase, there is a transition over a few days to a much slower exponential kill of persisting organisms, often at about log $_{10}$ 0.12 cfu / ml sputum / day. These two phases can be described in approximate terms by the following equation describing two exponential phases of killing:

Cfu count / ml sputum/ day at time t = $C_1e^{-k_1 t} + C_2e^{-k_2 t} + S$ … [1]

where C_1, C_2, k_1, k_2 and S are parameters to be fitted to the experimental curves.

Since the expression Cfu count = Ce^{-kt} can also be expressed as a straight line drawn between log count at time t_1 and log count at time t_2, it is evident that the equation 1 can be represented approximately by log counts charted against time during treatment to give straight lines with different slopes during the two phases (Figure 1). Taking account of the short period of a few days between days 2 – 5, when the two lines representing the two parts of the equation show a noticeable overlap, a visual inspection from day-5 onwards indicates the linearity of the exponential sterilizing phase.

4.2. Persister populations

It is reasonable to suppose that the bacilli in the lesions are initially composed of several different populations. These include actively dividing bacilli, probably killed during the early bactericidal phase, together with "persisters", a name coined by Bigger (11). They are bacilli that are metabolising at a slower rate and may be occasionally dividing. The peristers must be dividing occasionally because INH (in the absence of RMP in the regimen) and RMP continue to have slow bactericidal activity on them and would not do so if they were not dividing at all (12). They can be categorized according to the length of time that the culture remained under microaerophilic conditions and their phenotypic resistance to the antituberculosis drugs.

4.2.1. Persistence stages 1 and 2

In view of the probable importance of O_2 deprivation in cavitary and caseous lesions, it is reasonable to assume that some of the persister populations are in one or

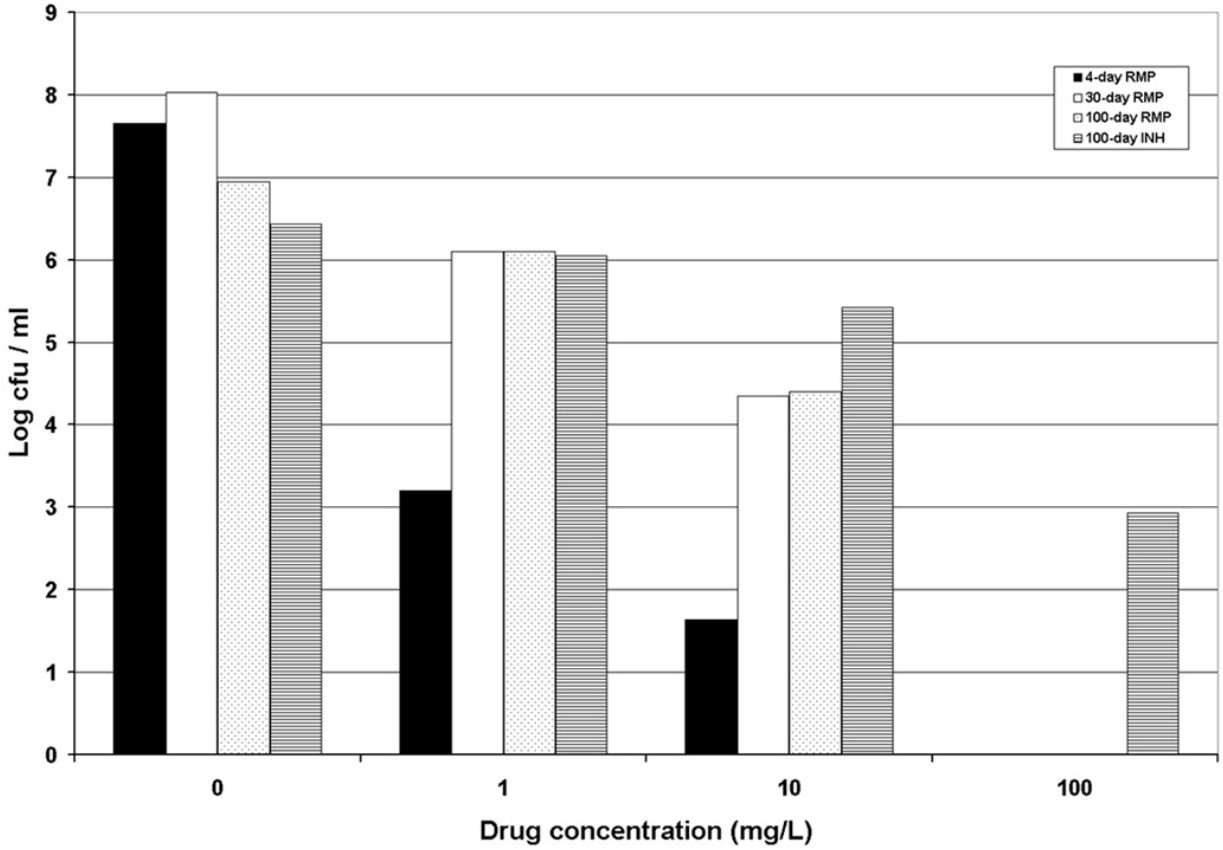

Figure 2. Hu/Coates model 1. Counts of colony forming units from 7H9 liquid medium cultures aged 4 days (log phase), 30 days or 100 days, to which were added 0, 1, 10 or 100 mg/L RMP or 100 mg/L INH. The cultures were then incubated for 5 days and plated out on 7H11 medium. The proportion of surviving organisms increases with RMP concentration and age of the initial culture, and is also higher for INH than for RMP. Data from reference 13 and unpublished, Hu YM and Coates ARM.

other of the two shift-down stages of adaptation to micro-aerophilic conditions described by Wayne in his non-replicating phases nrp1 and nrp2 (14). Growth could be obtained on solid medium subculture. All of these populations were more susceptible to the bactericidal action of RMP than to the action of INH. The effect on susceptibility to RMP and INH of extending the static incubation period from 4-days (log phase) to 30 days and to 100 days (15-17) is shown in Figure 2. The proportion of survivors decreases with increasing RMP concentration but it increases with increasing length of the initial static incubation period. Also in Figure 2, a 100-day culture exposed to INH shows a higher survival proportion at each INH concentration than at the corresponding RMP concentration. At 100 mg/L, about 10^3 organisms survive INH but none survive RMP.

4.2.2. Persistence stage 3

There may also be additional populations of bacilli that are completely tolerant to RMP (15-17). Tolerance is a phenotypic expression of adaptation to long continued incubation under static, micro-aerophilic conditions. The tolerant bacilli grew in liquid medium but not on solid medium plates. When RMP was added to such a culture, the tolerant organisms survived as a proportion of the bacilli detectable by liquid medium subcultivation; they do not multiply further. The extent of tolerance depended on the age of the static culture and the concentration of RMP. A population tolerant to 100 mg/L RMP was just detectable in 30-day cultures but was present as 0.01% of a 100-day culture that had been incubated under static conditions for 100 days (Figure 3). They could transcribe and had low levels of metabolism, as measured by [^{14}C] uridine uptake into RNA. During subcultivation in RMP-free liquid medium, they recovered the capacity to grow on plates after a few days. They were then essentially normal in behaviour; genetic resistance had not developed. Tolerance was thought to result from thickened cell walls that prevent drug penetration or from modification of the sensitivity of the RNA polymerase holoenzyme (15). Almost all of the 100-day culture was also tolerant to 100 mg/L INH (Figure 3). A proportion could grow on plates (Figure 2) but the remaining organisms could only be recovered from liquid medium subcultures (Figure 3).

4.2.3. Persistence stage 4

There is also the possibility of an even greater shift towards low metabolism and drug tolerance in the

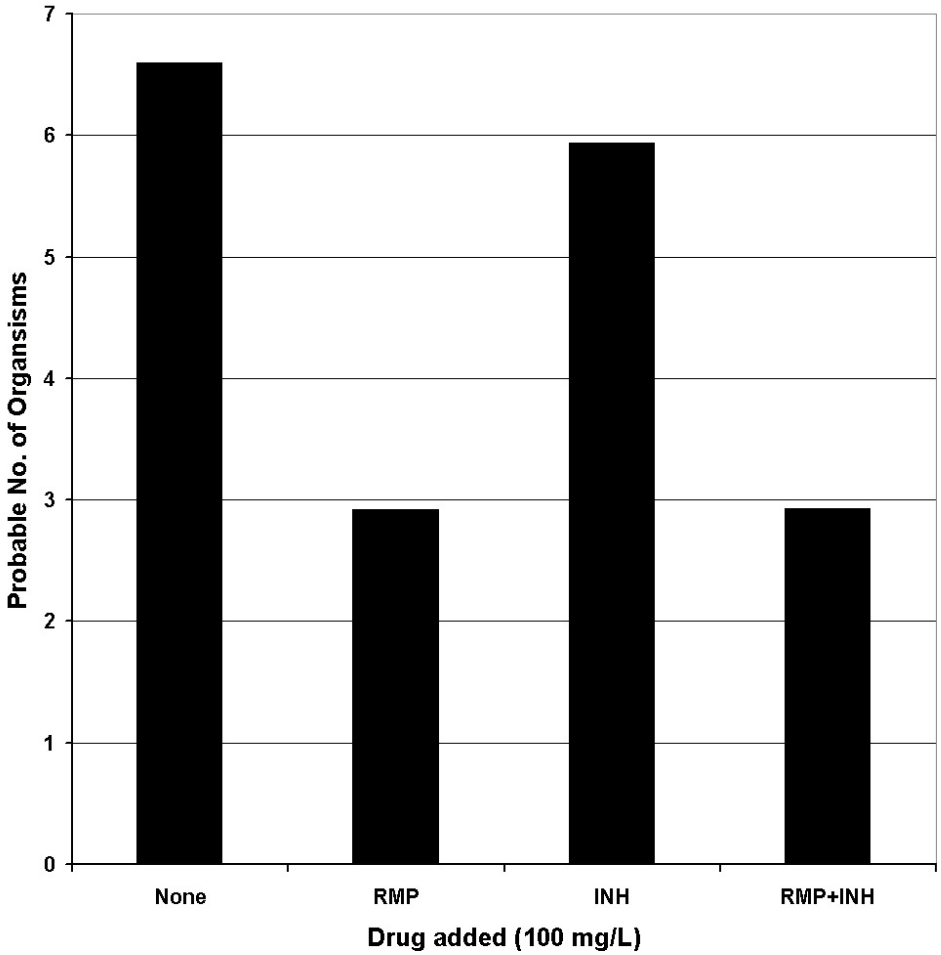

Figure 3. Hu/Coates model 1. The probable number of viable organisms estimated by liquid medium titration from the 100-day cultures to which had just been added 100 mg/L RMP or INH. These are the same cultures from which plate counts have been done (Figure 2). Data from reference 13 and unpublished, Hu YM and Coates ARM.

bacilli of the "sterile state" of Cornell model mice. The sterile state is the period after a 12-week period of treatment with INH and high dosage PZA (or RMP) when culture of whole organs in liquid medium failed to yield growth, yet the disease eventually re-activated, especially if the mice were treated with high dosage steroids. *M. tuberculosis* appeared to survive in a form that was non-culturable even in liquid medium since organs from mice that eventually relapsed with active disease were cultivated first in liquid medium in some Cornell model techniques (18). That they were resistant to INH is shown by the failure to produce the sterile state if the periods of dosages with INH and PZA were separated in time, and INH was given after PZA (19). However, they were still just susceptible to PZA since prolonging treatment beyond 12-weeks decreased the proportion of the mice that eventually relapsed.

In view of the relatively greater importance of the initial 2 months of treatment in determining the eventual outcome of treatment, as compared to the subsequent continuation phase (20), one can assume that the bacilli in human lesions are steadily slowing down in their rate of metabolism and occasional multiplication during the course of treatment. This would happen in any case as the more actively metabolising populations were killed, leaving more slowly metabolising persisters still alive.

5. STERILIZING ACTION OF CURRENT DRUGS

5.1. Modes of action of anti-tuberculosis drugs

Since we are looking for new drugs that would be given together with established drugs, if for no other reason than to prevent the emergence of drug-resistance, we need to know about the sterilizing activity of current drugs. In current standard short-course chemotherapy, INH, RMP, PZA and EMB are given together in the 2-month initial phase and are followed by 4 months of INH and RMP. This can be expressed in a condensed form as 2EHRZ/ 4RH. All of the drugs, with the exception of PZA, act on known sites in the genome of the tubercle bacillus to which they or their derivatives bind (21). EMB inhibits cell wall synthesis (EmbA, B and C) and a major target of INH action is also inhibition of cell wall synthesis (inhA and

kasA), while RMP inhibits transcription by binding to DNA-dependent RNA polymerase (RpoB) and SM inhibits protein formation at the ribosome (RpsL and 16S rRNA), though the site causing the important low level of resistance has not been identified. As we shall see, the two drugs responsible for the main sterilizing activity of standard therapy are RMP and PZA. Why are they so effective?

5.1.1. Rifampicin and isoniazid

If we take INH and RMP as examples, slowing of the entire bacterial metabolism by lowering incubation temperature or altering pH to the acid side decreased bactericidal activity of both drugs to a similar extent (13). Thus RMP was not a better sterilizing drug than INH simply because of increased activity against slowly metabolising bacilli. However, using changes in incubation temperature to obtain pulsed periods of growth, RMP was then appreciably more bactericidal than INH. RMP started to kill as soon as it was added to the culture, whereas INH took about 24 hours to start killing in these and other in vitro experiments (22). Thus, the sterilizing activity of RMP is probably due to the rapid start of killing as soon as a pulse of increased metabolism occurs. The rapidity of the kill is probably the result of tight binding of RMP to RpoB. We can imagine that bacilli are partially inhibited during treatment. While they are completely inhibited, neither INH nor RMP kills them, but as soon as they start to metabolise again, RMP kills before INH has been able to act.

5.1.2. Pyrazinamide

PZA appears to be remarkable in not having a binding site (23). It seems to act by causing accumulation of the protonated form of pyrazinoic acid (HPOA) in the bacterial cell, leading to acidification and membrane damage. However, HPOA diffuses passively into the bacterial cell but can only be excreted by an inefficient efflux pump. In consequence, as the metabolic activity of the cell decreases, the bactericidal activity of PZA increases. This is in contrast to other antibacterial drugs with binding sites, which become inactive against resting bacilli (12,22,24). It is probably the only anti-tuberculosis drug that can kill truly non-metabolising bacteria. This is the likely reason for its high efficacy as a sterilizing drug. In an in vitro system to model sterilizing activity, (Model 3, described in section 6.2.3, where the uptake of uridine into bacterial RNA was very low), PZA had remarkably rapid and complete sterilizing activity at pH 5.6, indicating that that it is not so much necessary to have great acidity in the lesions, as to have low bacterial metabolism (Hu, Coates and Mitchison, unpublished observations).

5.2. Role of drugs during treatment

Evidence on the role of the different drugs during treatment has been obtained partly from RCTs (20), partly from studies of early bactericidal activity (EBA) during which drugs are given in monotherapy or in various combinations during the first 14 days of treatment (9,10) and partly from counts of the viable bacilli in sputum during the first month of treatment (5).

In the early days of chemotherapy combinations of INH, SM and p-aminosalicylic (PAS) had to be given for periods of at least 12 months to avoid high rates of relapse (20). It is usually assumed that the drug responsible for the main sterilizing action of these regimens was INH, since the few studies of long-term treatment with only SM, PAS or thiacetazone all had high failure rates (25,26). Less well documented, the introduction of INH was also accompanied by improved cavity closure (27). In these studies, before the introduction of RMP and PZA, initial drug resistance to INH was responsible for poor outcomes, indicating its major effect in the drug combination (28).

The remarkable sterilizing activities of RMP and PZA were first demonstrated in models of long term treatment of experimental tuberculosis in mice (19,29,30). In the treatment of pulmonary tuberculosis, their addition in an RCT to a 6-month regimen of SM + INH greatly reduced the subsequent relapse rates from about 23% to 3-8% (31). In further RCTs, the addition of RMP only or PZA only allowed shortening of the treatment period from 12 months to 9 months (32,33), and regimens with both RMP and PZA could be given for only 6 months (34-37). PZA is almost as effective as RMP despite the evidence that it does not start sterilizing before about 14 days (10) and stops before the end of the 8-week initial period of treatment (38,39) whereas RMP acts throughout treatment (38,40). Thus, considering its short period of activity, PZA has remarkable sterilizing power, greater per unit of time even than RMP.

The properties of drugs in the early bactericidal and sterilizing phases are very different. The action of the five major anti-tuberculosis drugs, given alone or in a variety of combinations in the original EBA study lasting 14 days, were examined by multiple regression (10). During the first 2 days, there were large differences in the rate of kill produced by different drugs and different doses of the same drug. However, INH was the predominant drug with the greatest rate of kill, probably because its therapeutic margin is about 20 (the ratio between the usual dose of 300 mg and the minimal dose of about 15 mg that just gives a detectable EBA), whereas the therapeutic margin of other drugs was much lower (41). The addition of other drugs in combinations with INH did not increase the rate of kill. This early phase is thought to be due to drug action on dividing bacilli, though there is no proof of the hypothesis.

In contrast, drug action in the second sterilizing phase is quite different. INH had no detectible additional bactericidal activity in combination regimens. In its place, RMP, alone or in combination, was now the predominant drug. SM also had significant sterilizing activity, though only when all combinations, including monotherapy, were considered. However, the sterilizing activity of streptomycin arose particularly early in the study and the results of clinical trials clearly indicate that it might have measurable sterilizing activity early in treatment (up to perhaps the first month) but this was not maintained thereafter (references in 10). Hence it does not contribute to the overall sterilizing activity of a combined regimen. The action of ethambutol, when only combined regimens were analysed, seemed to be inhibitory. However, this

apparent antagonism with other drugs is not evident in the results of RCTs. PZA in monotherapy had little or no bactericidal activity during the first 2 days (10,42) and then killed at a steady rate of 0.054 $\log_{10}$ cfu/ml/day (10). It did not alter the rate of sterilizing activity of other drugs in combination. Since PZA is definitely bactericidal from day-2 onwards against extra-cellular bacilli that originate in cavities and are then excreted to sputum, it follows that it must be bactericidal against extra-cellular bacilli (43). To account for the steady bactericidal activity, these bacilli must have a mildly acidic environment, due to active inflammation during the initial phase. However, it is presumed that the pH returns to normality as inflammation dies down, so accounting for its inactivity during the continuation phase. PZA is often assumed to act on intra-cellular bacilli, but such activity may well be weak or non existent, since several studies have shown bacteriostatic but not bactericidal activity against bacilli in tissue culture cells (44-46).

In summary, RMP and PZA are responsible for most of the killing of persisting bacilli from 2 days onwards. This early onset of the sterilizing phase of treatment occurs when the great majority of bacilli are cavitary in origin. Since there are few professional immune cells near cavitary bacilli, the immune system does not play more than a minor part in creating the conditions for persistence during chemotherapy. However, the evidence from HIV infection indicates that immunity keeps organisms seeded by the blood stream spread from the original primary Ghon focus in a state of dormancy.

5.3. Predominance of drugs during treatment

The predominance of drugs in their bactericidal activity is important because we wish to find new drugs that can increase the sterilizing activity, while maintaining the prevention of drug resistance. To prevent drug resistance, any new drug will have to be given in combination with established drugs. A new drug might be given to replace one of the current four (INH, RMP, PZA, EMB), probably INH because of its very limited sterilizing role. It would very probably be given together with RMP and PZA, so synergism between their sterilizing activities is crucial. It is of interest that SM is a drug that inhibits the basic function of protein formation and has early sterilizing activity. However, it is not an effective sterilizing drug in combined regimens (10), probably because it cannot compete with RMP and PZA. This demonstrates the difficulty in choosing molecules that might be thought to be effective, but turn out not to be.

6. THE SEARCH FOR NEW DRUGS

6.1. Current procedures

Methods currently advocated have been outlined in some detail, though these have not yet been carried out in practice (47). They start with target selection using genomic information to choose for specificity. Genes chosen should not be involved with pathways that are found in the human cell, and may also be specific for mycobacteria or even *M. tuberculosis*. More difficult is to find targets that might select for potential sterilizing activity. If this is not done, the chances are that a cell wall inhibitor will be found. Amongst current drugs, there are five, INH, EMB, ethionamide, cycloserine and thiacetazone, that certainly or probably act by inhibition of the formation of the cell wall. None are effective sterilizing drugs, nor is it logical to expect a drug that only kills multiplying cells to be effective against metabolising, non-dividing cells. They may be chosen for potential sterilizing activity as part of a genomic system that controls bacterial dormancy. However, *M. tuberculosis* spends most of its life in a dormant state and it therefore seems probable that several genes control such a vital bacterial adaptation. Thus it may be difficult to find a single controlling gene, for instance a sigma factor, whose inhibition would be fatal to dormant bacteria. It will be particularly relevant to see whether drugs that inhibit isocitratelyase, shown to be upregulated in dormant cells in the Wayne model (48), are effective in complete inhibition of the enzyme and in killing dormant bacilli. Another theoretical possibility is to stimulate the change from dormancy to active growth, thus making the bacilli more rapidly susceptible to antibacterials. This idea has been investigated by the use of corticosteroids in models of bacterial dormancy (the Cornell model) in mice (49), and also by treating patients with high dosage steroids at the start of chemotherapy (50). Steroids undoubtedly stimulate dormant organisms to multiply in mice and probably also in humans. In neither the mouse nor the human experiments was steroid administration found to alter the speed with which the antibacterial drugs were bactericidal. It is also possible to choose a “basic” biochemical target, but here the lesson of SM is appropriate. What could be more basic than inhibition of protein formation, yet SM is not effective as a sterilizing drug (10,51).

Once a target has been selected, then an assay should be developed to measure the possible effects of inhibitor molecules. The assay can then be used in the high through-put screening of large numbers of molecules (often millions) to find lead compounds. These leads are optimised for efficiency in inhibiting the assay and also for other “drugable” properties. It is often possible to obtain inhibitory molecules but much more difficult to obtain those that bind sufficiently tightly. Tight binding, as shown by RMP, is probably essential for effective sterilizing activity. Only after much development work has been done is a test done to see whether the new molecule has sterilizing activity. The assessment is usually made in long-term experiments in murine tuberculosis. Such experiments are highly effective in demonstrating sterilizing activity but take some 6 months or more to complete. The problem about this approach is that there is a chance, possibly quite high, that one ends up with a drug that is not an effective sterilizer. The expensive procedure of high throughput screening followed by lead selection, optimisation and long-term mouse experiments would then be wasted. The only use for the new drug would be in the treatment of MDR tuberculosis.

There would seem to be an urgent need to develop methods in which sterilizing activity is measured early in the development procedure. This could be either at

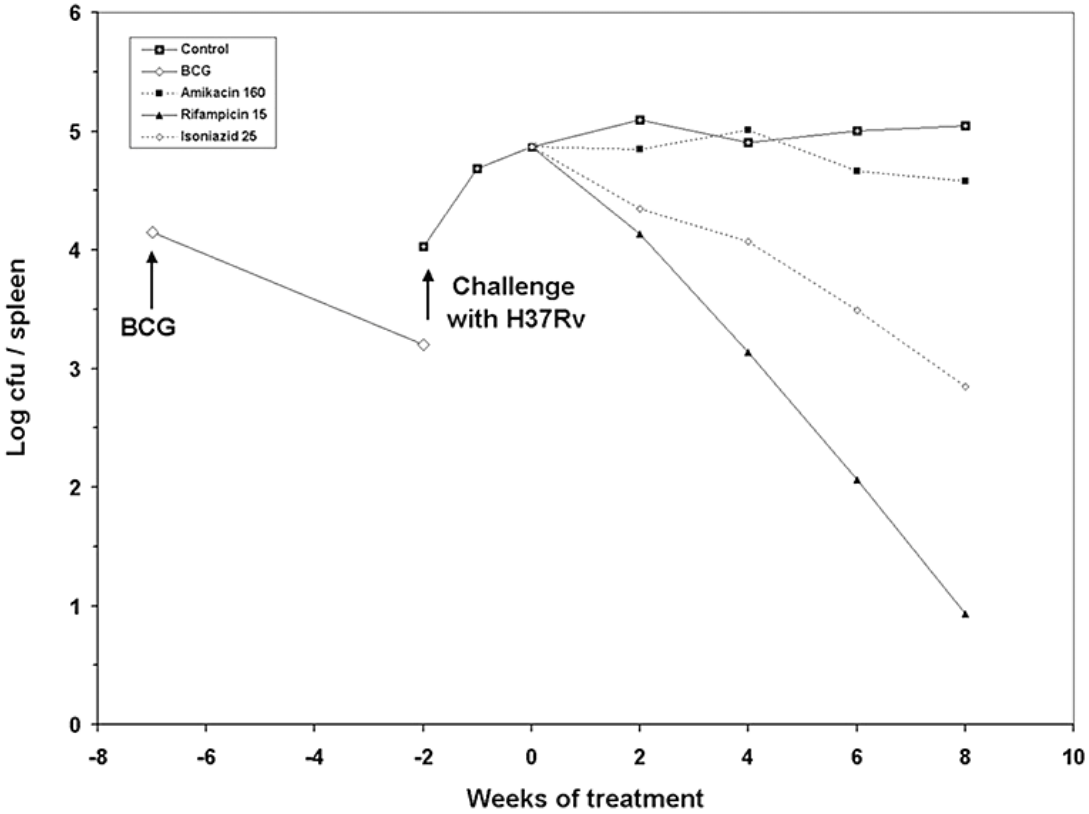

Figure 4. Counts of viable *M. tuberculosis* in the spleens of mice during a chronic tuberculosis experiment. Mice were first BCG vaccinated, and then challenged intravenously with the virulent H37Rv strain. Treatment was started 14 days after H37Rv challenge with either amikacin 150 mg/kg, RMP 15 mg/kg or INH 25 mg/kg. Amikacin, although actively bactericidal in acute model experiments (not illustrated) had almost no sterilizing activity. Both INH and RMP had sterilizing activity, but the rate of kill was greater with RMP. Data from reference 53.

the initial stage, using a whole bacterial cell assay, or early on during lead optimisation to select molecules that are sterilizing. In the past, all current drugs were developed using whole cell assays. It is argued that this is inefficient as compared to high throughput screening, but there is as yet no clear evidence that target selection on genomic evidence followed by high throughput screening is really as efficient as is claimed. It would certainly have failed to find PZA. Furthermore, automation of suitable whole cell assays has not been attempted. Even if sterilizing activity is to be measured during lead optimisation, there is an urgent need for effective assays to measure sterilizing activity.

6.2. Models for sterilizing activity early in drug development

We need simple and rapid models that are capable at least of demonstrating the sterilizing activities of RMP and PZA. This should be done in a way which shows their superiority to other drugs, such as INH, and if possible their potential when added to a regimen to increase its overall sterilizing activity. We should, as far as is possible, use in our models those factors that are likely to cause persistence in the bacilli in the human host. These seem to be micro-aerophilic adaptation and age of the culture with the possibility that quorum sensing systems may be involved. The screens should include models in which a new molecule can be tested against bacilli that are tolerant of RMP and even of PZA, since we would wish to use it together with the established sterilizing drugs to shorten treatment. Current interest is in mouse models and in vitro systems.

6.2.1. Classical mouse models

Mouse models are useful in indicating the oral absorption of new drugs and their activity in curing the lesions of tuberculosis. There is no doubt that prolonged experiments mimic the behaviour of drugs and drug combinations in humans, but the question is whether effective short term experiments can be developed to measure sterilizing activity. In all such experiments, the system has to slow down the rate of growth and metabolism of the challenge infection.

It is usual to allow high cellular immunity to slow growth. In the chronic tuberculosis model (52,53), mice are BCG vaccinated, challenged with a virulent strain some 4-6 weeks later, and then left for 2 weeks after challenge before treatment is started (Figure 4). In this model, immunity is sufficient to stop growth in the spleens of untreated control mice completely. The distinction between the sterilizing activities of INH and RMP is clear, as RMP kills more

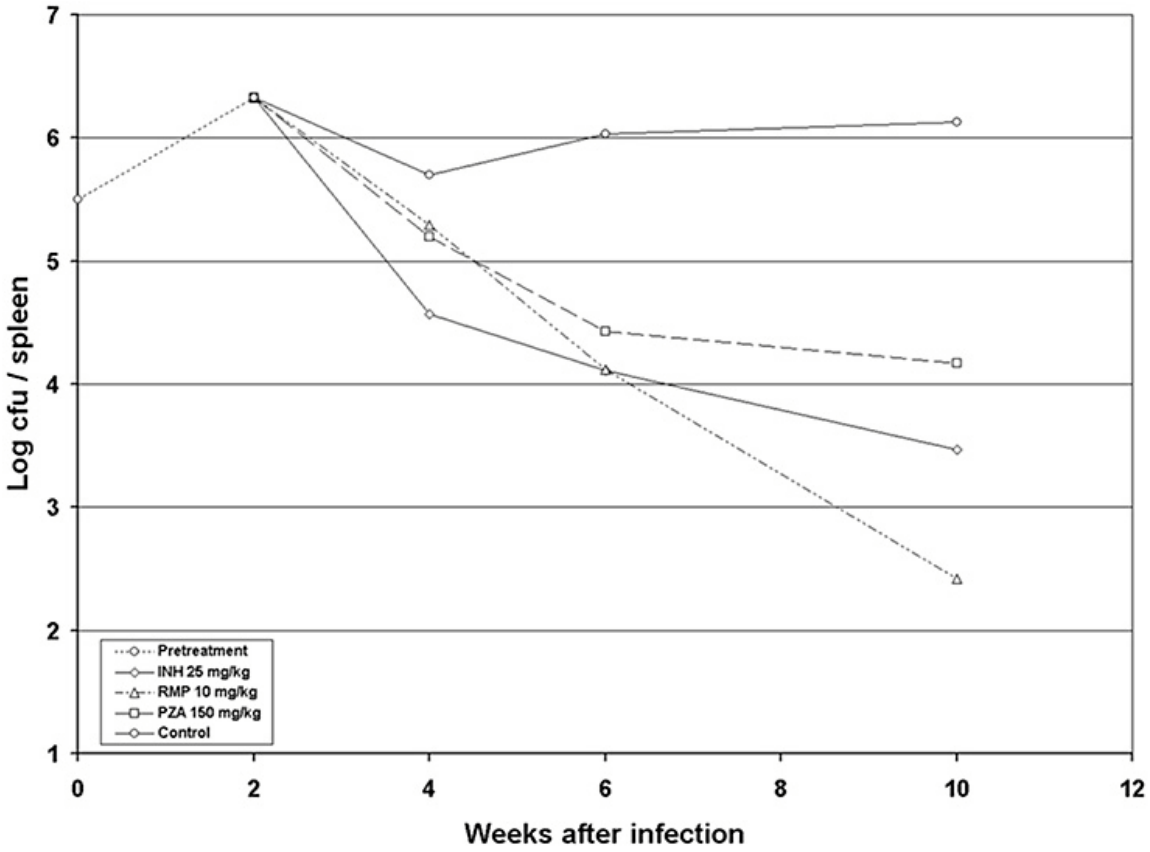

Figure 5. Counts of viable *M. tuberculosis* in the spleens of mice during a 10-week tuberculosis experiment. Mice were infected intravenously with M. tuberculosis, strain H37Rv and were left for 2 weeks before treatment was started with INH 25 mg/kg, RMP 10 mg/kg or PZA 150 mg/kg. Samples of 9-10 mice were sacrificed at each time point in each treatment group. The pre-treatment growth between infection and start of treatment is estimated. The speed of bactericidal activity is greater for INH than for RMP during the first 2 weeks of treatment but their relative activities then change over until after week 6, when there is a large difference between the continuing sterilizing activity of RMP and the almost static action of INH . This illustrates the necessity of a high degree of immunity for demonstrating sterilizing activity. PZA is however less bactericidal than INH indicating that its sterilizing activity is difficult to demonstrate in mouse models. Data from reference 54.

rapidly than INH, but PZA appears never to have been tested. While the high degrees of immunity necessary to distinguish a sterilizing drug can develop during the course of these long experiments, the question is whether more rapid and economical mouse systems could be evolved. In an experiment reported by the Grosset group (54), mice were infected and the disease allowed to develop for 2 weeks when a considerable degree of host immunity would be expected (Figure 5). The time course of cfu bacterial counts in the spleens of mice showed early bactericidal activity of INH greater than that of RMP during the first 2 weeks of treatment but during the next 4 weeks, RMP overtook INH. This indicates that the additional heightening of immunity during the 4 weeks from challenge is necessary to be able to distinguish the sterilizing activities of RMP and INH. However, even then, the sterilizing activity of PZA, while weakly present, was less than that of INH. The prolonged sterilizing activity of PZA, first demonstrated in the original Cornell experiments, was obtained with much higher dosage of PZA in the diet. Instead of the decreasing rate of kill found after 6 weeks with 150 mg/kg PZA (Figure 5), the kill continued to reach the "sterile state" when 2,000 mg/kg PZA was given in the diet with INH (Figure 6). These results show that it is appreciably more difficult to demonstrate the same degree of sterilizing activity of PZA in the mouse as is found in human disease.

6.2.2. Rapid mouse models

An interesting method developed by Kondo and Kanai used an SM-dependent strain of *M. tuberculosis* which only grew in the mouse if it received SM (55). With SM treatment of the mice, INH, RMP and kanamycin were all bactericidal, but without SM, when control counts were static, a difference between the bactericidal activity of RMP and no activity of INH or kanamycin was evident after only a few days of treatment. PZA was never tested. This seems a remarkably efficient and rapid model for sterilizing activity which merits further development.

Amongst more conventional methods, the programme for screening carried out at NIH uses an airborne initial infection with treatment started 20 days later when the rapid early rise in cfu counts from the lungs

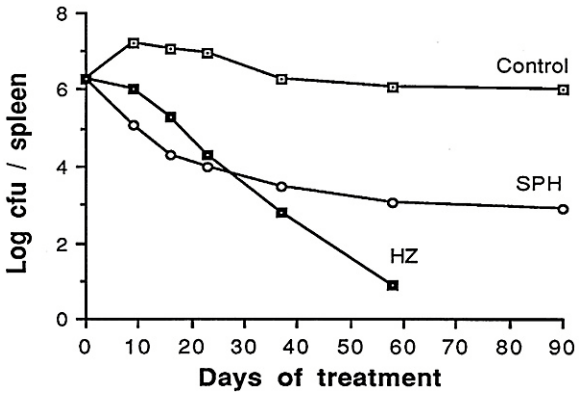

Figure 6. The sterilizing activity in mice of a regimen of PZA and INH. The regimens were 4,000 mg/kg PZA and 25 mg/kg INH in diet (HZ) compared to 200 mg/kg SM by daily injection, with 150 mg/kg PAS and 25 mg/kg INH in diet (SPH). Redrawn from Text-Fig 4 of reference 17.

has halted (56). This technique might not be adequate for demonstrating sterilizing activity since INH caused a fall in cfu counts of nearly one log unit during the first fortnight of treatment. Residual bacillary multiplication might not have decreased sufficiently, since the different sterilizing activities of INH and RMP in the experiment shown in Figure. 5 could only be demonstrated at least 4 weeks after challenge, when INH reduced counts by only half a log unit counts. No data have been provided to compare the activities of RMP or PZA with INH. A more rapid method uses mice with immunity reduced by gamma irradiation. This method may show early bactericidal activity but is unlikely to be able to demonstrate sterilizing activity (57). A method has been described in which mouse splenocytes are removed at various periods after intravenous challenge of the mice with virulent *M. tuberculosis* carrying a luciferase reporter gene (58). Drugs were then added and changes in light response and cfu counts noted after incubation for 2 days. It was difficult to show any greater reduction in either measure with RMP than with INH. PZA caused a slight reduction in light response and cfu counts only at 21 days after challenge probably when immunity was maximal. The response could be accelerated by BCG vaccination before challenge with the virulent strains, and then was found at 11 days. It is interesting that the timing of this response corresponded with the timing of the classic chronic model when BCG is given, then challenge and then a 14-day gap until treatment is started (Figure 4).

In summary, attempts to design a rapid mouse model to screen for sterilizing activity have not been particularly successful. Any model should be able to show clear superiority of RMP over INH and should also be able to demonstrate PZA activity. The limitation of these models probably arises because of inability to stop bacillary growth and slow down metabolism sufficiently to demonstrate the differences of the sterilizing activities of INH, RMP and PZA to the same degree as occurs in human disease.

6.2.3. *In vitro* models

There are three main advantages in the use of in vitro screens: (1) the probable causes for slow bactericidal activity in human lesions, which are low O_2 tension and old bacillary age, can be used rather than unnatural host immunity; (2) a requirement for only small amounts of the new drug, often less than 1 mg; (3) cheapness and easy adaptation to automation.

The bactericidal activities of RMP and INH are decreased to the same extent by simple slowing of growth of the entire bacterial population by, for instance, suspension in a non-nutritive medium or by lowering temperature or by making the environment acidic. Such systems are unlikely to be useful. We need systems in which there are spurts of metabolism or growth. Such spurts have traditionally been thought to occur in bacterial cultures in the stationary phase of growth. When using ill-defined stationary phase cultures, we have often failed to find any difference in the bactericidal activities of RMP and INH (unpublished data). However, the demonstration by Wallis that RMP is more bactericidal than INH in cultures tested at a growth index of 250 in the BacTec system indicates that the metabolic state of the bacilli needs careful definition (59). Wayne has also shown in his shift-down models that during incubation in the non-replicating phase for up to 22 days, there was a change in the bactericidal activities of INH, RMP, ciprofloxacin and metronidazole (12). INH and ciprofloxacin lost their activity after incubation for 8-14 days, while RMP retained its activity during incubation for at least 22 days, and the activity of metronidazole steadily increased with longer incubation periods.

A series of patented in vitro models have recently been described which seem able to demonstrate the bactericidal activity of drugs against *M. tuberculosis* in a state of low metabolism and also against a sub-population of old bacilli that are phenotypically resistant to RMP (Figure 7). The background to these models has already been described in section 4.2.2. All of the models start with a liquid medium (7H-9) culture incubated without shaking for 100 days. This static culture is micro-aerophilically adapted, probably in the Wayne nrp2 stage. In model 1, a range of concentrations of the drug is added to the old culture, which is then incubated for 5 days and a cfu count done. This model could be modified by incubating with drug for more than one incubation period thus giving a measure of the speed of bactericidal activity. Models 2 and 3 are designed round earlier observations that there is a bacterial sub-population within old static cultures that can survive in the presence of high RMP concentrations. This population cannot grow on solid medium plates but if subcultivated into liquid medium it recovers this capacity during the next 4 days and the cultures that have recovered in this way have the usual MICs of RMP. Thus, they are phenotypically resistant. The numbers of surviving organisms in the tolerant culture can be estimated at the start of recovery by doing a liquid medium titration with estimation of the most probable number of viable organisms. This estimate is much the same as the mean cfu plate count done after the 7-day recovery period, indicating that there has been little or no actual cell multiplication during the recovery period. There is, however, a sharp increase in bacterial metabolism, as

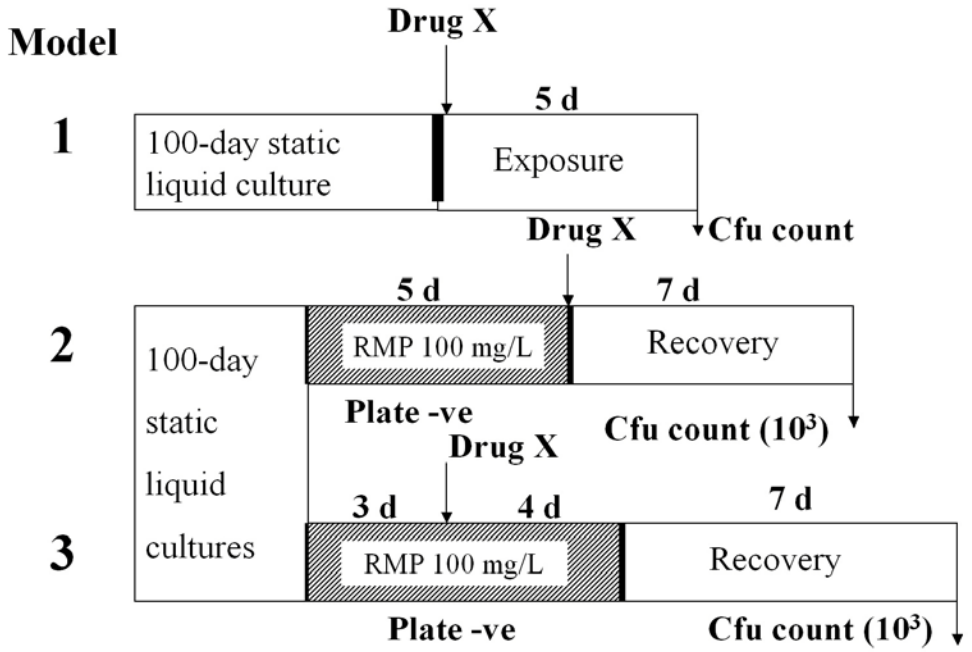

Figure 7. Hu/Coates patented models for persistence. All models start with dispersed bacilli from micro-aerophilically adapted 100-day static cultures in 7H9 liquid medium. In model 1, the drug to be tested (drug X) is then added and, after incubation for 5 days, a count of colony forming units (cfu) is set up on 7H11 medium plates. In models 2 and 3, the dispersed 100-day bacilli are resuspended in fresh7H9 medium containing 100 mg/L RMP and incubated for 5 days. After incubation for one day, all subcultures to plates from this fail to grow colonies, but the probable number of viable organisms (about 10^3) can be estimated by replicated serial dilutions in 7H9 medium. After incubation for 5 days, the bacilli are washed and resuspended in RMP-free 7H9 recovery medium, when they gradually recover the capacity to grow on plates. A cfu count after incubation for 7 days gives about 10^3 organisms indicating no multiplication during the recovery. The effects of drug X when added at the start of recovery (Model 2) or during exposure to 100 mg/L RMP (Model 3) is estimated from these final cfu counts. Redrawn from reference 17.

measured by ^{14}[C] uridine uptake, starting immediately on recovery. Drug can be added at the start of the recovery period in model 2 or during the exposure to RMP in model 3.

As measured solely by plate cfu counts, the bactericidal activity of RMP is greater than that of INH in all three models (Figure s 2 and 3). Models 1 and 3 can detect the sterilizing activity of PZA provided the tests are done in mildly acid culture medium (Hu, Coates and Mitchison, unpublished observations). In model 2, however, PZA has no sterilizing activity probably because of the burst of metabolic activity that occurs at the start of the recovery phase. Models 1 and 3 seem most suitable for screening sterilizing activity since the organisms are not only not multiplying but also have low metabolic activity. We do not know whether the RMP tolerant organisms of models 2 and 3 are also found in human lesions, but there is a reasonable possibility that they might be present during treatment with RMP and contribute to delay in sterilisation. These three models, and particularly models 1 and 3, seem promising for screening new molecules and even for the detection of target molecules. They need testing against a range of current drugs to establish whether they agree with gradings of sterilizing activity obtained in human studies. They could be elaborated further and are simple enough to warrant automation.

7. CONCLUSION

What should be our priorities in seeking new sterilizing drugs? First, we should develop *in vitro* screens for sterilizing action. These might be used in whole cell searches for new drugs, since there is little evidence as yet that the current fashionable approach to find genomic targets and then find inhibitor molecules is really as effective as is hoped. The development of automated whole cell screens has been hindered by fear of using a pathogen, *M. tuberculosis*, as the whole cell. Yet attenuated *M. tuberculosis* strain such as H37Ra seem not to have been used. Alternatively, a screen for sterilizing activity could be used at a very early stage after an inhibitor of a genomic target had been identified, or at least at a very early stage in drug discovery. Secondly, we should recognize the great merits of PZA as perhaps the most efficient sterilizing drug available in human lesions, whose activity is only limited by the need for an acid environment round the bacilli. Could we not start a search for another molecule that enters the cell by passive diffusion, is little influenced by pH, but is excreted by an inefficient efflux pump? Such a drug could revolutionize chemotherapy. Thirdly, we should stop or severely curtail the considerable body of work being undertaken at present to find new inhibitors of cell wall synthesis. INH is a remarkable drug

in many ways, having a very low MIC against *M. tuberculosis* and remarkably little toxicity, so that it can be given in a dose some 20 times higher than the minimal effective dose. Yet when given with RMP or PZA, it kills only during the first few days of chemotherapy and does not add to the sterilizing activity during almost all of the long treatment period. Finding another INH would be of little value, except for treating MDR disease, and would not contribute to the control of tuberculosis.

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