EVALUATION OF A RADIOMETRICALLY-DETERMINED REGROWTH MODEL FOR THE STUDY OF ANTI-TUBERCULOSIS DRUGS

by

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ABSTRACT

Background : A post-exposure regrowth model utilizing the well-tried Bactec radiometric system, which would simulate *in vivo* situations at the site of invasive disease, was developed to measure drug activity against multiplying *Mycobacterium tuberculosis*.

Aims : The aims of this dissertation were to (a) construct a radiometric model simulating drug efficacy relating to the combined bactericidal activity and delays in regrowth due to the action of anti-tuberculosis (TB) agents, (b) compare the killing kinetics of drugs singly and in combinations by the time-kill curve method, with the radiometrically-determined regrowth model, and (c) assess whether the Bactec radiometric regrowth model could predict likely bactericidal activities of drugs.

Design and methods : Drug concentrations in the time-kill curve method were in a range of achievable drug concentrations at the site of infection and in multiples of the minimal inhibitory concentration (MIC), (1x, 2x or 3x,and 8x). Exposure times of 6h, 24h, 48h and 72h were used and these were based on pharmacokinetic data reflecting likely periods of *in vivo* exposure in TB lesions. Standardized inocula of approximately 10^6CFU/ml of actively multiplying *M. tuberculosis* strains were used. The same concentrations, exposure times and bacterial concentrations were used for the assessment of radiometrically-determined post-exposure regrowth times of *M. tuberculosis*. Growth times were recorded as the number of days required to reach a predetermined growth index (GI) level in the Bactec system, and were expressed as T_{400} readings in days. Simple linear regression and a mathematical logistic model were used to assess whether the radiometric post-exposure

regrowth model could predict the bactericidal activity of the drugs.

For drug combination studies, 1MIC of isoniazid (INH) and rifampicin (RMP) were used singly and in combination while 2MIC of ethambutol (EMB), streptomycin (SM), ofloxacin (OFL) and amikacin (AMK) were used in combinations studies. Colony counts at 0h and following 24h exposures were performed and regrowth patterns were determined using the T_{400} method. *M. tuberculosis* H37Rv was tested and subsequently resistant strains.

Results : INH and RMP were highly bactericidal while EMB showed moderate activity in the time-kill curve method. The three drugs produced the best curves, showing longer regrowth times and markedly depressed rates of regrowth in the Bactec post-exposure regrowth model. Using simple linear regression, a linear relationship between bacterial survivors and the radiometric regrowth times, T_{400}, was achieved for all drugs tested. Even better agreement was found when control-related regrowth times, $(T-C)_{400}$, were used in the analysis. Conditions compromising the linear relationship in the radiometric regrowth model, for OFL and less markedly EMB and AMK, were likely postantibiotic effects (PAEs) brought on by the short exposure time (6h), and drug carry-over effects due to concentrations $\geq$ 8 MIC for INH, RMP and SM (10x and 20x MICs). The mathematical logistic model showed good correlation between bactericidal activity and regrowth for INH and RMP but not for EMB, SM, OFL and AMK.

Drug combination effects in the two techniques depended on the criteria used to describe synergy. Generally, it was found in drug combination experiments that the drugs did not influence each other to a meaningful extent.

Discussion and conclusions : For prediction of bactericidal activity, interpretation of the radiometrically-determined regrowth model needs to accommodate PAEs and the effect of subinhibitory concentrations. The validity of the mathematical logistic model is not clear. Technical aspects of future studies such as better organism dispersal, need to be improved to achieve a more reliable evaluation based on the logistic model. For drug combination studies, the radiometric regrowth model yielded findings that were difficult to interpret in relation to published data, reinforcing the need for the use of internationally standardized techniques which would give statistically reliable data.

The radiometrically-determined regrowth model showed good discrimination between the standard activities of anti-TB agents, correlating with clinical efficacy. It is simple to perform and could prove to be useful for the screening of candidate anti-TB drugs. Improved technical stringency and the evaluation of poorly active control drugs, are however needed before proof of validity of the model can be established.

Key terms

Anti-tuberculosis agents, *Mycobacterium tuberculosis,* post-antibiotic regrowth model, minimal inhibitory concentration, bactericidal activity, time-kill curve method, post-antibiotic effects, mathematical logistic model, drug carry-over effects, subinhibitory antimicrobial concentrations, drug combinations.

SAMEVATTING

Agtergrond :'n Na-blootstelling-hergroeimodel wat die aanwending van die gevestigde Bactec radiometriese stelsel benut en wat die *in vivo* omstandighede by die setel van indringende siekte naboots is ontwerp om die aktiwiteit van middels teen vermenigvuldigende *Mycobacterium tuberculosis* te bepaal.

Oogmerke : Die oogmerke van hierdie verhandeling was om (a) 'n radiometriese model wat middeldoeltreffendheid wat verband hou met die gesamentlike kiemdodende aktiwiteit en vertragings in hergroei as gevolg van die werking van anti-tuberkulose (TB) middels te konstrureer, (b) die kinetika van die kiemdodende werking van middels, alleen en in kombinasies, met behulp van die tyd-dodingskurwe metode, te vergelyk met die radiometriese-bepaalde hergroeimodel, en (c) bepaal of die Bactec radiometriese hergroeimodel die waarskynlike kiemdodende aktiwiteit van middels kan voorspel.

Ontwerp en Metodes : Middel-konsentrasies vir die tyd-dodingskurwe metode was binne die perke van behaalbare vlakke by die setel van infeksie en is in veelvoude van die minimum inhiberende konsentrasie (MIK), (1x, 2x or 3x, en 8x) gebruik. Blootstellingstye van 6u, 24u, 48u en 72u is gebruik en was gebaseer op farmakokinetiese data wat die waarskynlike duurtes van *in vivo* blootstelling in TB-letsels weerspieël. Gestandardiseerde inokula van ongeveer 10^6 kolonie-vormende eenhede (KVE)/ml van aktief vermeningvuldigende *M. tuberculosis* stamme is gebruik. Dieselfde middel-konsentrasies, blootstellingstye en bakterieële konsentrasies is gebruik vir die bepaling van radiometriese-bepaalde na-blootstelling-hergroeitye van *M. tuberculosis*. Groeitye is

aangeteken as die aantal dae wat verloop het om 'n vooropgestelde groei-indeksvlak (GI-vlak) in die Bactec-stelsel te bereik en is uitgedruk as T_{400}-lesings in dae. Eenvoudige liniêre regressie en 'n matematiese logistiese model is gebruik om te bepaal of die radiometriese na-blootstelling-hergroeimodel die kiemdodende aktiwiteit van middels kan voorspel.

Vir middelkombinasie-studies, is 1MIK van isoniasied (INH) en rifampisien (RMP) alleen en in kombinasie gebruik, terwyl 2MIK van etambutol (EMB), streptomisien (SM), ofloksasien (OFL) en amikasien (AMK) gebruik is in die kombinasie studies. Kolonietellings is gedoen na 0u en 24u blootstellings en hergroei-patrone is bepaal met die T_{400} metode. *M. tuberculosis* H37Rv is gebruik en daarna ook weerstandige stamme.

Resultate : INH en RMP was hoogs-kiemdodend terwyl EMB matige aktiwiteit in die tyd-dodingskurwe-metode getoon het. Die drie middels het die beste kurwes met die langste hergroeitye en meer onderdrukte groeitempo in die Bactec na-blootstelling-hergroeimodel vertoon. Met eenvoudige liniêre regressie is 'n liniêre verwantskap gevind tussen oorlewende bakterieë en radiometriese hergroei (T_{400}), vir al die middels wat getoets is. Selfs beter ooreenstemming is gevind wanneer kontrole-verwante hergroeitye, $(T-K)_{400}$, vir analise gebruik is. Toestande wat die liniêre verwantskap in die radiometriese hergroeimodel in die geval van OFL, en minder duidelik met EMB en AMK, gekompromiteer het, was waarskynlike postantibiotiese effekte (PAEs). Hierdie is deur die kort blootstellingstyd van 6u en middeloordrag effekte as gevolg van konsentrasies van $\geq$8MIK vir INH, RMP en SM (10 en 20 MIKs) teweeg gebring. Die matematiese logistiese model het goeie korrelasie met die kiemdodende aktiwiteit en die hergroei vir INH en

RMP, maar nie vir EMB, SM, OFL en AMK nie, getoon.

Middelkombinasie-effekte van die twee tegnieke het van die kriteria vir sinergisme afgehang. In die algemeen is gevind dat in middel kombinasie eksperimente, die middels mekaar nie noemenswaardig beinvloed het nie.

Bespreking en Gevolgtrekkings : Vir die voorspelling van kiemdodende aktiwiteit moet die interpretasie van die radiometriese-bepaalde hergroeimodel, die PAEs en die effek van subinhiberende konsentrasies akkomodeer. Die geldigheid van die matematiese logistiese model is nie duidelik nie. Tegniese aspekte in toekomstige studies, soos beter organisme verspreiding, moet verbeter word as 'n meer betroubare evaluering van die logistiese model verkry wil word. Vir middelkombinasie-studies, was die radiometriese hergroeimodel bevindinge moeilik om te interpreteer in die lig van gepubliseerde data, wat weereens die noodsaaklikheid beklemtoon van internasionaal-gestandardiseerde tegnieke wat statisties-betroubare data sal verseker.

Die radiometriese-bepaalde hergroeimodel het goeie diskriminasie tussen standaard aktiwiteite van anti-TB agense getoon en het goed gekorreleer met kliniese effektiwiteit. Dit is maklik om te gebruik en behoort handig te wees vir die sifting van anti-TB middels. Verbeterde tegniese strengheid, en die evaluering van kontrole middels met swak aktiwiteit is egter nodig voor die geldigheid van die model bevestig kan word.

Sleutelterme : Anti-TB middels, *Mycobacterium tuberculosis*, na-blootstelling hergroeimodel, minimum inhiberende konsentrasie, kiemdodende aktiwiteit, tyd-dodingskurwe metode, postantibiotiese

effekte, matematiese logistiese model, middel-oordrageffekte, subinhiberende konsentrasies, middelkombinasies.

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ABBREVIATIONS

ADC	Albumin-dextrose-catalase
AIDS	Acquired immunodeficiency syndrome
AMK	Amikacin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the killing curve
CERT	Control-related regrowth time
CFU	Colony forming units
CV	Coefficient of variation
d	day
EBA	Early bactericidal activity
EMB	Ethambutol
FDA	Food and Drug Administration
GI	Growth index
GV	Growth value
h	hour
HIV	Human immunodeficiency virus
INH	Isoniazid
LJ	Löwenstein Jensen
MDR	Multidrug resistant
MDR-TB	Multidrug resistant tuberculosis
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MRC	Medical Research Council
NJCIRM	National Jewish Centre for Immunology and Respiratory Medicine
OFL	Ofloxacin
PA	Postantibiotic phase
PAE	Postantibiotic effect

PAS	Para-aminosalicylic acid
PZA	Pyrazinamide
r	Correlation coefficient
R^2	Coefficient of determination
RMP	Rifampicin
SCC	Short course chemotherapy
SCID	Severe combined immunodeficiency
SD	Standard deviation
SEM	Standard error of the mean
SM	Streptomycin
s^2	Pooled error mean square
T_{400}	Time (in days) it takes for drug exposed and unexposed *Mycobacterium tuberculosis* cultures to reach a growth index reading (GI) in the Bactec TB-460 instrument of 400.
$(T-C)_{400}$	Difference between T_{400} growth times in days between drug exposed and non exposed *M. tuberculosis* cultures.
TBRP	Tuberculosis Research Programme

CHAPTER 1

LITERATURE REVIEW

1.1 HISTORY OF TUBERCULOSIS

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis*, an agent that has accounted for more human deaths than has any other pathogen (Cole,1994).

1.1.1 *Historical perspective of the disease*

TB is one of the oldest human diseases (Shaw, 1995). It is known to have existed at least since 2 000 BC, and perhaps much longer (Tynes, 1993). During the course of recorded history, TB had been referred to as consumption, phthisis, the white plague or wasting disease, in describing the wasting character, cough and fever of this disease (Rubin, 1995; Daniel *et al*, 1994; Tynes, 1993). In the 17th century, the term tubercle was first used by Fransiscus Silvius to describe the knobby lesions found in the lungs of patients who had died of the wasting disease. The term TB seems first to have been used in 1839 by Jonam Schöhlein (Anonymous, 1993).

TB did not become a major problem for humans until the industrial revolution (1750 - 1848), when urban overcrowding created an ideal environment for the spread of TB (Shaw, 1995) and led to an increase in cases (Daniel *et al*, 1994; Bates and Stead, 1993). However, at that time, the aetiology of the disease was not known. It was in 1882 that the German physician, Robert Koch, described the tubercle bacillus, *M. tuberculosis* (Daniel *et al*, 1994; Tynes, 1993; Anonymous, 1993), an organism still known to many as the Koch's bacillus. He convincingly demonstrated it to be the cause of

TB and introduced the famous Koch´s postulates required to prove pathogenicity (Daniel *et al,* 1994; Tynes, 1993).

TB was common in European cities during the first half of the 19th century and one-fifth to one-quarter of all deaths at that time were due to this disease (Tynes, 1993; Daniel *et al,* 1994). Despite the fact that TB was spreading rapidly in Europe, it remained essentially unknown in sub-Saharan Africa as late as the beginning of the 20th century. A number of medical observers reported complete absence of TB in the interior of sub-Saharan Africa and found no TB in parts of South Africa as late as the first half of the 20th century (Daniel *et al,* 1994; Bates and Stead, 1993).

1.1.2 *Treatment of tuberculosis*

Treatment of TB over the centuries has involved a variety of approaches. These can be divided into three eras, namely, the so-called classical approach, the sanatoria and the modern chemotherapy era, respectively (Pesanti, 1995; Shaw, 1995).

For centuries treatment of TB relied on combinations of dietary restrictions, choice of proper climate and cough palliation. With the development of chemistry as a science in the 19th century, a variety of specific treatments such as iodine, creosote and a host of others, were introduced and combined with these approaches, the so-called classical approaches. Later, came the movement to provide benefits of nutrition, rest and clean air in sanatoria, the first being founded in 1854 by Behmer in Germany (Pesanti, 1995). Pneumothorax or collapse therapy became almost an invaluable accompaniment to the sanatorium treatment of TB (Pesanti, 1995; Shaw, 1995).

In the 1930's experiments in guinea pigs showed that sulphonamide

inhibits the growth of *M. tuberculosis*. Although none of the animals was cured, experiments had suggested that chemotherapy was an achievable goal (Pesanti, 1995).

It was not until 1943 that Selman Waksman, a microbiologist who was a pioneer researcher of soil microorganisms (Shaw, 1995) isolated *Streptomyces griseus*, an organism that produced streptomycin (SM) (Tynes, 1993), the first antibiotic effective against *M. tuberculosis* (Shaw, 1995; Grange *et al*, 1994; Zhang and Young, 1994; Anonymous, 1993). Waksman won the 1952 Nobel Prize for Medicine (Anonymous, 1993).

When SM was first introduced as chemotherapy, it was soon recognised that treatment with a single drug led to selection of resistant strains of bacteria (Youmans *et al*, 1946). Subsequent experiments by investigators such as Crofton and Mitchison (1948), brought expansion of knowledge regarding SM resistance in mycobacteria and the understanding of the necessity for employing two effective drugs. Thus, the search for additional agents continued (Bates, 1995). Soon afterwards, para-aminosalicylic acid (PAS) (1949) and isoniazid (INH) (1952) followed (Bates, 1995; Tynes, 1993; Bloom and Murray, 1992).

Multidrug therapy was introduced and it then became possible to prevent the selection of resistant mutants in a majority of TB cases with a combination of SM, PAS and INH. In the 1950's treatment with this three-drug combination became the rule and constituted the major thrust of anti-tuberculosis (anti-TB) treatment (Grosset, 1990). Although the three-drug regimen of SM, PAS and INH was capable of preventing the selection of resistant mutants, it was not capable of killing rapidly all or almost all the organisms in the

lesions. A number of organisms still survived despite 6-12 months of treatment with the three-drug combination. As a result, treatment was prolonged to 18-24 months to prevent recurrence of the disease (Grange *et al,* 1994; Grosset, 1990). Despite such prolonged treatment, the relapse rate after discontinuation of treatment was found to be 4% indicating that INH, although active against intracellular organisms, has a poor bactericidal activity against persisters, that is, organisms responsible for relapses after treatment was stopped (Grosset, 1990).

The efficacy of chemotherapy improved in the early seventies when rifampicin (RMP) was introduced. RMP was found to be effective not only against multiplying organisms but also against persisting organisms. A combination of RMP, INH and SM effected the cure of almost 100% of patients when treatment was continued for 9 months. A few years later, pyrazinamide (PZA) was rediscovered and the combination of PZA, INH and RMP further increased the efficacy of chemotherapy, that is, the introduction of PZA made it possible to decrease the period of treatment to 6 months. These findings led to the recommendations of a 6-month short course chemotherapy (SCC) for TB. Chemotherapy became the sole treatment of almost all forms of TB, relegating other forms of medical or surgical care in institutions to past history (Grosset, 1990).

The SCC includes an initial intensive phase of treatment which lasts for 2 months, in which patients are treated with three or four drugs, depending on the extent of primary drug resistance : RMP, INH, PZA and ethambutol (EMB) or SM, to ensure that mutants resistant to a single drug will not emerge, and a continuation phase of 4 months of dual therapy with INH and RMP to kill any persisting organisms,

is being practised (Cole, 1994).

The introduction of effective treatment of TB in the 1950's and 1960's raised hopes that the disease would soon be controlled or eliminated. A reduction in incidence was achieved in most industrialized countries (Zhang and Young, 1994). However, today, 40 years after the introduction of chemotherapy for TB (WHO, 1993) it has remained the predominant cause of mortality and morbidity worldwide affecting 8-10 million (Strull and Dym, 1995) and killing 2-3 million people each year (Zhang and Young, 1994; WHO, 1993). In South Africa, it accounts for more than 80% of all communicable diseases notified and is regarded as one of the most serious health problems affecting the country (Weyer *et al*, 1995).

The re-emergence of this infectious disease can be attributed to and is associated with a variety of factors including, the growing epidemic of HIV infection, (Shaw, 1995; Zhang and Young, 1994; WHO, 1993; Van Scoy and Wilowske, 1992) poverty associated with overcrowding and poor nutrition, and increasing numbers of refugees (Shaw, 1995). Multidrug resistant (MDR) strains of *M. tuberculosis* have been reported before (Canetti, 1965). However, the recent active transmission of MDR strains poses new challenges to the effective treatment of TB (Zhang and Young, 1994).

1.2   EVALUATION OF TREATMENT

1.2.1 *Properties of drugs required for optimal treatment*
Optimal use of antimicrobial agents in the chemotherapy of TB demands consideration of a number of important factors that may influence the choice of an appropriate agent.

1.2.1.1   General properties

An ideal anti-TB agent needs to be of low cost and low toxicity (Grassi and Peona, 1995; WHO, 1991). In addition, it must have a high degree of potency and a long half-life, one of the properties that make intermittent schedules possible, that is, schedules where drugs are given less frequently than daily (Mitchison, 1992).

Intermittent schedules have several potential advantages over daily schedules : full supervision of drug toxicity is easier to arrange, drug costs are reduced and chronic toxicity is often lower (Mitchison, 1992). To be suitable for intermittent dosage, a drug ideally should possess the ability to produce a postantibiotic effect (PAE) on bacteria (Heifets, 1991a; Dickinson and Mitchison,1966). PAE is defined as a period of persistent suppression of bacterial growth after a short antimicrobial exposure (Heifets, 1991a). It means that even though not all microorganisms are killed, they will not begin to proliferate again for a number of hours after exposure to a concentration above the minimal inhibitory concentration (MIC) (Harold, 1987).

It should however be emphasized that drugs with very long half-lives such as rifapentine would be suitable for intermittent therapy irrespective of PAE, provided they have sufficient activity against the infecting *M. tuberculosis* strains, but PAE could to some extent compensate or enhance the efficacy of the drugs with less optimal pharmacokinetic properties (Koornhof , personal communication).

One of the decisions that must be made in the chemotherapy of infectious diseases is whether a bacteriostatic agent is adequate or whether bactericidal therapy is required (Harold, 1987). In TB infections an ideal antimicrobial agent needs two important

properties. One is to possess potent early bactericidal activity (EBA), that is, the ability to decrease rapidly the number of metabolically active bacilli in sputum during the first days of therapy, an important factor for shortening the period during which the patient is most infectious to others (Heifets, 1994; WHO, 1991). The second property is the sterilizing activity of a drug which involves the ability to eliminate or substantially decrease the number of bacteria in semi-dormant subpopulations (Heifets, 1994; WHO, 1991), thus shortening the overall period of therapy (WHO, 1991). In addition, emergence of resistant organisms should not develop readily to an ideal drug, that is, the mutation rate to resistance should be low (Grassi and Peona, 1995; WHO, 1991). Treatment with bacteriostatic agents has an unacceptable relapse rate. In contrast, when a bactericidal agent (with potent bactericidal and sterilizing activity) is used in schedules, the relapse rate can be reduced to a minimum (Davidson and Le, 1992).

Another important factor in the optimization of TB therapy is that an antimicrobial agent should be able to address conditions in lesions, for example, environmental pH at which the drug should be active, and metabolic activity of tubercle bacilli in the lesion. The ability to penetrate the tissues including macrophages which habour viable tubercle bacilli, would also be advantageous. The differing actions of drugs in the chemotherapy of TB is dependent on such conditions which differ in different sites (Kucers and Bennett,1987)

Examples of anti-TB agents whose activity is pH-dependent are SM and PZA. SM is only active in the neutral or slightly alkaline environment of pulmonary lesions and is virtually inactive in macrophages because of poor penetration into cells. On the other hand, PZA has no activity in cavities or in the neutral environments

of closed caseous lesions, but is uniquely active in acidic environments in TB lesions (Kucers and Bennett, 1987)

Tubercle bacilli are susceptible to most anti-TB drugs only when they are replicating. They multiply actively in areas where the oxygen tension is high such as extracellularly in pulmonary cavitary lesions. In macrophages or intracellularly in closed caseous lesions, the oxygen tension is low and as such tubercle bacilli multiply slowly or intermittently. An ideal anti-TB drug should thus not only be effective against extracellular organisms which may be multiplying actively, but should also be active against intracellular organisms that multiply slowly or intermittently (Kucers and Bennett,1987).

Many infections are caused by microorganisms that are readily destroyed when they are ingested by macrophages. In contrast, *M. tuberculosis* can survive within these phagocytic cells. An ideal antimicrobial agent should thus be able to penetrate phagocytic cells to be successful in eradicating infection caused by *M. tuberculosis* (Harold, 1987).

However, no known chemotherapeutic agent possesses all of these properties to a degree that renders it sufficient for monotherapy of TB cases. Hence, at least 3 drugs are administered for the first 2 months in particular, to meet these conditions as well as to prevent emergence of resistance, and treatment is continued with at least 2 drugs for several months longer before there can be reasonable assurance for cure (WHO, 1991). It follows therefore that the secret of achieving optimal therapy lies in combining the various anti-TB drugs correctly.

1.2.2 *Properties of standard anti-tuberculosis drugs*

There are nine drugs approved by the Food and Drug Administration (FDA) in the United States of America and most developed countries for the treatment of TB. These are PAS, capreomycin, cycloserine, EMB, ethionamide, INH, PZA, RMP and SM while there are a few additional experimental agents available (Peloquin, 1993).

Approved antimicrobial agents are divided into two major categories, that is, first line and second line agents. The first line or standard agents combine the greatest level of efficacy with an acceptable degree of toxicity and include INH, RMP, PZA, EMB and SM. A large majority of patients can be treated successfully with these drugs. Occasionally, however, because of microbial resistance especially when patient-related factors such as HIV infection or AIDS play a role, second line agents may be used in addition so that treatment may be initiated with 5 or 6 drugs. This category of alternative or supplementary agents include, ofloxacin (OFL), ciprofloxacin, ethionamide, PAS, cycloserine, amikacin (AMK), kanamycin and capreomycin (Mandell and Petri, 1996).

1.2.2.1   Isoniazid

INH, also referred to as isonicotinic acid hydrazide (Barber *et al,* 1995; WHO, 1993; Van Scoy and Wilowske, 1992) is a relatively inexpensive, well tolerated and a very valuable agent for the treatment of TB (Barber *et al,* 1995; Houston and Fanning, 1994). It has remarkable specificity for mycobacteria, in particular, *M. tuberculosis* (Zhang and Young, 1994) and concentrations in excess of 500 *u*g/ml are required to inhibit other microorganisms (Mandell and Petri, 1996). Among the non-tuberculous or atypical mycobacteria, only *M. kansasii* is susceptible but at higher concentrations than those effective for *M. tuberculosis* (Mandell and

Petri, 1996; Van Scoy and Wilowske, 1992).

It penetrates cells with ease and is effective against bacilli growing intracellularly (Mandell and Petri, 1996). INH is mycobactericidal (Barber *et al*, 1995; Strull and Dym, 1995; Yokishawa and Fujita, 1982) with its greatest activity being against actively replicating mycobacteria. Resting or slowly growing organisms are only inhibited but not killed by this drug (Mandell and Petri, 1996; Barber *et al,* 1995; WHO, 1993; Yokishawa and Fujita, 1982). It has been found to be more active than other first line agents in the EBA against TB (Barber *et al*, 1995).

Its mechanism of action is unknown (Mandell and Petri, 1996; Barber *et al,* 1995). However, several hypotheses have been proposed. These include effects on lipids, nucleic acid biosynthesis and glycolysis (Mandell and Petri, 1996). Inhibition of the biosynthesis of mycolic acids, unique constituents of the mycobacterial cell wall, has been suggested as the primary action of INH (Mandell and Petri, 1996; Barber *et al,* 1995; Van Scoy and Wilowske, 1992; Yokishawa and Fujita, 1982). Because mycolic acids are unique to mycobacteria, this action would explain the narrow spectrum of INH activity (Mandell and Petri, 1996; Barber *et al,* 1995; Zhang and Young, 1994).

Development of resistance to INH has been associated with point mutations within two mycobacterial genes, that is, the Kat G gene, encoding the catalase peroxidase enzyme (Zhang and Young, 1994) and the inhA gene encoding a 32 Kd protein with homology with a bacterial enzyme involved in mycolic acid biosynthesis (Mandell and Petri, 1996; Zhang and Young, 1994).

INH is relatively non-toxic and may be used as intermittent chemotherapy of TB (Mandell and Petri, 1996). It is readily absorbed when administered orally or parenterally (Mandell and Petri, 1996; Barber *et al*, 1995) with peak levels of 3-7 *u*g/ml (Barber *et al*,1995) being achieved 1 to 2 hours (Mandell and Petri, 1996; Van Scoy and Wilowske, 1992) after an oral dose of 300 mg (Barber *et al*, 1995). INH diffuses readily into all body tissues and fluids (Mandell and Petri, 1996; WHO, 1993) and is metabolised in the liver by acetylation which is genetically determined (Strull and Dym, 1995; Van Scoy and Wilowske, 1992; Yokishawa and Fujita, 1982).

The plasma half-life varies from less than 1 hour in fast acetylators to more than 3 hours in slow acetylators (WHO, 1993). Hepatotoxicity is the most common side-effect associated with INH (Strull and Dym, 1995; Houston and Fanning, 1994; Yokishawa and Fujita, 1982).

1.2.2.2   Rifampicin

RMP is one of the rifamycin antibiotics, a group of agents produced by *Streptomyces mediterranei*, and is a semisynthetic derivative of rifamycin B (Mandell and Petri, 1996; Davidson and Le, 1992; Van Scoy and Wilowske, 1992; WHO, 1991; Yokishawa and Fujita, 1982). It is an essential element of modern SCC (Houston and Fanning, 1994) and along with INH is central to current TB therapy (Zhang and Young, 1994). Unlike INH, RMP has a broad spectrum activity (Barber *et al*, 1995; Houston and Fanning, 1994; Zhang and Young, 1994; Van Scoy and Wilowske, 1992; Goldberger, 1988). However, it is best known for its activity against mycobacteria (Goldberger, 1988). Widespread use of RMP for non-mycobacterial infections could result in increasing resistance to *M. tuberculosis* (Barber *et al*, 1995; Houston and Fanning, 1994). *In*

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vitro, this drug has the broadest spectrum of any of the primary anti-TB drugs. It inhibits almost all strains of *M. tuberculosis, M. kansasii, M. marinum* and a few strains of *M. avium* organisms (Van Scoy and Wilowske, 1992).

RMP has bactericidal action (Barber *et al,* 1995; Strull and Dym, 1995; WHO, 1991) and potent sterilizing effect against tubercle bacilli in both cellular and extracellular locations (Strull and Dym, 1995; Houston and Fanning, 1994; Davidson and Le, 1992; WHO, 1991) and is noted for its rapid onset of bactericidal activity (Strull and Dym, 1995; Davidson and Le, 1992). It is effective against rapidly dividing mycobacteria (Yokishawa and Fujita, 1982) and is able to kill semi-dormant organisms that have short periods of active metabolism (Barber *et al,* 1995; Yokishawa and Fujita, 1982).

Its bactericidal activity derives from its capacity to inhibit DNA-dependent RNA polymerase (Mandell and Petri, 1996; Heifets, 1994; Davidson and Le, 1992; Yokishawa and Fujita, 1982). More specifically, RMP acts on the ß-subunit of this enzyme by binding to it and thus forming a stable drug-enzyme complex (Zhang and Young, 1994). Initiation of RNA synthesis rather than the synthesis of RNA in progress, is suppressed (Mandell and Petri, 1996; Barber *et al,* 1995; Yokishawa and Fujita, 1982). Development of resistance to this drug may be rapid as a one-step process (Drobniewski *et al,* 1994; Mandell and Petri, 1996) and is due to an alteration of its target DNA-dependent RNA polymerase (Mandell and Petri, 1996; Barber *et al,* 1995). More specifically, microbial resistance is conferred principally by point mutations within the rpo B gene which encodes the ß-submit of the enzyme (Cole, 1994; Zhang and Young, 1994).

RMP is well absorbed when taken orally and peak serum concentrations of about 7 ug/ml (Mandell and Petri, 1996; Van Scoy and Wilowske, 1992) with a range of 4-32 ug/ml (Barber *et al*, 1995) are achieved 2 to 4 hours after administration of a 600 mg dose (Van Scoy and Wilowske, 1992). It is distributed throughout the cellular tissues and body fluids (Strull and Dym, 1995; Davidson and Le, 1992; WHO, 1991) and reaches therapeutic levels in many body fluids and organs including the lungs (Mandell and Petri, 1996; Barber *et al*, 1995; Van Scoy and Wilowske, 1992).

RMP is metabolised in the liver by deacetylation into an active metabolite and its half-life is 2 to 3 hours (Barber *et al*, 1995; WHO, 1991). It is suitable for intermittent administration schedules (Goldberger, 1988) and is 80%-90% protein bound (Barber *et al*, 1995). This anti-TB agent is generally well tolerated (Mandell and Petri, 1996; Houston and Fanning, 1994). It displays a variety of types of toxicity and like INH, it can also cause hepatotoxicity (Strull and Dym, 1995; Houston and Fanning, 1994; Van Scoy and Wilowske, 1992; Goldberger, 1988), which can be enhanced with INH (Davidson and Le, 1992; Van Scoy and Wilowske, 1992; Goldberger, 1988). Other adverse effects (skin rashes, fever, influenza - like syndrome et cetera, (Mandell and Petri, 1996; Strull and Dym, 1995; Davidson and Le, 1992) are more likely to occur with intermittent administration (WHO, 1991).

1.2.2.3 Pyrazinamide

PZA is a synthetic pyrazine analogue of nicotinamide (Mandell and Petri, 1996; Barber *et al*, 1995). It is now the third most important drug in modern therapy of TB after INH and RMP (Heifets, 1994; Goldberger, 1988). PZA has a high degree of activity, is inexpensive and can be taken orally (Goldberger, 1988).

It was first introduced in 1952, but it was not considered an important component in TB therapy until after 1980 when its unique role in accelerating the sterilizing effect, in combination with INH and RMP, was reported (British Thoracic Association, 1984; Snider *et al*, 1982; SingaporeThoracic Service/British Medical Research Council, 1981). PZA is highly effective during the first two months of treatment while acute inflammatory changes persist (WHO, 1991) and is less effective afterwards. Hence it is often discontinued after two months of therapy (Barber *et al*, 1995).

It is particularly active against tubercle bacilli in an acid environment both intracellularly and extracellularly in highly inflamed tissue (Davidson and Le, 1992). PZA is weakly bactericidal against *M. tuberculosis* but has potent sterilizing activity (WHO, 1991). The effect of this drug is usually associated with its activity against the semi-dormant bacterial population persisting in the low pH of the intracellular environment, in early acute inflammation sites (Barber *et al*, 1995; Strull and Dym, 1995; Heifets, 1994; Houston and Fanning, 1994; WHO, 1991). It has little, if any, activity at higher pH levels (Barber *et al*, 1995).

PZA is a highly specific agent for *M. tuberculosis*, but not *M. bovis*. It is inactive against most non-mycobacterial organisms (Barber *et al*, 1995). Its mechanism of action is not known (Mandell and Petri, 1996; Heifets, 1994), however, it has been suggested that susceptible *M. tuberculosis* strains produce an enzyme, pyrazinamidase which converts PZA to pyrazinoic acid, and it is proposed that this enzyme-generated product is the actual antibacterial moiety of the drug. Strains resistant to PZA do not produce this enzyme and are therefore not vulnerable to PZA (Heifets, 1994).

Pyrazinoic acid has anti-TB activity and lowers intracellular pH, inhibiting mycobacterial growth. It has therefore been suggested that the antimicrobial activity of PZA is a combined effect of the specific activity of pyrazinoic acid and its ability to lower the pH below the limits of tolerance of *M. tuberculosis* (Barber *et al*, 1995; Heifets, 1994). However, such hypotheses require experimental confirmation (Heifets, 1994).

Resistance develops rapidly when PZA is used alone (Mandell and Petri, 1996) and it may be due to the loss of activity of the enzyme, pyrazinamidase (Barber *et al,* 1995). Little is known about the molecular basis of resistance to PZA (Cole, 1994).

PZA is readily absorbed from the gastrointestinal tract and is rapidly distributed throughout all body tissues and fluids including the liver, lungs and CSF (Mandell and Petri, 1996; Barber *et al*, 1995; Strull and Dym, 1995; Van Scoy and Wilowske, 1992; WHO, 1991). Peak plasma concentrations of 45 ug/ml occur approximately 2 hours after an oral dose of lg (Van Scoy and Wilowske, 1992) and the plasma half-life is about 10 hours (Barber *et al,* 1995; WHO, 1991). PZA penetrates well intracellularly and its activity at acid pH makes it uniquely qualified in the treatment of intracellular tubercle bacilli (Barber *et al,* 1995).

It is metabolised in the liver and is excreted largely in the urine (WHO, 1991). The most common and serious side effect is hepatotoxicity which appears in 15% of patients (Mandell and Petri, 1996; Goldberger, 1988). Other side effects include arthralgias, anorexia, nausea and vomiting, dysuria, malaise and fever (Mandell and Petri, 1996; Strull and Dym, 1995).

1.2.2.4   Ethambutol

EMB is a synthetic, orally administered agent that has replaced PAS as a second, third or fourth drug in multidrug regimens (Van Scoy and Wilowske, 1992). It is not essential to the 6-month treatment regimens that have become first line treatment in most industrialized countries except when the incidence of resistance to primary treatment drugs (INH, RMP and PZA) is high (Houston and Fanning, 1994; Combs *et al*, 1990).

Its main use is as a companion drug, to prevent development of resistance to more potent agents, that is, INH and RMP (Houston and Fanning, 1994; Mitchison, 1985). According to Jindani *et al*, 1980), EMB is considered to be one of the anti-TB drugs with substantial EBA, second to INH (Heifets, 1994; Davidson and Le, 1992).

It is highly specific for mycobacteria (Mandell and Petri, 1996; Barber *et al*, 1995) and is effective against only actively multiplying cells (Heifets, 1994; Van Scoy and Wilowske, 1992; Yokishawa and Fujita, 1982). Nearly all strains of *M. tuberculosis* and *M. kansasii* as well as the majority of *M. avium* complex strains are sensitive to EMB. The sensitivities of the other atypical mycobacteria are variable (Mandell and Petri, 1996).

Various reports have suggested that it is bacteriostatic (Mandell and Petri, 1996; Barber *et al*, 1995; Strull and Dym, 1995; Van Scoy and Wilowske, 1992). On the other hand other studies (Gangadharam *et al*, 1990; Crowle *et al*,1985) have shown that EMB has a bactericidal effect both intracellularly and extracellularly at a concentration of 5 to 10 *u*g/ml which can be achieved clinically.

Although the precise mechanism of action of EMB is not known, this drug has been shown to inhibit the incorporation of mycolic acids into the mycobacterial cell wall (Mandell and Petri, 1996). Little is known about the molecular basis of resistance to this drug (Cole, 1994).

EMB is administered by the oral route and is suitable for use in regimens whereby chemotherapy is given intermittently for the treatment of TB (Davidson and Le, 1992). It is well absorbed after oral administration (Van Scoy and Wilowske, 1992) and is rapidly and widely distributed into most cells, tissues and body fluids (Davidson and Le, 1992).

Peak serum concentrations of 2 to 5 *u*g/ml are achieved 2 to 4 hours after administration of a single dose of 15 mg/kg (Barber *et al*, 1995; Heifets, 1994; Van Scoy and Wilowske, 1992). EMB is metabolized in the liver (Barber *et al*, 1995; Strull and Dym, 1995) and its half-life is 3 to 4 hours (Mandell and Petri, 1996; Barber *et al*, 1995; Davison and Le, 1992). It is a relatively safe drug. The major side effect is optic neuritis which occurs in 5% and 15% of patients taking 25 and 50 mg/kg/day, respectively, but rarely or in 1% of patients taking a dose of 15 mg/kg/day (Mandell and Petri, 1996; Houston and Fanning, 1994). Other side effects include rash, headache, gastrointestinal upset, reduction in visual acuity, fever, pruritis and joint pain (Mandell and Petri, 1996; Strull and Dym, 1995).

1.2.2.5 Streptomycin

SM was the first effective anti-TB agent (Mandell and Petri, 1996; Barber *et al*, 1995; Goldberger, 1988). It is now the least used of the first line agents in the therapy of TB (Mandell and Petri, 1996).

At present it is utilized for treatment of resistant organisms and multidrug SCC (Goldberger, 1988). It belongs to a group of antibiotics known as aminoglycosides (WHO, 1991).

SM has a broad spectrum of activity being active against mycobacteria and a variety of Gram-positive and Gram-negative bacteria (Davidson and Le, 1992; WHO, 1991). It was found to be active only against actively multiplying mycobacteria (Heifets, 1991a) and is bactericidal against tubercle bacilli (Barber *et al,* 1995; Strull and Dym, 1995).

Its activity is pH-dependent, with potent anti-TB activity against bacterial populations in lesions where the pH is neutral or alkaline, but not intracellularly, due to poor penetration (Barber *et al,* 1995; Strull and Dym, 1995; Houston and Fanning, 1994).

It achieves its bactericidal activity by binding irreversibly to the 30S subunit of the ribosome, resulting in a misreading of the mRNA codon and consequently inhibiting protein synthesis (Barber *et al,* 1995; Strull and Dym, 1995; Edson and Terrell, 1991).

As in other eubacteria, high level resistance to SM in *M. tuberculosis* results predominantly from missense mutations in the rps L gene, which encodes the ribosomal protein S12. A small minority of clinical isolates have mutations in conserved loops of the 16S rRNA, which is encoded by the rrs gene. However, in 30% of SM resistant isolates of *M. tuberculosis,* there are no mutations in either the rps L and rrs genes. Such strains have been found to have a low level of resistance by MIC determination (Cole, 1994).

SM is poorly absorbed after oral administration because of its highly

polar water soluble structure. Therefore, like other aminoglycosides, it must be given parenterally to achieve adequate serum levels (Mandell and Petri, 1996; Davidson and Le, 1992; Edson and Terrell, 1991; Driver and Worden, 1990). Disadvantages to the use of this drug include the need for the parenteral route of administration as well as its significant ototoxicity (Houston and Fanning, 1994; Goldberger, 1988).

It is not absorbed from the gastrointestinal tract but, after intramuscular administration it diffuses readily into the extracellular component of most body tissues and attains bactericidal concentrations, particularly in TB cavities (WHO, 1991). It does not readily enter living cells and thus cannot kill intracellular organisms (Mandell and Petri, 1996; Barber *et al,* 1995).

Peak plasma concentrations of 25 to 30 *u*g/ml are achieved 30 to 90 minutes after an intramuscular injection of 1g of SM (Davidson and Le, 1992). The plasma half-life is 2 to 3 hours (Mandell and Petri, 1996; Davidson and Le, 1992; WHO, 1991). The most frequent adverse reactions are hypersensitivity and ototoxicity, occurring mostly in patients over 40 years of age and in infants (Davidson and Le, 1992).

1.2.3 *Properties of alternative drugs for tuberculosis*
There are instances where anti-mycobacterial therapy results are inadequate as a result of drug toxicity, poor compliance by the patient, and primary drug resistance. Therefore, agents with low toxicity, low cost and high efficacy against MDR-TB strains are needed (Mandell and Petri, 1996).

Antimicrobial agents that may hold promise in the treatment of TB,

alternative drugs, include members of the rifamycin, fluoroquinolone, aminoglycoside and macrolide (in particular roxithromycin) drug classes, as well as ß-lactam/ß-lactamase combinations (Blumberg, 1995). Only the first three drug classes will be discussed.

1.2.3.1   Rifamycins

RMP has been one of the most effective drugs for the treatment of TB for decades. However, recently, the incidence of RMP resistant strains of *M. tuberculosis* has been rising (Dickinson and Mitchison, 1981). Furthermore, with the search of widely spaced intermittent dosage regimens of TB treatment, it has been shown to have serious side effects when administered less than twice weekly (Parenti, 1989; Girling, 1977; Poole *et al*, 1971), but was relatively safe when given two or three times a week (Dutt *et al*, 1979). This led to the development of new rifamycins (Grassi and Peona, 1995) such as rifabutin and rifapentine which have been shown to be active against *M. tuberculosis* (Bermudez and Young, 1995).

Rifapentine, also known as cyclopentyl rifamycin SV, DL473 and MDL 473, is a derivative of rifamycin SV (Dickinson and Mitchison, 1987). Like RMP, it has activity against a broad spectrum of pathogenic microorganisms (Bermudez and Young, 1995). Its half-life in humans is much larger than that of RMP (Grassi and Peona, 1995; Bermudez and Young, 1995; Houston and Fanning, 1994; Dickinson and Mitchison, 1987).

Experiments performed by Dickinson and Mitchison (1987) have suggested that rifapentine might be useful in the treatment of pulmonary TB if regimens employing widely spaced intermittent dosage are used. In experimental TB infection in mice, rifapentine is as active given once per week as RMP given daily (Davidson and Le,

1992; Truffot-Pernot *et al*, 1983).

Another advantage of rifapentine over RMP is its ability to accumulate within macrophages, which is reported to be approximately 60 times greater than in the extracellular fluid, whereas RMP accumulates only five fold (Bermudez and Young, 1995). However, rifapentine shows no activity against RMP-resistant strains (Grassi and Peona, 1995; Woodley and Kilburn, 1982).

Rifabutin is a semisynthetic derivative of rifamycin S (Bermudez and Young, 1995). Like rifapentine, it has a larger elimination half-life than RMP (Houston and Fanning, 1994). It has been considered for use in patients with MDR-TB. Because it shares the same mechanism of action with RMP and, because, like RMP, a single-step mutation is responsible for resistance, rifabutin should not be relied upon to treat RMP-resistant isolates (Peloquin and Berning, 1994).

It has been shown that approximately 50% to 60% of *M. tuberculosis* strains that develop resistance to RMP appear to be resistant to rifabutin as well (Bermudez and Young, 1995; Woodley and Kilburn, 1982). RMP-resistant *M. tuberculosis* strains have various degrees of susceptibility to rifabutin (Heifets *et al*, 1988b).

Thus, the new rifamycin derivatives are more likely to be used as alternatives to RMP in the treatment of RMP-susceptible TB, particularly in intermittent dosing than as new drugs for the treatment of MDR-TB (Peloquin and Berning, 1994).

1.2.3.2   Fluoroquinolones

Fluoroquinolones are a class of orally absorbed antimicrobial agents (Wolfson and Hooper, 1989; Wolfson and Hooper, 1991) with a broad spectrum of activity including activity against mycobacteria (Davidson and Le, 1992; Rastogi and Goh, 1991; Wolfson and Hooper, 1989; Berlin *et al,* 1987; Smith, 1986).

They are bactericidal (Barber *et al,* 1995; Wolfson and Hooper, 1989) and achieve this bactericidal activity by inhibiting the function of DNA gyrase (Strull and Dym, 1995; Wolfson and Hooper, 1989) (an essential enzyme for DNA replication) (Houston and Fanning, 1994) which is to catalyze the introduction of negative supercoiling into circular bacterial DNA. The absence of such circular DNA in mammalian genomes explains the lack of an effect in human cells (Barber *et al,* 1995). Resistance to fluoroquinolones in *M. tuberculosis* is associated with mutations in the gyrA gene which encodes the A subunit of DNA gyrase (Cole, 1994).

Fluoroquinolones show good penetration into macrophages where they are both concentrated and retain a high degree of activity : a particularly important property in view of the ability of mycobacteria to survive and multiply within these phagocytic cells (Garcia-Rodriguez and Gomez-Garcia, 1993; Leysen *et al,* 1989).

Their bioavailability after oral administration, penetration into human macrophages, concentration in the respiratory tract (Houston and Fanning, 1994), long serum half-lives combined with preliminary clinical investigations, suggest that these drugs represent alternatives to standard agents for the treatment of infectious diseases (Walker and Wright, 1991) such as infections caused by mycobacteria, particularly when there is resistance to the latter

group of drugs (Garcia-Rodriguez and Gomez-Garcia, 1993; Berlin *et al*, 1987). However, the fluoroquinolones are much more expensive than most of the anti-mycobacterial drugs (Peloquin and Berning, 1994) and resistance may develop against them quite readily (Gillepsie and Kennedy, 1998).

Among the newer fluoroquinolones tested against mycobacteria, ciprofloxacin, OFL and sparfloxacin showed the highest activity against *M. tuberculosis* (Rastogi and Goh, 1991; Leysen *et al*, 1989). These drugs were found to penetrate and actively concentrate inside mammalian cells and were bactericidal against intracellularly growing tubercle bacilli but toxicity may preclude their general use (Bates, 1995). OFL seems to possess the best properties : levels in tissues and elimination half-lives are higher (Leysen *et al*, 1989).

Various studies have been done to understand the role of OFL in the therapy of TB. Studies performed by Kohno *et al* (1992), Tsukamura (1985) and Tsukamura *et al* (1985) have suggested that this drug may be useful in the treatment of MDR-TB. In these studies (Kohno *et al*, 1992; Tsukamura, 1985;Tsukamura *et al*, 1985) patients with isolates resistant to various drugs were treated with OFL, given once daily as monotherapy for 6 to 8 months, and culture conversion to negative was observed in some patients. However, these studies emphasized the need for clinical trials to further study the role of this drug in the treatment of MDR-TB.

Other aspects that warrant further investigation are the early bactericidal effects and the sterilizing activity of OFL (Kohno *et al*, 1992; Tsukamura *et al*, 1985). Because OFL has distinct activity against intracellular *M. tuberculosis* (Crowle *et al*, 1988) and that it

is concentrated within alveolar macrophages (Perea, 1990), it is likely that, like PZA, it has sterilizing activity, a characteristic that contributes to the success of SCC (Yew *et al*,1994).

Like other fluoroquinolones, OFL is well absorbed after oral administration. It has a bioavailability of near 100% (Barber *et al*, 1995). There is no standard duration or dosage of OFL for the treatment of TB (Sahoo, 1993) and peak serum concentrations of 10 *u*g/ml are achieved 4 hours after an oral administration of a 500 mg dose (Davidson and Le, 1992; Leysen *et al*, 1989). The serum half-life of OFL is 5 to 8 hours (Wolfson and Hooper, 1991). It is reported to rarely induce adverse effects such as nausea, insomnia, headache, dizziness and diarrhoea (Davidson and Le, 1992).

1.2.3.3   Aminoglycocides

The activities of various aminoglycocides, AMK, kanamycin, capreomycin as well as SM, have been previously tested and compared against *M. tuberculosis in vitro*. All four drugs were found to be bactericidal against this organism (Heifets and Lindholm-Levy, 1989).

AMK has been found to inhibit *M. tuberculosis* at concentrations lower than those for kanamycin or SM *in vitro* and to be more active than either of them in experimental TB in guinea pigs. It has therefore been suggested that a possibility exists that this drug would be of value in the treatment of MDR-pulmonary TB (Sanders *et al*, 1982).

Very few clinical studies on the efficacy of AMK in the treatment of pulmonary TB have been carried out. However, those that have been done could not give a clear picture of the efficacy of this drug

because very few patients were included in the studies. In addition, most of the patients developed serious side effects as a result of administration of AMK and/or did not respond to AMK (Allen *et al*, 1983).

AMK is a semisynthetic derivative of kanamycin A (Barber *et al*, 1995; Ristuccia and Cuhna, 1985). Like other aminoglycosides, it is bactericidal and its mode of action is similar to that of SM and other aminoglycosides (Ristuccia and Cuhna, 1985).

Little is known about the mechanisms of drug resistance involved (Cole, 1994). AMK is a broad spectrum antibiotic with activity against a variety of Gram-negative and Gram-positive organisms. It also has activity against mycobacteria including the rapidly growing species (Barber *et al*, 1995).

As an aminoglycoside, AMK is administered parenterally. It is rapidly and nearly completely absorbed when administered intramuscularly (Ristuccia and Cuhna, 1985) and peak serum levels of 10-30 ug/ml are reached after a 0.5-1g parenteral dose, with a postantibiotic residual effect (Barber *et al*, 1995).

AMK shares the same toxicities as other aminoglycosides, including nephrotoxicity and ototoxicity, the latter manifesting as hearing loss (Barber *et al*, 1995; Ristuccia and Cuhna, 1985). It is relatively inexpensive. In addition, like other aminoglycosides no oral form is available. This may limit its use in the treatment of MDR-TB (Allen *et al*, 1983).

Injectable drugs other than SM may represent an alternative in cases of resistance to this agent, but the choice should be based on the

actual data of drug susceptibility to any of these drugs, taking into account the possibility of cross-resistance among aminoglycosides (Heifets, 1994).

1.3   EVALUATION OF ANTI-TUBERCULOSIS DRUGS

1.3.1 *Background*

Emerging problems with the treatment of infections caused by *M. tuberculosis* require the development of new models *in vitro* as well as *in vivo* in which chemotherapeutic approaches can be tested.

The evaluation of the activity of anti-TB drugs against *M. tuberculosis* has been previously performed in various ways including : observations in patients, experiments *in vitro* and in animals (Paramasivan, 1994; Heifets, 1991a).

In vitro and animal experiments are designated as models because the various experimental components can be modified to evaluate their importance for the test outcome and the relevance to human TB. These experimental models have played and still play an eminent role in the development of anti-TB chemotherapy (Trnka and Mison, 1988a).

One of the principal tasks of these models is the screening of substances for antimicrobial activity. Positive results provide the basis for further research. The second principal task of these experimental models is the formulation of basic principles to guide a rational application of anti-TB drugs in man. Within this framework hypotheses are tested for the optimization of drug regimens from the aspects of efficacy, duration of treatment, type of drug application, prevention of bactericidal resistance et cetera (Trnka

and Mison, 1988a).

Animal models constitute the second level (second to *in vitro* models) of evaluation in the search of anti-TB drugs. Whereas *in vitro* the anti-mycobacterial activity of a given substance is tested, what is checked in the animal is its therapeutic activity (Trnka and Mison, 1988a). The mouse (Orme and Collins, 1994) and the guinea pig (McMurray, 1988) are examples of the several infection models that have been used in the study of experimental TB infection (McMurray, 1994; Orme and Collins, 1994).

In the early 1800s and 1900s the guinea pig was the most used experimental infection model for the study of infectious diseases and for more than 100 years this animal model has contributed to the battle against TB in both the clinical (its use in diagnosis is no longer widely employed) and research arenas. Evidence for the efficacy of new anti-TB drugs or drug combinations has been previously obtained with this model (McMurray, 1994).

The use of the mouse model in the chemotherapy of TB emerged in the 1940s at the time when the first effective chemotherapy for TB was under active development. At this time, it was realized that this model was cost-effective for the evaluation of drugs such as SM and that it was more accurate as a readout of drug than other animal models such as the guinea pig. This era soon became the golden age for TB research (Orme and Collins, 1994). The model has contributed a great deal to the development of SCC. It showed that when PZA and RMP were given with another drug to prevent emergence of resistant organisms, were uniquely able to sterilize the organs (Jindani *et al*, 1980).

More recently, with the advent of AIDS, animal models using immunodeficiency animals such as SCID and beige mice have been used to evaluate drugs. These models have however been used mainly against *M. avium* (Koornhof, personal communication).

In vitro, tubercle bacilli are cultivated in artificial media, which facilitates a direct study of the influence of drugs. In contrast to experiments in animals, *in vitro* models allow the investigation of many strains, study of the speed and pattern with which resistance to various drugs develops, study of cross-resistance with already known anti-TB drugs. Studies of effects of drug combinations on organisms can also be performed using the *in vitro* methods. This model remains indispensable at the first level of evaluation in search of anti-TB drugs (Trnka and Mison, 1988b).

In recent years experimental approaches using cultured murine or human phagocytic cells or cell lines have been developed. This type of *in vitro* model can be particularly useful for studies of the effect of anti-TB drugs on phagocytosed, intracellularly located tubercle bacilli. As facultatively intracellular parasites, tubercle bacilli are able to survive and multiply within these cells and even destroy them (Orme *et al*, 1994; Trnka and Mison,1988b).

The systematic investigation of anti-TB drugs includes as a last step controlled clinical trials. Such trials are time consuming, technically demanding and very expensive but are an absolute requirement of medicines control agencies worldwide. Their design depends to a large extent on the information derived from *in vitro* and *in vivo* experimental models (Trnka *et al*, 1988a).

1.4 OBJECTIVE AND FORMAT OF PRESENT STUDIES

In vitro techniques can be useful in the formulation of basic principles to guide a rationale application of antimicrobial drugs (Trnka and Mison, 1988a). Such methods were used in the present study to evaluate six anti-TB drugs against clinical isolates of *M. tuberculosis*.

The overall objective of this study was to explore laboratory approaches and methodologies for the evaluation of anti-TB drugs and drug combinations, with particular emphasis on constructing a simple, safe and rapid model which measures bacterial cell metabolism radiometrically. The model was used to determine drug efficacy relating to the combined bactericidal activity and rates of regrowth following exposure to anti-TB drugs.

An aminoglycoside, AMK, and a fluoroquinolone, OFL, are the two alternative drugs that were tested in the present study. Previous studies have suggested that these, among other drugs, may be useful in the treatment of MDR-TB. The performance of established agents with known clinical efficiency were used as a basis of comparison.

Chapter 2 of this dissertation deals with the conventional *in vitro* techniques used to evaluate anti-TB drugs. Quantitative bacteriostatic and bactericidal tests covered in this chapter, formed the basis of experiments discussed in Chapter 4 to Chapter 10, the latter dealing with drug combinations. Chapter 3 provides the background and principles behind the radiometric bacteriopausal regrowth model developed in this study. The conventional time-kill curve technique is discussed in Chapter 4 while the potential

contribution of bactericidal activity in the radiometric regrowth technique (covered in Chapter 5) was evaluated in Chapter 6 and Chapter 7. Chapter 8 comprises a critical appraisal of the radiometric post-exposure regrowth model and in Chapter 10 the significance of the findings of the present study is discussed.

CHAPTER 2

THE BACTERIOSTATIC AND BACTERICIDAL ACTIVITIES OF SIX ANTI-TUBERCULOSIS DRUGS AGAINST *MYCOBACTERIUM TUBERCULOSIS*

2.1 BACKGROUND

2.1.1 *The inhibitory activity of drugs*

The inhibitory or bacteriostatic activity of an antimicrobial agent can be determined by qualitative or quantitative tests (Craig, 1993b; Heifets,1988a). The principles employed in these methods are the same except that the so-called critical or breakpoint concentrations are used in the former and a wide range of drug concentrations, in the latter (Heifets, 1988a).

2.1.1.1 Critical concentrations

The conventional way to guide the chemotherapy of tuberculosis (TB) requires testing of *Mycobacterium tuberculosis* isolates with critical concentrations of standard anti-TB drugs (Canetti *et al*, 1963; Canetti *et al*, 1969). These critical concentrations were initially developed for *M. tuberculosis* empirically by finding the highest minimal inhibitory concentration (MIC) in Löwenstein-Jensen (LJ) medium at or below which strains were shown to be susceptible based on clinical grounds. Such concentrations were therefore selected as the susceptibility thresholds that were best able to reflect the clinical outcome of the treatment of TB (Canetti *et al*, 1969; NJCIRM, 1993). Later, equivalent concentrations were found for Middlebrook 7H10 (Lee and Heifets, 1987; NJCIRM, 1993), 7H11 agar, and 7H12 broth (Lee and Heifets, 1987). By using this fixed concentration, qualitative method, *M. tuberculosis*

strains are classified as sensitive or resistant to the anti-TB drugs (Hoel and Eng, 1991).

For many years, this qualitative testing approach has proved to be generally efficacious in the management of TB and in laboratory testing (Lee and Heifets, 1987). The reason to this is that *M. tuberculosis* strains that had not been previously exposed to the antimicrobial agents were uniform in the degree of their susceptibility to these drugs (NJCIRM, 1993; Heifets, 1996).

Although efficacious, qualitative tests have certain weaknesses including: (1) there are no established critical concentrations for new experimental drugs; (2) inconsistent results with a series of isolates when the bacterial population is in an intermediate state between susceptible and resistant; and (3) occasional discrepancies between the patient's response to chemotherapy and the results of drug susceptibility tests (Chen *et al*, 1994).

2.1.1.2 Determination of the minimal inhibitory concentration

The MIC is defined as the lowest concentration of a drug that inhibits growth of more than 99% of the bacterial population present at the beginning of the test within a specified period of cultivation (Heifets, 1988a; Heifets *et al*, 1985b; Suo *et al*, 1988).

Evaluation of the MIC provides an opportunity to quantitate the susceptibility of clinical *M. tuberculosis* isolates as well as of other bacteria, that is, it provides full information on the degree of resistance of a particular isolate and on the proportion of resistant organisms at each drug concentration (Heifets, 1988a). Bacterial strains are usually characterised as susceptible, intermediate and

resistant against an antimicrobial agent (Soussy *et al*, 1994; Heifets, 1988a; Heifets, 1991a). In addition, MIC values are important as a basis for drug combination studies (Lee and Heifets, 1987).

The two major approaches to susceptibility testing (including MIC determination) for bacteria in general are, the agar disc diffusion method and the drug dilution method. These methods differ significantly. Each tests a different population of the microorganism (Isenberg, 1988).

The dilution method tests the most resistant segment of the microbial isolate that will be completely inhibited by the drug concerned. The disc diffusion method (the application of drug-containing discs to an agar plate inoculated with the bacterium under scrutiny) on the other hand, tests the average susceptibility of that microbial population to an antimicrobial agent by establishing a zone of inhibition that is interpreted as meaning the organism is susceptible, has intermediate susceptibility or is resistant (Isenberg, 1988). Inhibition diameters that can be shown to correlate with MICs of strains and diameter breakpoints are based on this correlation. This forms the basis of the E-test which is a diffusion test from which MICs are derived (Sanchez and Jones, 1993). The agar disc diffusion method is much easier to perform than the dilution test in the average laboratory. However, the dilution method is usually selected as the method of choice (Isenberg, 1988).

There are three widely used drug dilution methods for MIC determination. Many laboratories use the modified proportion method (first described by Canetti *et al*, 1969) on Middlebrook agar according to the proposed standard of the National Committee for Clinical Laboratory Standards (NCCLS, 1991) for determining

antimicrobial susceptibility of *M. tuberculosis* strains. Resistance with this method is defined by growth on drug-containing media that represents 1% or more of the colonies observed on the drug-free medium (Salfinger and Pfyffer, 1994; Heifets, 1988a; Heifets, 1991a). Explicit in this system is the assertion that below a certain proportion, the strain is classified as sensitive, and above as resistant (Heifets, 1988a).

Other methods for determining antimicrobial susceptibility of *M. tuberculosis* strains are the absolute concentration method, also referred to as the MIC breakpoint method, and the resistance ratio method (Salfinger and Pfyffer, 1994). The latter does not provide a quantitative endpoint but compares growth of the clinical isolate with that of the standard control H37Rv strain on selected drug concentrations, expressing the result as a ratio of the lowest inhibitory concentration affecting the two strains (Canetti, *et al*, 1963; Canetti *et al*, 1969; Heifets, 1988a). In the absolute concentration method, resistance is defined as the presence of 20 or more colonies on the drug-containing medium (Canetti *et al*, 1963). Briefly, in the dilution method, a suspension of bacteria is mixed with various concentrations of the drug (usually serial doubling dilutions) and the capacity of the inoculum to grow is determined (Orme *et al*, 1994).

MICs determined by the dilution method can be assessed in liquid (broth) or solid (agar) media (Heifets, 1988a; Vogelman and Craig, 1986). The most popular technique of MIC determination is based on the use of either Middlebrook 7H10 or 7H11 agar media to which anti-TB agents have been added (Stratton, 1993; Garcia-Rodriguez and Gomez-Garcia, 1993). These agar media are transparent and thus permit early microscopic detection of colonies (Rastogi *et al*,

1989; Vareldizis *et al*, 1994; Wayne,1994). Growth of tubercle bacilli on these agar media is stimulated by inclusion of a 5 to 10% CO_2 supplement to the air in the incubator (Vareldzis *et al*, 1994; Wayne, 1994).

The other solid media used throughout the world for susceptibility testing is the LJ medium (Siddiqi *et al*, 1985). LJ medium is an inspissated egg-based medium. Although simple to prepare and cheap, it is complex in composition and lacks reproducibility because of variation in the quality of its ingredients and the effects of heat in its preparation. Therefore, it is not especially good for research purposes (Wayne,1994).

On solid media, a certain proportion of drug incorporated into the media is degraded during preparation of the medium and during long periods of cultivation required for obtaining sufficient growth in drug-free controls. The degree of degradation depends on the stability of the drug and the specific features of the medium (Heifets, 1988a). The test on solid media therefore cannot give an exact MIC value because of unknown levels of binding, absorption and deterioration (Heifets, 1988a; Heifets *et al*, 1986).

The use of liquid media helps to avoid some of the problems (mentioned above) inherent in the use of solid media. In broth media, MICs can be determined in various ways including: detection of growth or its inhibition by plating, turbidometrically or radiometrically (NJCIRM,1993).

Turbidometric methods are cheaper and easier to perform. However, they are not accurate because of the long periods of incubation required for slow growing mycobacteria such as *M. tuberculosis.*

Furthermore, the turbidity of the culture continues to increase after the exponential phase and during the stationary phase of growth reflecting the total number of bacteria rather than the number of viable bacteria (NJCIRM, 1993). Broth-determined MICs by plating on the other hand are costly and labour intensive (NJCIRM, 1993; Heifets, 1988a). Moreover, components of liquid media can have a profound effect on the test results (Heifets, 1988a).

The 7H12 broth medium is used for the radiometric susceptibility testing of mycobacteria using the Bactec system (Stratton, 1993; Inderlied, 1994). This medium does not contain Tween 80 which can effect the results of susceptibility testing (;Naik *et al*, 1989;Youmans and Youmans, 1948; Heifets *et al*, 1986). A major advantage to the use of the 7H12 broth is that it requires a relatively short period of incubation, thus decreasing the length of exposure of the drug to 37°C and yielding quick results which may assist the clinician in the management of patients (Heifets *et al*, 1986).

The 7H12 broth medium contains a radiolabelled (^{14}C-labelled) fatty acid substrate, palmitic acid. Growth is based on the demonstration of metabolic activity which releases ^{14}C-labelled CO_2. This can be measured with the Bactec TB-460 instrument (Siddiqi, 1988; Stratton, 1993; Inderlied, 1994). Metabolic activity is determined quantitatively and is expressed as a growth index (GI) in units on a scale of 0 to 999 (Heifets, 1991a). The rate and amount of CO_2 production is directly proportional to the extent of growth and therefore can be used as a measure of growth inhibition (Siddiqi *et al*, 1981).

The Bactec radiometric susceptibility testing method, also suitable

for MIC testing, was first described by Siddiqi *et al* in 1981 and is a variant of the proportion method using Middlebrook 7H10 or 7H11agar (Siddiqi *et al*, 1981; Klietman, 1995; Stratton, 1993; Inderlied, 1994). It is reliable and has a rapid turnaround time with results generally being available in approximately one week or less (Siddiqi *et al*, 1981; Chen *et al*, 1994; Stratton, 1993; Inderlied, 1994; Salfinger and Pfyffer, 1994; Vareldzis *et al*, 1994). According to Inderlied (1994), the rapidity of results available using this method justifies the higher cost compared with the conventional proportion method which takes approximately four weeks. Another advantage to the determination of MICs in 7H12 broth is that some drug resistant cultures do not grow sufficiently well on agar plates and require more than three weeks of incubation, possibly invalidating the results (Chen *et al*, 1994).

Standardization of the experimental conditions is of utmost importance in *in vitro* evaluation of an antimicrobial agent (Vareldzis *et al*,1994). The degree of activity of a drug depends on many factors, for example, inoculum size and growth phase, kind of medium and its pH, techniques of measurement of growth and its inhibition et cetera (Garcia-Rodriguez and Gomez-Garcia, 1993; Heifets, 1991a).

Standardization of the size of inoculum is critical. There may be large differences between cultures reflected in the number of colonies that grow from similar inocula (Canetti *et al*, 1964). This is largely due to clumping of the bacilli. Variability in the inoculum size is the major cause of variability in MIC results. A basic requirement for reliability of testing techniques is information on the number of viable bacilli on the drug-free versus drug-containing media (Vareldzis *et al*, 1994).

2.1.2 *Determination of the minimal bactericidal concentration*

Minimal bactericidal concentration (MBC) determinations are quantitative measures of the bactericidal activity of an antimicrobial agent (Jacobs, 1991). In clinical microbiology, MBC is usually defined as the lowest concentration of drug that kills 99.9% of the bacterial population within a limited period of cultivation in a liquid medium (Handwerger and Tomasz, 1985). However, in the field of mycobacteriology, the 99% has been previously found to be a more appropriate criterion than 99.9% killing (Heifets and Lindholm-Levy, 1989; Heifets *et al*, 1986).

Estimation of the bactericidal activity is an important assessment of any new antibacterial drug. In addition, re-evaluation of the bacterial potency of conventional anti-TB drugs may provide valuable insight into the chemotherapeutic potential of these drugs (Heifets, 1988a). A rational and commonly accepted approach to quantification of the bactericidal activity of an antimicrobial agent *in vitro*, is the determination of the MBC/MIC ratio (Heifets, 1988a). Because the MIC depends on the method of determination, the most accurate MBC/MIC ratio is obtained if both the MIC and MBC are determined in the same medium and by the same basic technique (Heifets, 1988a; Heifets and Lindholm-Levy, 1989).

Determination of the MBC is performed in liquid media by using the conventional sampling and plating method (Heifets, 1988a), or radiometrically (Heifets, 1991a). In most studies, MBC determinations are performed in liquid media as a two-step procedure. The initial standard broth dilution assay of the MIC is followed by performing subcultures on all dilution tubes without obvious turbidity onto agar plates which contain no antimicrobial agent (Daniel, 1991; Isenberg, 1988; Heifets, 1988a;

Schoenknecht *et al*, 1985). The proportion of survivors after a specified period of incubation in broth-containing antimicrobial agents is determined (Schoenknecht *et al*, 1985).

Another method of MBC determination, described by Heifets (1991a), makes use of the Bactec radiometric system. Briefly, the inoculum is grown in Bactec vials until growth is in the logarithmic phase. Graded concentrations of antimicrobial agents are then added to the vials, followed after different exposure times by sampling and plating, and counting the number of colony forming units (CFUs) in the drug-free and drug-containing vials. Both methods involve sampling and plating, therefore, they are costly and labour intensive (Heifets, 1991a).

Like the MIC, a number of variables are important in the determination of the MBC (Daniel, 1991). Problems of poor reproducibility have beset this test from early on, and this has not been helped by the considerable differences in the MBC tests employed in routine practice. Recent studies have emphasised the effect of several technical factors on the outcome of MBC tests and their reproducibility. The most important of these include antibiotic carry-over which gives low counts of survivors at higher antibiotic concentrations, phase of growth where there may be increased survivors in the stationary phase and, insufficient contact between the test organism and the antimicrobial agent (Schoenknecht *et al*, 1985; Sherris, 1986).

Antimicrobial agents which are relatively bactericidal in their mode of action will often be lethal at a level equal or close to the MIC and thus give MBC endpoints which are identical with or only slightly (one or two dilutions) higher than the MIC. Antimicrobial agents

which are relatively bacteriostatic in their mode of action show MBC endpoints many dilutions higher than the MIC (Daniel, 1991).

The objectives covered in this chapter were: (1) to evaluate the degrees of resistance and, the susceptibility patterns of *M. tuberculosis* strains to four standard drugs, isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and streptomycin (SM) and two alternative drugs, ofloxacin (OFL) and amikacin (AMK), (2) to compare the susceptibility patterns and MICs determined by using the agar plate with those determined by the Bactec broth method, (3) to determine the MIC as an initial step to subsequent investigations, (4) to compare the bactericidal activities of the six drugs, and (5) to determine the MBC/MIC ratios.

2.2   MATERIALS AND METHODS

2.2.1 Minimal inhibitory concentration determination

Antimicrobial agents

The drugs were received in powder from the following suppliers: INH (Noristan, Pretoria), RMP (Ciba-Geigy, Johannesburg), EMB (Rolab, Pretoria), SM (Novo-Nordisk, Johannesburg), OFL and AMK (Bayer, Johannesburg). Stock solutions were prepared after factoring in the potency of each antimicrobial powder.

INH, EMB, SM, OFL, and AMK were dissolved in sterile distilled water. RMP was dissolved initially in 95% ethanol and subsequently in sterile distilled water. After the drugs were completely dissolved, the solutions were filter sterilized through a 0.22 *u*m pore size polyethersulfone membrane filter (Whatman). The stock solutions were frozen in 2-3 ml aliquots at 70°C. At the time of testing,

working solutions were made in sterile distilled water.

2.2.1.1 *The agar dilution method*

2.2.1.1.1 Standard drugs

Test strains

Sixteen *M. tuberculosis* strains obtained from the Tuberculosis Research Programme (TBRP) of the Medical Research Council (MRC) were used and were previously identified as *M. tuberculosis* using conventional standard methods (Kent and Kubica, 1985) by the TBRP laboratories. For each strain colonies were scraped off from 2-weeks old LJ cultures. Colonies were subcultivated in Middlebrook 7H9 broth (Bacto) enriched with albumin-dextrose-catalase (ADC, Biolab) for 7-10 days at 37°C or until the turbidity of the culture reached that of the McFarland no.1 standard (Appendix 1), equal to a density of 10^7 CFU/ml (NCCLS,1991).

The standardized cultures were preserved at 70°C in 2 ml aliquots until needed for testing. Before an experiment, for each strain, a single frozen culture vial was thawed, mixed thoroughly and subcultivated in 7H9 broth (Bacto) for 7-10 days or until the turbidity reached that of the McFarland no.1 standard.

Procedure

A modified version (as reported by Kleeberg *et al*, 1985) of the indirect proportion method by Canetti *et al* (1969) was used. Middlebrook 7H10 medium was prepared in 200 ml aliquots in 500 ml Erlenmeyer flasks from the commercially available Middlebrook 7H10 agar base (Bacto). The agar base powder (3.8g) was suspended in distilled water (approximately 180 ml, depending on

the amount of drug that was added), autoclaved at 121°C for 10 minutes, and cooled to 50-55 °C in a water bath.

ADC enrichment was asceptically added at this stage, in the amount of 10 % of the volume of the cooled agar solutions (20 ml). Each flask was used to prepare either the drug-free control or drug-containing medium. Using Eppendorf automated micropipetts, working solutions of drugs were added to the cooled agar media to ensure the final concentrations of 0.2; 1.0; 2.5; 5.0; 10; 20 and 40 ug/ml for INH, 1.0; 2.0; 4.0; 8.0; 16 and 32 ug/ml for RMP, 2.5; 5.0; 10; 20 and 40 ug/ml for EMB, and 0.025; 0.5; 1.0; 2.0; 4.0; 8.0; 16 and 32 ug/ml for SM. One flask in which drugs were not added was used as a control. The contents of the flasks were thoroughly mixed and were dispensed (approximately 20 ml) into standard petri dishes (15 mm).

To prepare the inoculum, a frozen culture was thawed, mixed thoroughly and subcultured in 7H9 broth (Bacto) for 7-10 days or until the turbidity of the culture was equivalent to that of the McFarland no. 1 standard. The culture was diluted 10^{-3} and 10^{-5} to provide approximately 2×10^4 and 2×10^2 CFU/ml, respectively, using a sterile 7H9 broth without ADC.

The test plates were inoculated in duplicate with a 0.01 ml volume of the 10^{-3} dilution for each culture strain. Two duplicate sets of drug-free control plates were prepared for each culture. One set was prepared in the same way as the test plates and the other with 0.01 ml of the 10^{-5} diluted bacterial suspension (approximately 2×10^2 CFU/ml) to achieve a 1 : 100 control or 1% of the bacterial suspension in the test plates.

The 7H10 plates were sealed in plastic bags to prevent media from drying out and were wrapped in black plastic bags to protect from light. The plates were incubated at 37°C for 3 weeks in the presence of a 5-10% CO_2 atmosphere and were read after 3 weeks of cultivation. The results were reported as the proportion or percentage of bacteria in the population resistant to each concentration of drug versus the number of colonies grown in the 1 : 100 control. The MIC was the lowest concentration of drug that inhibited more than 99% of the bacterial population.

2.2.1.1.2   Alternative drugs

Test strains

Eight *M. tuberculosis* strains were selected based on their MIC values and their susceptibility patterns against the standard drugs.

Procedure

The same procedure as for the standard drugs (Section 2.2.1) was used. The final concentrations of drugs in agar media were 0.125; 0.25; 0.5; 1.0; 2.0; 3.0 and 6.0 *u*g/ml for OFL and, 0.25; 0.5; 1.0; 2.0; 4.0; 8.0 and 16 *u*g/ml for AMK.

2.2.1.2   *The radiometric 7H12 broth dilution method*

Test strains

Due to costs involved in using the Bactec radiometric system, four of the eight selected strains were used in this experiment. These include strains TB 0368/93 and MR 84452 which have a low level of resistance to INH and RMP, respectively; a strain TB 0552/93 fully resistant to INH and moderately resistant to RMP and, the standard reference strain H37Rv ATCC 27294.

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Procedure

The radiometric MIC determination was done to compare results obtained by this method with those obtained by the agar dilution method. Prior to inoculation, the 7H12 vials (Becton Dickinson) were tested on the Bactec TB-460 instrument (Johnston Laboratories Inc., Maryland) to establish a 5% CO_2 atmosphere inside the vials and to check for contamination in the vials.

The inoculum was prepared by inoculating a seed vial of 7H12 medium (4 ml) with a 0.1 ml volume of a 1 : 2 dilution of a fresh 7H9 *M. tuberculosis* culture having the McFarland standard no. 1 optical density. The culture seed vial was homogenized thoroughly with a 1.0 ml tuberculin syringe (Becton Dickinson) with a permanently attached needle and was read daily on the Bactec TB-460 instrument.

When growth in this vial reached a GI reading of 400-500, the 7H12 broth culture was used undiluted to inoculate 0.1 ml into a set of drug-containing 7H12 vials. These inocula provide an initial concentration of 10^4 to 10^5 CFU/ml in the test vials (Heifets *et al*,1985b; Heifets *et al*,1986). Two drug-free vials were used as controls. One was inoculated in the same way as the test vials and the other with a 1 : 100 diluted inoculum to produce 10^2 to 10^3 CFU/ml (Heifets,1988a; Heifets *et al*, 1985b; Chun-nin and Heifets,1987). The 1 : 100 control was prepared by inoculating 0.1 ml of the initial 7H12 broth culture into a vial containing 9.9 ml of Bactec diluting fluid (Becton Dickinson). A 0.1 ml portion of the diluted inoculum was inoculated into a 7H12 vial.

Working solutions of drugs were prepared in such a way that when a volume of 0.1 ml of drug was injected into standard 7H12 vials

(4.0 ml), the desired concentration was achieved. The following final concentrations were achieved in the 7H12 vials : 0.025 - 20 ug/ml for INH, 0.0625 - 16 ug/ml for RMP, 0.475 - 8.0 ug/ml for EMB, 0.5 - 8.0 ug/ml for SM, 0.125 - 2.0 ug/ml for OFL and, 0.25 - 2.0 ug/ml for AMK.

The 7H12 vials were incubated at 37°C and were read and recorded daily on the Bactec TB-460 instrument at approximately the same time. Vials were read until a GI of 30 or greater was found for two or three consecutive days in the 1 : 100 control vials (usually 7 to 10 days of cultivation).

The radiometric MIC was defined as the lowest concentration of drug in the presence of which the daily GI increase (change in GI) and the final GI reading are lower than those in the 1 : 100 control vials, indicating that this concentration inhibited more than 99% of the bacterial population (Snider *et al*, 1981; Siddiqi *et al*, 1985). If the MIC of the drug fell out of the range used in the primary titrations, the test was repeated with higher or lower concentrations of the drugs.

2.2.2 Radiometric susceptibility testing : critical concentrations

Antimicrobial agents

The drugs were the same and prepared as in Section 2.2.1 above.

Test strains

Four strains (the remaining 4 of the 8 selected strains that were not used in the radiometric MIC determination): MR 83173, MR 83507, MR 84882 and 249/941, were used.

Procedure

The procedure was the same as for the radiometric MIC determination above except that breakpoint concentrations adapted for Bactec 7H12 medium were used as opposed to a wide range of concentrations required for MIC testing. Antimicrobial concentrations of 0.1 and 0.4 ug/ml; 2.0 ug/ml; 2.5 and 7.5 ug/ml; and 2.0 and 6.0 ug/ml; 1.0 ug/ml, and 2.0 ug/ml, were used for INH, RMP, EMB, SM, OFL and AMK, respectively.

2.2.3 Minimal bactericidal concentration determination

Antimicrobial drugs

Stock solutions (10,000 ug/ml) of INH, RMP, EMB, SM, OFL and AMK, and working solutions of the drugs were prepared as in Section 2.2.1 above.

Test strains

Previous studies on the bactericidal activity of anti-TB drugs have been done with one strain only (Dickinson *et al*, 1977). We selected for our study the same *M. tuberculosis* strain, H37Rv. In many reports by other authors, the MBC for *M. tuberculosis* was usually tested with one strain only (Heifets *et al,* 1991a).

Procedure

The MBC was determined in the radiometric model in 7H12 medium as described by Heifets (1991a). Duplicate 7H12 vials (Becton Dickinson) for control and each drug concentration were inoculated in the same way as described for radiometric MIC determination except for the 1 : 100 control which is not required for MBC determination.

The 7H12 vials were allowed to incubate drug-free at 37°C until growth was in the exponential phase with the number of CFU/ml being 10^5 to 10^6. According to Heifets *et al* (1985b) this bacterial concentration is achieved when the GI reading is approximately 500 but in a later publication (Heifets *et al*,1986) the authors recommended a GI reading of approximately 400. In the present study, a GI reading of 400-500 was used. At this stage, 0.1 ml of appropriate drug solutions was added into the labelled 7H12 vials to achieve 1, 2, 4, 8 and 16 times the MIC. Two drug-free 7H12 vials were used as controls.

The 7H12 vials were incubated at 37°C and were read daily on the Bactec TB-460 instrument (Johnston Labs. Inc., Maryland) for 15 days. In order to determine the number of CFU/ml of surviving bacteria, 0.1 ml samples were taken from drug-free control vials after initial inoculation, when drugs were added and from alternate drug-containing and drug-free control vials at various time points (days 0, 3 or 4; 7 or 8; 12 and 15).

Samples were diluted serially (five 10-fold dilutions) and 0.01 ml of the diluted and undiluted samples were inoculated onto duplicate drug-free 7H10 agar plates. The 7H10 agar plates were incubated at 37°C in a 5-10% CO_2 atmosphere for 14 days. Colonies were counted after 14 days of cultivation and CFU/ml were calculated. A 10^{-3} dilution was used to determine CFU/ml and this dilution factor was sufficient to reduce the concentration of drugs below the previously determined MIC, thus obviating the possibility of drug carry-over effect.

The final interpretation of the MBC was based on comparison of CFU/ml in the drug-free vials on the day when drugs were added

(day 0) with CFU/ml in the drug-containing vials after 1-15 days of cultivation in 7H12 medium. The MBC was the lowest concentration of drug that killed more than 99% of the bacterial population (present when the drug was added) within 15 days of cultivation in 7H12 broth.

2.3 RESULTS

2.3.1 Minimal inhibitory concentration

2.3.1.1 *The agar dilution method*

2.3.1.1.1 Standard drugs

The MICs obtained with solid 7H10 medium ranged from <0.2 to 20 *ug*/ml, <1 to >32 *ug*/ml, 2.5 to 20 *ug*/ml, and 1 to >32 *ug*/ml, for INH, RMP, EMB and SM, respectively, against 16 strains of *M. tuberculosis* (Table 2.1). Most (11) of these strains were resistant to at least one standard anti-TB drug (see Appendix 2 for breakpoint concentrations of standard anti-TB drugs that have been proposed internationally for susceptibility testing).

Based on MIC results, it has been suggested that *M. tuberculosis* strains can be categorized into completely susceptible, intermediately resistant or completely resistant strains to a drug (see Appendix 2 for definitions of the levels of resistance according to Heifets *et al* (1991a), when Bactec susceptibility testing is used).

Eight strains (Table 2.2) were selected from those listed in Table 2.1 and selection of these strains was based on their susceptibility patterns as well as on their level of resistance to INH or RMP. These strains were kept for further study. Two strains with a low level of

resistance to INH and RMP were found. One strain (TB 0368/93) showed a low level of resistance to INH (MIC = 2.5 ug/ml) and the other strain (MR 84452) showed a low level of resistance to RMP (MIC = 4.0 ug/ml).

Table 2.1 MICs (ug/ml) for the standard drugs; isoniazid, rifampicin, ethambutol and streptomycin by using 7H10 agar medium.

Strain	MIC[a] (ug/ml)			
	INH	RMP	EMB	SM
H37Rv	<0.2	1.0	5.0	2.0
TB 1984/92	20	>32	20	1.0
TB 0294/93	10	>32	2.5	2.0
TB 0368/93	2.5	>32	2.5	32
TB 0552/93	20	1.0	5.0	8.0
MR 83173	10	<1.0	5.0	32
MR 83366	10	8.0	20	>32
MR 83507	10	>32	10	>32
MR 84452	20	4.0	20	4.0
MR 84882	10	<1.0	5.0	>32
MR 85901	5.0	>32	20	2.0
2	<1.0	<1.0	5.0	4.0
6	<1.0	<1.0	5.0	4.0
10	<1.0	<1.0	10	4.0
12	<1.0	<1.0	10	4.0
249/941	10	>32	5.0	>32

[a]MIC = minimal inhibitory concentrations for INH (isoniazid), RMP (rifampicin), EMB (ethambutol), SM (streptomycin), OFL (ofloxacin) and (AMK) amikacin.

Table 2.2 Susceptibility patterns (as determined by the 7H10 agar method) of the selected *M. tuberculosis* strains.

Strain	MIC[a] (*u*g/ml) and susceptibility patterns[b]			
	INH	RMP	EMB	SM
H37Rv	<0.2 (S)	1.0 (S)	5.0 (S)	2.0 (S)
TB 0368/93	2.5 (MS)	>32 (R)	2.5 (S)	32 (R)
TB 0552/93	20 (R)	1.0 (S)	5.0 (S)	8.0 (MR)
MR 83173	10 (R)	<1.0 (S)	5.0 (S)	32 (R)
MR 83507	10 (R)	>32 (R)	10 (S)	>32 (R)
MR 84452	20 (R)	4.0 (MR)	20 (R)	4.0 (MS)
MR 84882	10 (R)	<1.0 (S)	5.0 (S)	>32 (R)
249/91	10 (R)	>32 (R)	5.0 (S)	>32 (R)

[a] MIC = minimal inhibitory concentrations for INH (isoniazid), RMP (rifampicin), EMB (ethambutol), SM (streptomycin), OFL (ofloxacin) and AMK (amikacin), [b] S = susceptible, R = resistant, MS = moderately susceptible and MR = moderately resistant.

2.3.1.1.2 Alternative drugs

MICs for the two alternative drugs, OFL and AMK, were determined on 7H10 solid media against the 8 selected strains and MIC ranges of 0.5 to 1.0 *u*g/ml and 0.25 to 1.0 *u*g/ml were obtained for OFL and AMK, respectively (Table 2.3).

All the strains tested were completely susceptible to the two alternative drugs irrespective of their susceptibility patterns to the standard drugs. MICs for these drugs were lower than those for the four standard drugs, for all but the fully susceptible standard strain, H37Rv. In the case of the latter, MICs for the two alternative drugs were the same as those of RMP, greater than those of INH, and less than those of EMB and SM.

Table 2.3 MICs for alternative drugs, ofloxacin and
amikacin by using 7H10 agar medium against
eight selected *M. tuberculosis* strains.

Strain	MIC[a] (ug/ml)	
	OFL	AMK
H37Rv	0.5	0.5
TB 0368/93	1.0	1.0
TB 0552/93	1.0	1.0
MR 83173	0.5	0.25
MR 83507	1.0	1.0
MR 84452	1.0	1.0
MR 84882	0.5	0.5
249/91	0.5	0.25

[a]MIC = minimal inhibitory concentrations for OFL (ofloxacin)
and AMK amikacin.

2.3.1.2 *Comparison of MIC results obtained by 7H12 broth and 7H10 agar dilution methods*

A total of four of the 8 selected strains was tested by both 7H12 broth and agar dilution methods. The Bactec radiometric and agar MIC determinations were done twice. The repeated studies showed consistency in results for both methods; when MIC titrations were repeated, they never varied by more than one dilution. The comparative results for the various anti-TB drugs are shown in Table 2.4.

2.3.1.2.1 *Isoniazid*

The MIC range for INH was found to be <0.2 to 20 ug/ml and 0.05 to 10 ug/ml with solid media and 7H12 broth, respectively. MICs obtained with Bactec 7H12 broth were the same or one dilution

lower than those obtained with 7H10 agar media.

2.3.1.2.2 *Rifampicin*

Broth-determined MICs were lower than the agar-determined MICs and were either the same or more commonly one dilution lower than the agar-determined MICs (Table 2.4). MIC ranges of <1 to >32 ug/ml and 0.5 to 16 ug/ml were obtained with 7H10 agar and 7H12 broth, respectively.

2.3.1.2.3 *Ethambutol*

The 7H12 broth-determined MICs were 2.5 to 5 times lower than the agar-determined MICs (Table 2.4); MICs of 2.5, 5.0 and 20 ug/ml (MIC range of 2.5 to 20 ug/ml) in 7H10 agar correlated with those of 0.5, 1.0 or 2.0, and 4.0 ug/ml (MIC range of 0.5 to 4.0 ug/ml) in 7H12 broth.

A strain completely susceptible or resistant with 7H12 broth was also completely susceptible or resistant with 7H10 agar for all but one strain, MR 84452. This strain was found to be completely resistant with 7H10 agar (MIC = 20 ug/ml) but moderately resistant with the 7H12 broth (MIC = 4 ug/ml). See Appendix 2 for levels of resistance.

2.3.1.2.4 *Streptomycin*

Broth-determined MICs were either the same or one dilution lower than the agar-determined MICs. An MIC range of 2 to >32 ug/ml and 1 to >8 ug/ml was obtained in solid media and in 7H12 broth, respectively (Table 2.4).

2.3.1.2.5 *Ofloxacin*

Broth-determined MICs were the same or one dilution lower than the

agar-determined MICs (Table 2.4). An MIC range of 0.5 to 1.0 ug/ml and <0.5 to 0.5 ug/ml was found with solid media and 7H12 broth media, respectively. One of the four strains (MR 84452) tested showed an MIC in 7H12 broth being more than one dilution lower than that obtained with 7H10 agar.

2.3.1.2.6 *Amikacin*

Broth-determined MICs were one, fourfold or more than fourfold less than the agar-determined MICs with a range of 0.5 to 1.0 ug/ml and <0.25 to 0.25 ug/ml in 7H10 agar and 7H12 broth, respectively (Table 2.4).

2.3.2 Comparison of agar and Bactec broth susceptibility testing by critical concentrations

Bactec radiometric susceptibility testing by breakpoint concentrations was determined against four strains. The susceptibility patterns obtained with this method were compared with those obtained with the 7H10 agar method. Strains completely susceptible by the conventional method were also completely susceptible by the Bactec radiometric system. Strains resistant by the conventional method were also resistant by the Bactec radiometric method (See Appendix 2 for breakpoint concentrations). Results were obtained in 7 to 10 days using the Bactec method as opposed to 21 days in the agar plate method.

Table 2.4 Comparative MICs of four *M. tuberculosis* strains using 7H10 agar and 7H12 broth media against six anti-tuberculosis drugs.

Drug[a]	MICs[b] in (ug/ml) of *M. tuberculosis* strains							
	H37Rv		TB 0368/93		TB 0552/93		MR 84452	
	7H10	7H12	7H10	7H12	7H10	7H12	7H10	7H12
INH	<0.2	0.05	2.5	2.5	20	10	20	10
RMP	0.5	0.5	>32	16	1.0	0.5	4.0	2.0
EMB	5.0	2.0	2.0	0.5	5.0	1.0	20	4.0
SM	2.0	1.0	>32	>8.0	8.0	4.0	4.0	4.0
OFL	0.5	0.5	1.0	0.5	1.0	0.5	1.0	<0.5
AMK	0.5	0.25	1.0	<0.25	1.0	0.25	1.0	<0.25

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin and AMK = amikacin, [b]MICs = minimal inhibitory concentrations

2.3.3 Minimal bactericidal concentration

The MBCs of the six anti-TB drugs were determined against the standard strain, H37Rv ATCC 27294, only. Results describing the bactericidal action of INH, RMP, EMB, SM, OFL and AMK are summarized in Table 2.5. MBCs of 0.05; 1.0; 16; 2.0; and 1.0 ug/ml were found for INH, RMP, EMB, SM, OFL and AMK. These MBCs were achieved at days 2.5, 2.9, 14, 12, 10.75, and 4.75 for INH, RMP, EMB, SM, OFL and AMK, respectively. Comparison with broth-determined MICs gives MBC/MIC ratios of 1; 2; 8 and 4 for INH, RMP and SM, EMB, and, OFL and AMK respectively.

All except EMB showed good bactericidal activity against the H37Rv strain. INH, RMP and SM were highly bactericidal with MBC/MIC ratios of 1 or 2. EMB on the other hand showed moderate bactericidal activity with an MBC/MIC ratio of 8. The kinetics of killing of the six anti-TB drugs at the MBCs are graphically demonstrated in Figure 2.1.

Table 2.5 Bactericidal activity of six anti-tuberculosis drugs against *M. tuberculosis* H37Rv.

Drug[a]	MIC[b] : CFU/ml[c] when drug was added	MBC[d] (ug/ml) and CFU/ml with drug at MBC	Day at MBC[e]	MBC/MIC ratio[f]
INH	$0.05 : 4.5 \times 10^4$	$0.05 : 1.5 \times 10^2$	2.5	1
RMP	$0.5 \ : 4.5 \times 10^4$	$1.0 \ : 2.5 \times 10^2$	2.9	2
EMB	$2.0 \ : 2.1 \times 10^6$	$16 \ : 2.0 \times 10^4$	14	8
SM	$1.0 \ : 2.1 \times 10^6$	$2.0 \ : 5.0 \times 10^3$	12	2
OFL	$0.5 \ : 4.5 \times 10^4$	$2.0 \ : 3.9 \times 10^2$	10.75	4
AMK	$0.25 \ : 2.1 \times 10^6$	$1.0 \ : 3.0 \times 10^3$	4.75	4

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin and AMK = amikacin, [b]MIC = minimal inhibitory concentration, [c]CFU = colony forming unit, [d]MBC = minimal bactericidal concentration, [e]MBC/MIC ratio is a quatitative measure of bactericidal activity.

(a)

(b)
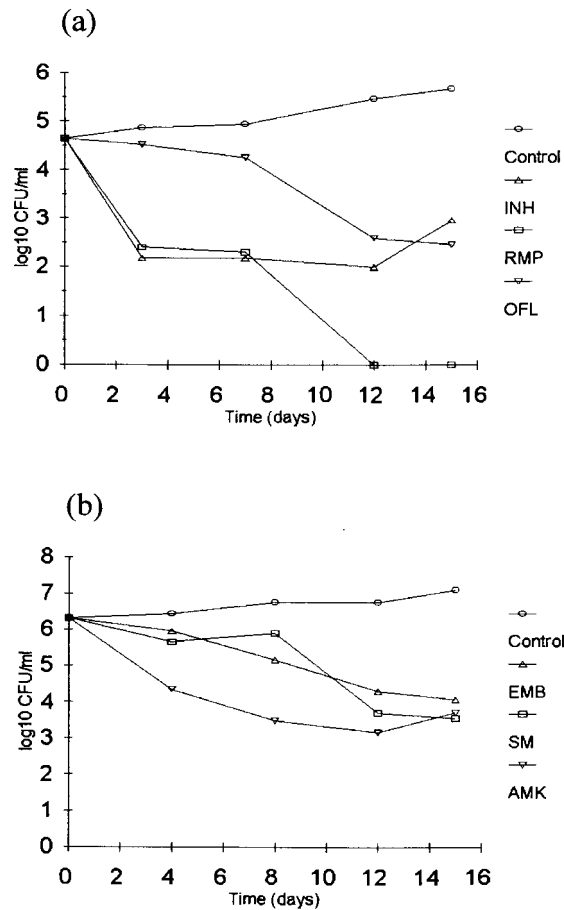
Figure 2.1: Kinetics of killing derived from the MBC experiments of (a) 0.05 ug/ml of isoniazid (INH), 1.0 ug/ml of rifampicin (RMP) and 2.0 ug/ml of ofloxacin (OFL); (b) 16 ug/ml of ethambutol (EMB), 2.0 ug/ml of streptomycin (SM) and 1.0 ug/ml of amikacin (AMK) against *M. tuberculosis* H37Rv.

2.4   DISCUSSION AND CONCLUSIONS

2.4.1 Minimal inhibitory concentration

When MICs for the four standard drugs were determined against 16 *M. tuberculosis* isolates (from a panel of strains with different susceptibility patterns) by the conventional agar dilution method, it was found that most of the strains were resistant to at least one

drug. MICs were then determined for OFL and AMK by the same method against 8 strains. The strains were completely susceptible to these drugs irrespective of their susceptibility patterns to the standard drugs. Berlin *et al* (1987) and Leysen *et al* (1989) have also found that the activity of OFL like other fluoroquinolones against *M. tuberculosis* is unrelated to the susceptibility of this species to other anti-TB drugs. The MIC range found for this drug (Berlin *et al,* 1987; Leysen *et al,* 1989) as well as AMK (Heifets, 1991a; Sanders *et al*, 1982) against the 8 strains tested in the present study, was within the same range as that found previously (Berlin *et al,* 1987; Leysen *et al*, 1989). MICs obtained for the two alternative drugs were lower than those obtained for the four standard drugs against all but the fully susceptible reference strain, H37Rv.

In the Bactec radiometric susceptibility testing of mycobacteria, the 7H12 broth medium is used (Stratton,1993; Inderlied,1994). Employment of this broth medium offers an opportunity to compare results of MICs determined by the agar dilution method with results of daily measurement of growth radiometrically expressed as GI readings. Such comparative studies (Trimble *et al*,1987; Rastogi and Goh,1991; Heifets and Lindholm-Levy,1987 ; Heifets *et al*, 1986; Heifets *et al*, 1985a) have previously shown a good correlation between the Bactec broth and the agar dilution MICs for *M. tuberculosis*.

In the present study, comparison of MICs determined for the six anti-TB drugs in 7H12 broth and 7H10 agar method has demonstrated that the broth-determined MICs were lower than those obtained on the 7H10 media. This is in agreement with previous findings when MICs for standard TB drugs (INH, RMP,

EMB, SM and ethionamide) against *M. tuberculosis* were determined using liquid and/or solid media (Lee and Heifets, 1987; Heifets *et al* 1991) and compared with the 7H12 broth-determined MICs. Lee and Heifets (1987) used wild *M. tuberculosis* strains to compare results obtained by the two methods. In the present study, strains resistant to at least one standard drug were used in order to determine whether the differences in MICs occur over a wide range of MICs.

The differences between 7H12 broth- and agar-determined MICs indicate that 7H12 broth MICs were generally lower in this study: approximately 2 times lower in the case of INH, RMP, SM and OFL, and 2 to 5 times and 2 to 4 times lower in the case of EMB and AMK, respectively. A study by Lee and Heifets (1987) also showed similar differences when MICs determined by the Bactec radiometric method and the agar dilution method were compared for INH, RMP, EMB and SM. Heifets *et al* (1986) found the broth-determined MICs for EMB to be 4 to 8 times lower than the agar-determined MIC and this was the case irrespective of the susceptibility pattern of the *M. tuberculosis* strains to EMB. These authors used 7H11 agar medium in their study. In the present study 7H10 agar medium was used. According to Rastogi *et al* (1989) results obtained by the 7H10 and 7H11 agar media are comparable.

Broth-determined MICs for OFL were either the same or one dilution lower than the agar-determined MICs. Previously, agar-determined MICs of this drug were found to be equal (Rastogi and Goh,1991; Heifets,1987) or less than one dilution higher than those observed with 7H12 broth. However, in both studies 7H11 agar as opposed to 7H10 was used. Heifets (1991a) found the MIC range for AMK to be the same in 7H12 broth and 7H11 agar media. In this study,

this was not the case. AMK MICs in broth were 2 to 4 times lower than in agar. The reason for this discrepancy is not clear.

Strains found to be susceptible, intermediately resistant or completely resistant by the conventional method were also found to exhibit similar susceptibility or resistance ratings by the Bactec radiometric method. The only discrepancy found was when MICs for EMB were compared, and one of the four strains (MR 84452, Table 2.4) was found to be completely resistant by the agar dilution method (MIC of 20 ug/ml) and moderately susceptible (MIC of 4 ug/ml) by the 7H12 broth method.

The 7H12 broth is useful as different levels of susceptibility or resistance can be expressed based on previously agreed (but not yet internationally approved) criteria. With regard to EMB it is accepted that its susceptibility findings are influenced by the medium used as different types of media vary in their levels of absorption and inactivation of the drug (Gangadharam and Gonzales,1970). Although there was a discrepancy in EMB results for one strain, results obtained with the other three strains by the two methods were in good agreement.

Heifets *et al* (1985b) discussed the differences in MICs between the 7H12 broth and the agar methods and suggested that the actual concentrations in the agar medium are probably much lower than those originally incorporated into the solid medium This may be due to different levels of absorption, binding and degradation of the drugs in this medium (Lee and Heifets, 1987; Heifets, 1988a; Heifets *et al*, 1986).

In 7H12 broth, MIC determination requires a significantly shorter

period of incubation than in agar plates at 37°C thus reducing the time for heat-related degradation of the drugs (Lee and Heifets, 1987). Furthermore, 7H12 broth does not contain Tween 80 which could affect results of the MIC (Naik *et al*, 1989;Youmans and Youmans, 1948). Thus susceptibility testing in 7H12 broth more accurately reflects MIC levels, since this method requires a few days for the detection of the inhibitory activity, in contrast to the agar plate method which requires at least 12 days of incubation to obtain visible growth in controls and to detect the effect of inhibition, whereas the effect in 7H12 broth can be detected by radiometric readings within a few days of the addition of drugs (Heifets *et al*, 1985b).

This study confirmed results of MIC testing in both 7H12 broth and 7H10 agar media, obtained previously by other investigators. It confirmed the low MIC values for OFL (Heifets and Lindholm-Levy, 1987; Rastogi and Goh, 1991; Fehlon and Cynamon, 1986) and AMK (Heifets and Lidholm-Levy, 1989) previously found by other authors. In addition, it also showed that the agar-determined MICs for OFL and AMK were lower than those obtained for the standard drugs for all but the fully susceptible H37Rv strain. Furthermore, the study confirmed that the 7H12 broth- and 7H10 agar-determined MICs are comparable and that the Bactec method has a rapid turnaround time than the agar plate technique. Results of MICs obtained from the experiments in this chapter were used in the subsequent investigations.

2.4.2 Susceptibility testing by critical concentrations

Comparison of results obtained by the radiometric 7H12 broth and by the conventional agar procedures for susceptibility testing using

critical or breakpoint concentrations showed a good correlation when six drugs were tested against *M. tuberculosis* in this study. A strain which was completely susceptible or resistant by the conventional method was also found to be completely susceptible or resistant by the radiometric 7H12 broth method. Hoel and Eng (1991) also showed that the radiometric 7H12 broth is comparable to the conventional method for testing the susceptibility of *M. tuberculosis* strains to drugs when using comparable and established breakpoint concentrations.

The Bactec radiometric method for susceptibility testing is thus a good method for testing the susceptibility of *M. tuberculosis* strains to drugs first because results obtained by this method are comparable in accuracy with those obtained by the conventional method and, secondly because it is significantly faster. Furthermore, some drug resistant cultures do not grow sufficiently well on agar plates and require more than three weeks of incubation, possibly invalidating the drug susceptibility results (Chen *et al*, 1994).

2.4.3 Minimal bactericidal concentration

The MBCs and MICs were determined in the same medium (7H12) to obtain the most accurate MBC/MIC ratios (Heifets, 1988a; Heifets and Lindholm-Levy, 1989). Due to costs involved in using the Bactec system and, because the MBC technique is tedious and time consuming, the MBC was only determined against one strain, H37Rv. This study confirmed results obtained previously (Heifets, 1994; Heifets, 1991a) that INH is highly bactericidal with an MBC/MIC ratio of 1. Previously (Heifets *et al*, 1990), an MBC/MIC ratio of 2 was found for RMP against H37Rv using 7H12 broth. The

same ratio was found for H37Rv in the present study. When the bactericidal activities of RMP and INH were compared, in terms of the MBC/MIC ratio, RMP had a lower bactericidal activity than INH. This has also been reported previously (Dickinson *et al*,1977).

An MBC/MIC ratio of 1 to 4 has been previously reported for SM (Heifets and Lindholm-Levy,1989; Heifets, 1991a). Furthermore, the authors found that in terms of the MBC/MIC ratio, the bactericidal activity of SM was lower than that of INH. The same results were found in the present study.

OFL and AMK showed MBC/MIC ratios of 4 against the H37Rv strain. Previously MBC/MIC ratios of 2 to 4 (Leysen *et al*, 1989; Heifets and Lindholm-Levy,1987; Rastogi and Goh,1991) and 1 to 4 (Heifets,1994) were found for OFL and AMK, respectively. EMB was less bactericidal than INH, RMP, SM, OFL and AMK with an MBC/MIC ratio of 8. The same results were obtained previously (Heifets *et al*,1986) when the bactericidal activities of EMB, INH, RMP and SM were compared.

The bactericidal activities of the six drugs tested in this study are well known. This study confirmed previous findings by other investigators. All but EMB showed high bactericidal activities the latter being only moderately bactericidal.

The technique described by Heifets (1991a) has the added advantage of demonstrating the kinetics of killing which can be demonstrated graphically from the data obtained. INH and RMP achieved MBCs earlier (day 2.5 and 2.9, respectively) than the other test drugs (see Figure 2.1).

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The MBC technique is time consuming and labour intensive (Heifets, 1991a). Furthermore, because the use of the 7H12 radiometric method for MBC determination is expensive as compared to the conventional methods, 7H12 vials and 7H10 plates were only used in duplicate and not in triplicate (or more) in this study. The technique is very laborious and is therefore mainly suited for the testing of new anti-TB drugs or for research purposes, for example, when testing drug combinations and demonstrating degrees of tolerance. It is not suitable for routine testing of wild strains from patients.

CHAPTER 3

FEATURES AND DESIGN OF A RADIOMETRIC MODEL FOR THE EVALUATION OF *MYCOBACTERIUM TUBERCULOSIS* REGROWTH PATTERNS FOLLOWING EXPOSURE TO ANTI-TUBERCULOSIS AGENTS

3.1 BACKGROUND

3.1.1 *Drug evaluation in relation to patient management*

Compared with rapidly growing bacteria, *in vitro* models for the assessment of the activity of mycobacterial agents against *Mycobacterium tuberculosis* have been slow and cumbersome to perform and often difficult to interpret.

Drugs are evaluated in routine susceptibility testing to predict probable clinical outcome. In such cases, critical or breakpoint concentrations are incorporated into the media (Canetti *et al*, 1963; Canetti *et al*, 1969). The evaluation of drugs in relation to patient management has been extensively discussed in Section 2.1.1.1 in Chapter 2.

3.1.2 *Experimental models for the evaluation of candidate and conventional anti-tuberculosis drugs*

With regard to assessment of antimicrobial activity and experiments designed to gain information which could form the basis of rational treatment, several approaches have been used over the years. These included tests for measuring growth inhibition and ability to kill, including minimal inhibitory concentration (MICs) and minimal bactericidal concentration (MBC) determinations (Heifets, 1988b; Heifets and Lindholm-Levy, 1989).

Conventional *in vitro* determination of the inhibitory and bactericidal activity is essential and these basic investigations are performed early in the evaluation of candidate drugs for the treatment of infections. However, MIC and MBC determinations offer only a limited view of antimicrobial activity over a period of time. They do not reflect effects of drug concentrations below these values, which may have inhibitory or bactericidal activity when organisms are exposed to the antimicrobial agents for longer than the specified times for MIC or MBC determinations. In addition, continuous exposure in MIC and MBC testing does not allow for the determination of antimicrobial effects that persist after drug exposure, that is, postantibiotic effects (PAEs) (LeBel and Spino, 1988; Vogelman and Craig, 1985; Vogelman and Craig, 1986). Furthermore, although giving extremely useful information, the standard MBC model for *M. tuberculosis* as described by Heifets (1991a) is time consuming, labour intensive and expensive.

A very important aspect in drug evaluation which had a profound impact on the modern approach to the management of TB concerns PAE studies relating to the effects of drug exposure at various concentrations and exposure periods on regrowth of *M. tuberculosis* strains. These models generally incorporate a filtration step to remove the test drug from the organisms (Mitchison and Dickinson, 1971; Dickinson and Mitchison,1966; Dickinson and Mitchison,1987). The experiments relied on colony counts to establish the size of the inocula, the numbers of surviving bacteria and the rates of regrowth. The tendency of *M. tuberculosis* to clump created a real problem in the quantification of viable organisms but the use of surface active agents such as Tween 80 or Triton WR 1339 in liquid growth media, and standardization of clump dispersal by mechanical shaking, tended to yield acceptable results

(Lefford,1984; Wayne,1962). Another potential problem of regrowth assessment in some experimental designs was that the killing effect of the test drug and the resulting reduction on the number of surviving organisms available for regrowth, was not taken into account in the process of quantifying post-exposure regrowth (Paramasivan *et al*,1993; Dickinson and Mitchison,1987). This problem and terminology aspects related to it, has received considerable attention in the case of rapidly growing bacteria (Mackenzie *et al*, 1994; Hanberger *et al*, 1995).

Because of the complexity of the previously described models, it was decided to design a simple post-exposure regrowth system which would be easy to perform and would give reproducible results and at the same time be useful for the evaluation of candidate anti-TB drugs. The availability of the well-tried Bactec system which would be safe and could obviate the need for colony counting for regrowth assessment, was an attractive option which was finally employed in the design used in the present study.

3.2 DESIGN FEATURES

The envisaged model was based on the original approach used by Dickinson and Mitchison (1987) which determined the post-exposure lag period, and also on more recent concepts relating to post-exposure regrowth of rapidly growing bacteria. It was designed to meet defined criteria and incorporate the following features :

(1) The use of a closed system which would minimise the risks of contamination and optimise safety. (2) Within the limitations of a simple laboratory model, it ideally should simulate *in vivo* situations at the site of invasive disease. Therefore, as anti-

mycobacterial agents exert their action at the site of infection by (a) inhibiting multiplication, (b) killing bacteria in the case of bactericidal drugs and (c) delaying regrowth, it was decided that a model which caters for all three of the activities simultaneously should yield useful information. (3) The model should also be able to accommodate the study of drug combinations.

Although there is a great need for new anti-TB agents with a sterilizing effect which will also act on slowly replicating or semi-dormant bacilli, the model was designed primarily to determine efficacy on actively replicating organisms. As will be discussed in Chapter 8, an extension of the proposed model could also address drug effects on semi-dormant tubercle bacilli.

The model envisaged the use of (a) a standardized inoculum of an *M. tuberculosis* strain, (b) realistic concentrations of the drugs evaluated in terms of multiples of the MICs in a range within achievable concentrations at sites of infection, (c) exposure times based on pharmacokinetic data reflecting likely periods of *in vivo* exposure in TB lesions, (d) exclusion of filtration and washing of the exposed inoculum , before transfer into the regrowth medium, (e) post-exposure regrowth kinetics measured radiometrically in Bactec vials and (f) final dilutions of drugs following exposure to ensure concentrations in the regrowth media which would be below (by at least 5-fold) the MIC of the test organism. At relatively low dilutions, regrowth may be influenced by sub-MICs of the drug which will have to be taken into consideration in the evaluation process.

As an essential aspect of the evaluation of the model, the bactericidal activity of the test drugs at the concentrations and

exposure times used for the radiometric regrowth experiment would be determined by conventional techniques based on counting of colony forming units (CFUs). The effect of bactericidal activity on regrowth patterns would then be studied. The performance of the time-kill curve experiments in the present study would be used to assess to what extent patterns of regrowth in the radiometric model would be influenced by and correlate with the magnitude of bactericidal activity of the test drug.

The kinetics of killing will be described first in the next chapter and will be followed by the newly designed radiometric post-exposure regrowth experiments.

CHAPTER 4

KINETICS OF KILLING OF *MYCOBACTERIUM TUBERCULOSIS* AFTER EXPOSURE TO SINGLE ANTI-MYCOBACTERIAL AGENTS

4.1   BACKGROUND

The time-kill curve method used to assess the kinetics of killing of antimicrobial agents against actively dividing bacteria, is a standard technique to simulate the time course of bactericidal activity in *in vivo* situations (Shaw *et al*, 1988; Craig and Ebert,1991). This quantitative method has been widely used on rapidly growing organisms for the evaluation of new drugs, against clinically important bacterial isolates (Schoenknecht *et al*, 1985).

Like the MBC, the time-kill curve technique measures the microbicidal activity of the antimicrobial agent (Krogstad and Moellering, 1986). This approach however, confers a dynamic aspect to the evaluation of bactericidal activity, involving the kinetics of killing over a specified period of exposure (Mattie, 1981; Canton *et al*, 1993).

This technique can be performed *in vitro* as well as *in vivo*. *In vivo* experiments have been performed in animals by serial quantification of bacteria in infected tissue (Craig, 1993a; Vogelman and Craig, 1986). Modified *in vivo* kill-kinetics studies have also been conducted in guinea pigs infected with *M. tuberculosis* (Dickinson and Mitchison, 1976). In humans, *ex vivo* time-kill curve studies are performed by repeated sampling of an infected fluid such as cerebrospinal fluid (Craig, 1993a; Vogelman and Craig, 1986). The early bactericidal activity (EBA) test of *M. tuberculosis* strains is an

example of an *ex vivo* test where the ability of a drug to kill tubercle bacilli in a patient is measured at 12-hour intervals by performing bacterial counts on sputum samples (Botha *et al*, 1996; Sirgel *et al*, 1993).

The *in vitro* time-kill curve method involves incubation of a standardized inoculum in broth medium, that contains various concentrations of the drugs (usually reflecting clinically achievable concentrations) alone or in combination (Isenberg, 1988; Young, 1980; Jacobs, 1991; Woolfrey *et al*, 1987b)). A measured aliquot of the test organism is removed at predetermined intervals to follow in time the dynamics of multiplication or death of the organism (Trnka and Mison, 1988b; Isenberg, 1988). From these data curves can be constructed to demonstrate the change in the number of viable bacteria based on colony counts, with time as a function of exposure to the antimicrobial agent alone or in combination (Young, 1980).

Turbidimetric, biochemical or quantitative culture techniques can be used to monitor bacterial growth or death (Trnka and Mison, 1988b). A good correlation exists between the optical density and the number of particles per unit volume. A deficiency of turbidimetric techniques however, relates to the problem that both viable and dead bacteria are measured (Mattie, 1981). A method that can circumvent the counting of dead organisms is the estimation of viable organisms by means of a bioluminiscence assay (Hanberger *et al*, 1995), which measures the total living bacterial cell mass by determining the intracellular adenosine triposphase (ATP). The counting of live microorganisms based on colony forming units (CFUs), however, is still regarded as the most reliable method for determining growth in the presence of antimicrobial agents

(Mattie, 1981).

When antimicrobial agents are tested singly, time-kill curve experiments compare the number of bacteria at time zero with the number of surviving bacteria after a specified period of drug exposure (Jacobs, 1991). The killing kinetics of an antimicrobial agent are dependent on certain factors, for example, drug concentration and exposure time (Nishida *et al*, 1978). To quantitate the bactericidal effects of increasing concentrations and exposure time, a variety of techniques have been used. These include, determination of bactericidal rate, that is, the slope of the plot of viable survivors at various time intervals (Jawetz, 1967), the area under the killing curve (AUC) (Tisdale *et al*, 1989), the percentage of inoculum killed at various exposure times (Woolfrey *et al*, 1987a), and mathematical modelling (Thonus *et al*, 1982; Gueriloot *et al*, 1993; Li *et al*, 1997).

Results of these studies suggest three categories of antimicrobial agents based on their pattern of bactericidal activity, namely: (1) those with marked concentration-dependent bactericidal activity such as aminoglycosides and quinolones. Increasing levels of these drugs have a major effect on the rate and extent of bactericidal activity, (2) those with little concentration-dependent bactericidal activity. Bacterial killing with these drugs is more time-dependent with little relationship to the magnitude of the drug concentration above the killing threshold, and (3) drugs that are significantly affected by both concentration and duration of exposure and (4) those that are predominantly bacteriostatic (Craig, 1993a; Vogelman and Craig, 1986).

Like most techniques, the time-kill curve method has its limitations.

Repetitive colony counts are tedious and thus limit the number of antimicrobial concentrations as well as combinations that can be tested with any one isolate. (Moellering, 1979a, Krogstad and Moellering, 1986). Another disadvantage to the use of this technique is that large populations of bacterial organisms may not be uniformly sensitive to antimicrobial agents. Resistant subpopulations can arise during drug exposure and confound the results (Vogelman and Craig, 1986). Additional potential problems with the time-kill curve method include drug carry-over and whether inocula used are in the exponential or stationary phase of growth (Wolfson and Hooper, 1989).

4.2   OBJECTIVE

In vitro techniques that have been used in the management of TB and in formulating basic principles to guide a rational application of anti-TB drugs have been discussed in Chapter 2. The objective of the experiments described in this chapter was to determine the kinetics of killing of *M. tuberculosis* following drug exposure.

The findings of the time-kill curve experiments would subsequently be used in the appraisal of the newly designed radiometric regrowth model, by comparing bactericidal activity with regrowth time. Drug combination studies in which kill kinetics of single drugs are compared with those of drug combinations, would also be performed. These will be covered in Chapter 5 to Chapter 8.

4.3   MATERIALS AND METHODS

Antimicrobial agents

Stock solutions of isoniazid (INH), rifampicin (RMP), ethambutol

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(EMB), streptomycin (SM), ofloxacin (OFL) and amikacin (AMK) (1,000 *u*g/ml) and working solutions of drugs were prepared as in Chapter 2, Section 2.2.1.

Test strains

One strain, the standard reference strain, H37Rv ATCC 27294, was used.

Procedure

The inoculum was prepared by subculturing into 50 ml of 7H9 broth (Bacto) with constant magnetic stirring (to prevent or reduce aggregate formation (Wayne,1994) for 7 to 8 days, until the turbidity of the culture equalled that of the McFarland no. 1 standard (Appendix 1). This is approximately 10^7 CFU/ml (NCCLS,1991). Using 7H9 broth, the standardized culture was diluted 10-fold to provide an initial inoculum of approximately 10^6 CFU/ml. This suspension was used to perform assays in sterile 15 ml screw capped plastic test tubes containing 3 to 4 glass beads (diameter 3 mm).

The assay mixture contained 4.5 ml of the bacterial suspension and 0.5 ml of the working drug solutions. The final concentrations of drugs were selected based on the previously determined minimal inhibitory concentrations (MICs) and also on achievable serum levels and were multiples of the MIC (½ x MIC, 1 x MIC, 3 x MIC and 8 x MIC for INH; 1 x MIC, 3 x MIC and 8 x MIC for RMP, SM and AMK and, 1 x MIC and 2 x MIC for EMB and OFL). Drug solutions were prepared in 0.5 ml volumes which resulted in the desired concentrations when added to 4.5 ml of bacterial suspensions. A test tube containing 5 ml of bacterial suspension not exposed to a test drug was used as a control.

After the addition of drugs, test tubes were incubated at 37°C. Sampling to determine the number of bacterial survivors was performed at 0, 6, 24, 48 and 72 hours after exposure to the drugs. The test tubes were immediately reincubated following each sampling procedure. CFUs were used to determine the number of surviving bacteria following drug exposure.

A 0.1 ml sample was taken from each tube and was serially diluted (10^{-1} to 10^{-5}) in sterile 7H9 broth without albumin-dextrose-catalase (ADC). A 0.01 ml portion of each diluted and undiluted sample was inoculated onto duplicate drug-free 7H10 plates. The spots were allowed to dry and the plates were incubated at 37°C in the presence of 5-10% CO_2 atmosphere for 3 weeks. After 3 weeks of incubation CFU/ml were calculated. When regrowth occurred, the MICs of the drugs were determined in 7- to 8-day old cultures and compared with MICs initially determined for the *M. tuberculosis* strain.

Results were interpreted by comparing CFU/ml obtained in the drug-free assay tube at time zero with those obtained with the drug-containing assay tubes at 6, 24, 48 and 72 hours of exposure. From this comparison the magnitude of killing was calculated. Kill curves were constructed by plotting $log_{10}CFU$ of viable bacteria remaining per millilitre versus time of incubation at the corresponding drug concentration.

To minimize and check for possible antibiotic carry-over effects on subculture plates, two methods were used. Samples were diluted 10^{-1} to 10^{-5} (Lacey *et al*, 1983) at the different time intervals from zero to 72h of incubation. When the concentrations of bacteria calculated from undiluted or low-dilution subculture samples were

less than those calculated using results from the higher dilutions, the possibility of antibiotic carry-over was entertained. The bacterial concentration was then calculated by using results of CFUs on the plates inoculated with higher dilutions, which would exhibit no or negligible residual antibiotic activity.

Using another method, a 0.1 ml sample of each drug dilution was plated onto 7H10 agar plates in triplicate. The plates were incubated at 37°C until resorption of the liquid was complete. Each plate was inoculated with approximately 100 CFU of the culture strain. Control plates without antimicrobial agents were prepared under the same conditions. Plates were incubated at 37°C and colonies were counted, and CFU/ml calculated after 21 days of cultivation. CFU/ml in drug-containing plates were compared with those in drug-free plates to demonstrate at which dilutions a carry-over effect may occur.

Standard errors of the mean were calculated for each drug exposed and unexposed control cultures. Since CFU counts were only done in duplicate, these were based on relatively few observations and can vary widely. In order to give an indication of such variation, standard errors of the mean were derived from the pooled error mean square (s^2) calculated. This approach assumes that the variance among drug concentrations and exposure time remains constant.

4.4 RESULTS

The bactericidal kinetics in terms of the mean $\log_{10}$ decrease in viable counts of the various anti-mycobacterial drugs are given in Table 4.1 and are graphically illustrated in Figure 4.1. The actual

viable counts in terms of log_{10}CFU/ml, with the pooled standard errors of the mean are given in Table 4.2. The coefficient of variation (CV), expressed as a percentage of standard deviations divided by the means of the CFU/ml findings, varied from 12,13% for EMB, to 28.44% for SM, with a mean of 19.45% for the six drugs tested.

4.4.1 *Isoniazid and rifampicin*

Results show that both INH and RMP are highly bactericidal, reducing the exposed inoculum by 2-3 log_{10} CFUs over a period of 48-72 hours. The bactericidal action of INH is less dose-dependent than that of RMP. RMP acts more rapidly than INH and already shows a reduction in viable counts of approximately 1 log_{10} at 1 MIC and 3 MIC and, a log_{10} CFU/ml reduction of 1.77 at 8 MIC, after 6 hours of exposure, while even at 8 MIC, INH only showed log_{10} reductions of approximately 0.5 and 0.6 log_{10} CFU/ml at both 6 and 24 hours of exposure, respectively.

After 48 hour exposure, however, INH showed activity equal to or marginally better than RMP at 1 MIC and 3 MIC and similar but marginally less activity than RMP at 8 MIC (1.99 log_{10} versus 2.43 log_{10} CFU/ml, respectively). The bactericidal activities of the two drugs at 72 hours were very similar, varying between 1.89 log_{10} to 3.02 log_{10} CFU/ml for INH and 1.79 log_{10} to 2.49 log_{10} CFU/ml for RMP, for the different drug combinations, giving INH an apparently marginal edge over RMP.

From the kill curves it is clear that for INH, exposure time is more important than concentration, at least up to 48 hour exposure. At 72 hours however, increased concentrations resulted in increased killing. In the case of RMP on the other hand, concentration is more

important than exposure time. After 24 hour exposure, the rates of killing by RMP remained the same but the levels of surviving bacterial populations were still related to exposure concentrations although the differences were not marked. The concentration effect appears to be very important as evidenced by the low count of surviving bacteria following exposure at 8 MIC for 6 hours which was already lower than that seen with 1 MIC at 72 hour exposure time. Exposure times of longer than 24 hours did not substantially increase RMP killing.

4.4.2 *Ethambutol and streptomycin*

Like RMP, EMB already showed bactericidal activity at 6 hours which generally increased with increasing time. The killing effect which was considerably less than that of INH and RMP did not appear to be dose-dependent in the range tested (up to 2 MIC).

Virtually no killing was observed with SM at the concentrations (maximum 8 MIC) and exposure time (maximum 72 hours) used in this study. Because of the poor bactericidal activity found at 1, 3 and 8 MICs, it was decided to extend the experiments to include concentrations of 10 *u*g/ml (10 MIC) and 20 *u*g/ml (20 MIC). Definite bactericidal activity was already present after 24 hours exposure time at both 10 MIC and 20 MIC. A 1.4 $\log_{10}$ CFU/ml killing was achieved after 72 hours when sustainable levels (20 *u*g/ml) were used.

4.4.3 *Ofloxacin and amikacin*

These two agents showed similar kill kinetics. They showed delayed bactericidal activities compared with EMB but after 24 hour exposure, the bactericidal effects of the three drugs were similar.

Table 4.1 Bactericidal kinetics of anti-tuberculosis drugs against *M. tuberculosis* H37Rv using the conventional time-kill curve technique.

Drug[a]	Conc[b] (xMIC)	Mean $\log_{10}$ CFU/ml[c] decrease in viable counts after exposure (hours)[d]			
		6	24	48	72
INH	½	0.28	0.35	1.61	1.89
	1	0.27	0.83	1.78	2.29
	3	1.05	0.92	1.70	2.47
	8	0.45	0.56	1.99	3.02
RMP	1	0.73	1.33	1.25	1.79
	3	1.02	2.01	1.69	2.39
	8	1.77	2.26	2.43	2.49
EMB	1	0.44	0.51	0.64	0.92
	2	0.55	0.59	0.97	1.08
SM	1	0.08	-0.01[e]	-0.01	-0.02
	3	-0.08	-0.11	-0.09	-0.15
	8	0.1	0.01	0.29	0.06
	10	ND[f]	0.44	0.68	0.84
	20	ND	0.80	0.88	1.37
OFL	1	0.25	0.54	0.52	0.61
	2	-0.16	0.65	0.83	1.04
AMK	1	0.19	0.45	0.09	0.23
	3	0.23	0.45	0.48	0.46
	8	0.24	0.76	1.09	0.66
Control	0	0.07	-0.07	-0.16	-0.13

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin. [b]Conc (x MIC) = concentration in multiples of the MICs equal to 0.05 *u*g/ml, 0.5 *u*g/ml, 2.0 *u*g/ml, 1.0 *u*g/ml, 0.5 *u*g/ml and 0.25 *u*g/ml for INH, RMP, EMB, SM, OFL and AMK, respectively. [c]CFU = colony forming units. [d]The mean decrease in viable counts was calculated from the initial concentration of the control cultures = 6.091 $\log_{10}$ CFU/ml, [e]A minus sign denotes an increase in viable counts. [f]ND = not done.

Table 4.2 The killing kinetics for six anti-tuberculosis drugs against *M. tuberculosis* H37Rv. Standard errors of the mean are given in parentheses.

Drug[a]	Conc[b] (xMIC)	Viable counts in $\log_{10}$ CFU/ml[c] at various exposure times (hours)							
		6		24		48		72	
INH	½	5.813	(0.375)	5.746	(0.375)	4.482	(0.375)	4.205	(0.375)
	1	5.825	(0.375)	5.258	(0.375)	4.314	(0.375)	3.793	(0.375)
	3	5.042	(0.375)	5.175	(0.375)	4.39	(0.375)	3.619	(0.433)
	8	5.639	(0.375)	5.532	(0.375)	4.105	(0.375)	3.072	(0.375)
RMP	1	5.356	(0.191)	4.759	(0.191)	4.838	(0.191)	4.291	(0.191)
	3	5.068	(0.191)	4.081	(0.191)	4.398	(0.191)	3.699	(0.191)
	8	4.324	(0.191)	3.834	(0.191)	3.659	(0.191)	3.602	(0.382)
EMB	1	5.648	(0.163)	5.578	(0.163)	5.449	(0.163)	5.17	(0.163)
	2	5.545	(0.163)	5.496	(0.163)	5.117	(0.163)	5.007	(0.163)
SM	1	6.01	(0.091)	6.098	(0.091)	6.103	(0.091)	6.109	(0.091)
	3	6.169	(0.091)	6.197	(0.091)	6.187	(0.091)	6.238	(0.091)
	8	5.99	(0.091)	6.083	(0.091)	5.802	(0.091)	6.029	(0.091)
	10	ND[d]	ND	5.646	(0.091)	5.414	(0.091)	5.249	(0.091)
	20	ND	ND	5.286	(0.091)	5.211	(0.091)	4.724	(0.091)
OFL	1	5.836	(0.247)	5.552	(0.247)	5.567	(0.247)	5.477	(0.247)
	2	6.245	(0.247)	5.436	(0.247)	5.259	(0.247)	5.046	(0.247)
AMK	1	5.896	(0.21)	5.645	(0.21)	6.001	(0.21)	5.858	(0.21)
	3	5.858	(0.21)	5.645	(0.21)	5.607	(0.21)	5.626	(0.21)
	8	5.855	(0.21)	5.326	(0.21)	5.001	(0.21)	5.431	(0.21)
Control[e]	0	6.018	(0.104)	6.162	(0.09)	6.249	(0.09)	6.215	(0.09)

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin. [b]Conc (xMIC) concentration in multiples of the MICs. [c]CFU = colony forming units. [d]ND = not done. [e]The initial bacterial concentration was 6.091 with a standard error equal to 0.09.
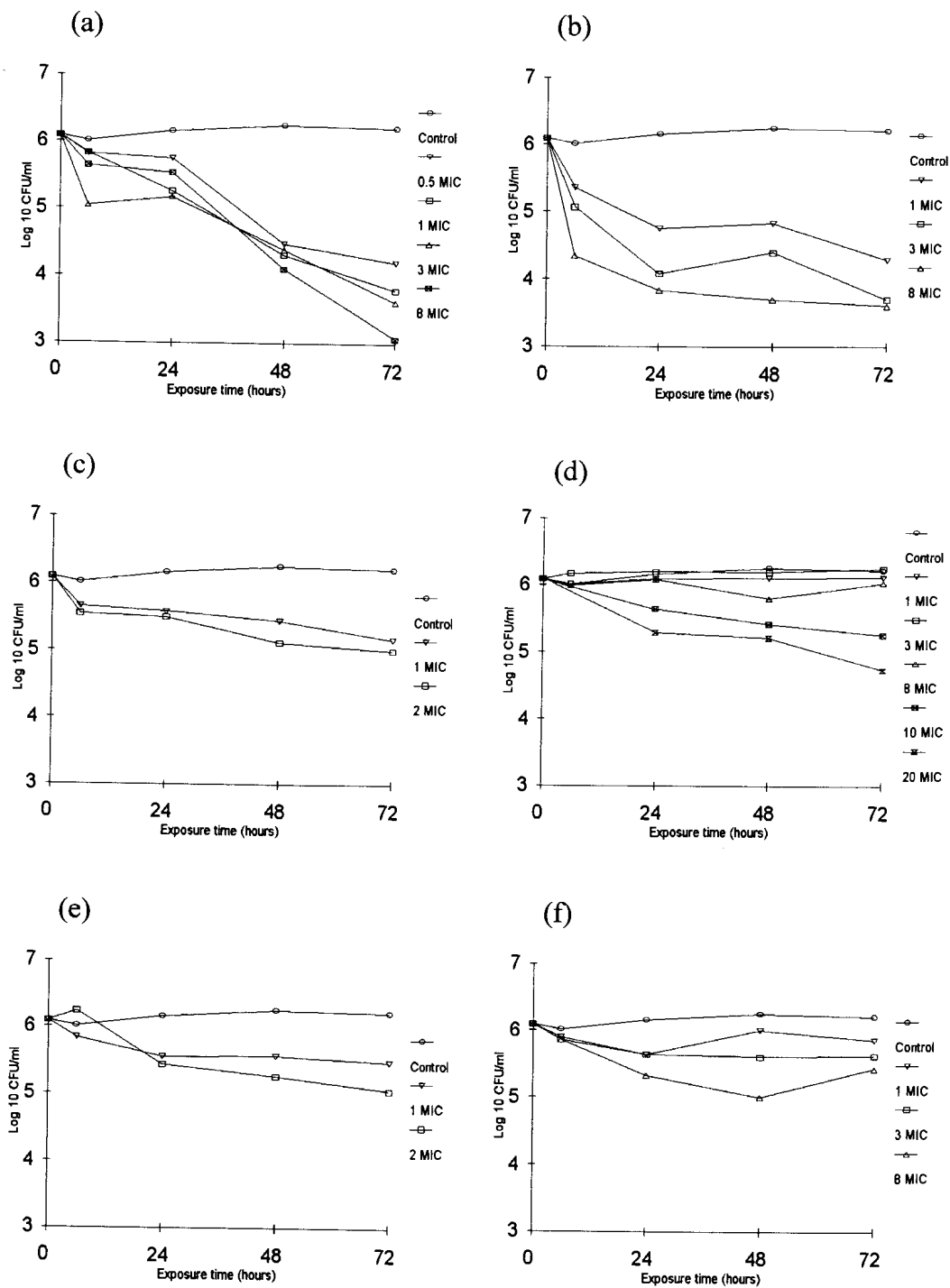
Figure 4.1: Kinetics of killing for isoniazid (INH, MIC = 0.05 *u*g/ml), (b) rifampicin (RMP, MIC = 0.5 *u*g/ml), (c) ethambutol (EMB, MIC = 2.0 *u*g/ml), (d) streptomycin (SM, MIC = 1.0 *u*g/ml). The initial bacterial concentration for the same for 1, 3, 8, 10 and 20 MIC experiments while the 6 hour exposure time was not done in the 10 and 20 MICs experiments, (e) ofloxacin (OFL, MIC = 0.5 *u*g/ml) and (f) amikacin (AMK, MIC = 0.25 *u*g/ml) against *M. tuberculosis* H37Rv using the conventional time-kill curve model. The standard errors of the mean are presented in Table 4.2.

4.5 DISCUSSION AND CONCLUSIONS

4.5.1 *Comparison with other in vitro studies employing colony forming units*

The findings reported in this chapter are in general agreement with those in the literature and are summarized in Table 4.3. The only obvious discrepancy concerns the bactericidal activity of SM which was exhibited only at 8-20 MICs (concentrations which are achievable in the blood and tissues) as opposed to activity at 5 *u*g/ml (5 MIC) in other studies (Dickinson *et al*, 1977).

INH and RMP were the two most bactericidal drugs at all exposure times and concentrations tested in the present study. RMP showed the greatest rate of CFU decrease during the early hours of exposure followed by INH, EMB, OFL and AMK, and SM respectively. This is in agreement with Mitchison and Dickinson (1978) who reported that killing with RMP may start within one hour of exposure of a culture of *M. tuberculosis* to the drug which compares with a period of at least a day for INH. At 48 and 72 hours, INH and RMP generally showed the same activities although INH was slightly more active than RMP. EMB was third to INH and RMP in bactericidal activity. At all exposure times tested, SM was the least active drug at concentrations up to 8 MIC. The use of higher concentrations which are achievable *in vivo* (10 and 20 MICs), increased the activity of SM. EMB, SM, OFL and AMK were weakly bactericidal at the concentrations and exposure times tested.

Similar experiments have been performed previously (Dickinson and Mitchison, 1966; Dickinson *et al*, 1977; Dickinson and Mitchison, 1987) in which log phase cultures of *M. tuberculosis* H37Rv were exposed to anti-TB drugs for short periods of time. In Dickinson *et*

al (1977) experiments, *M. tuberculosis* cultures were exposed to INH, RMP, pyrazinamide and EMB alone and in combination at various concentrations that are achievable in the body. Drug activity was measured as the decrease in viable counts at 4 and 7 days. The authors found INH, RMP and SM to be the three most actively bactericidal drugs, SM being more bactericidal than either INH or RMP after prolonged exposure of up to 96 hours. Results obtained by these authors with the latter drugs compare well with those obtained in the present study since INH and RMP were found to be the most actively bactericidal drugs in both studies. However, in the case of SM, results disagree. In the present study this drug was found to be much less active than the activity reported by Dickinson *et al* (1977).

As in the present study, the authors found INH to be slightly more bactericidal than RMP (at the longer exposure times) but the difference between the two drugs in bactericidal activity was not significant. Dickinson *et al* (1977) showed that EMB had little bactericidal activity and allowed growth to occur during the first four days. EMB was found to be moderately bactericidal in the present study however, it did not allow growth to occur in the exposure times tested. In EBA studies, EMB was however found to be more bactericidal than SM or ciprofloxacin (Sirgel *et al*, 1993; Mitchison, 1998). Findings of the present study therefore agree better with those of EBA studies compared with earlier *in vitro* reports (Dickinson *et al*, 1977; Mitchison, 1998)

The difference in the findings obtained by Dickinson *et al* (1977) and those obtained in the present study may be due to the different experimental conditions of the two studies. The main differences were the filtration-washing procedure which contained Tween 80.

Table 4.3 Comparison of kill kinetics of anti-tuberculosis drugs, over 72 hours. Comparison with published *in vitro* and early bactericidal activity (EBA) studies.

Drug[a]	Killing in relation to						
	Dependence on		Overall effect				
	Concentration	Exposure time (hours)[b]	Present study (exposure time in hours)				Literature (*in vitro* and EBA)[c]
			6	24	48	72	
INH	+	+ + +, (24h)	0 - +	+ + +	+ + + +	+ + + +	+ + + +
(EBA : 0.5-0.722[c])	NS[d]						+ + + +
RMP	+ + + +	+, (6h)	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
(EBA : 0.20[c])	+ +,P<0.05						+ +
EMB	±	+, (6h)	+ +	+ +	+ + +	+ + +	+
(EBA : 0.18[c])	±, NS						+ + +
SM	+ + +,	+ + +, (<24h)	0[e]	0(+ +)[f]	+(+ + +)[f]	±(+ + +)[f]	+ + + +
(EBA : 0.09[c])	NA[g]						+
OFL	±	+, (24h)	0	+ +	+ + +	+ + +	+ + + +
(EBA cipro : 0.1-0.2[h])	+ +,P<0.001						+ +
AMK	+	+ +, (24h)	0	+	+ + +	+ + +	+ + + +
(EBA : NA[d])	NA						NA

[a]INH = isoniazid (MIC = 0.05 ug/ml), RMP = rifampicin (MIC = 0.5 ug/ml), EMB = ethambutol (MIC = 2.0 ug/ml), SM = streptomycin (MIC = 1.0 ug/ml), OFL = ofloxacin (MIC = 0.5 ug/ml), AMK = amikacin (MIC = 0.25 ug/ml). [b]Exposure time in parentheses indicate the time required for drugs to show bactericidal activity. [c]References : Dickinson and Mitchison (1976), Dickinson *et al* (1977), Jindani *et al*, (1980), Mitchison (1992), Sirgel *et al*, (1993), Mitchison, (1998). EBA expressed as decrease in $\log_{10}$ CFU/ml per day during the first 2 days. [d]NS = not significant. [e]10 MIC and 20 MIC of SM were not tested at 6 hour exposure time. [f]Activity of SM at 10 MIC and 20 MIC is given in brackets. [g]NA = not available. [h]EBA of ciprofloxacin which has similar *in vitro* activity to OFL.

The latter has been shown to have an effect on the activity of some drugs (Naik *et al*, 1989). The possibility that the SM powder used in our experiments was degraded with diminished anti-mycobacterial activity cannot be excluded.

The technique used in the present study was able to demonstrate the time course of bactericidal activity during the early hours of exposure. Studies done to determine the minimal bactericidal concentrations (MBCs) of the six test drugs in the present study (Chapter 2) compare well with results obtained by Dickinson *et al* (1977) as continuous and not pulsed exposure is used in these studies. As shown by Dickinson *et al* (1977), INH, RMP and SM were found to be the three most bactericidal drugs by the MBC technique. Moreover, SM achieved the MBC only after 12 days of exposure, as opposed to 3 days in the case of both INH and RMP. Although the rate and amount of killing for SM, OFL and AMK were slow and low during the 72 hours of exposure, respectively, in terms of the time-kill curve studies, MBC experiments done in the present study showed that the drugs are bactericidal against *M. tuberculosis.* The time-kill curve technique performed in this study could not demonstrate the high bactericidal activity of SM reported by Dickinson *et al,* (1977). Exposure times of longer than 72 hours used in our time-kill curve studies for SM, which achieved an MBC at 2 MIC, and/or higher concentrations for OFL and AMK which achieved the MBCs at 4 MIC, are required for these drugs to show significant bactericidal activity.

In another study, Dickinson and Mitchison (1966) tested the activity of six anti-TB drugs, INH, SM, ethionamide, cycloserine, thiacetazone and thiocarlide, against *M. tuberculosis.* The drug concentrations used in their study were 10 times the inhibitory

concentrations. In the case of INH, the authors observed a slight rise in CFU counts during the first 24 hours of exposure and bactericidal activity was only achieved after this exposure period. Colony counts were reduced by 3 $\log_{10}$ after 96 hours of exposure. In the present study a slight decrease in viable counts was observed even after 6 and 24 hours of exposure and a 3 $\log_{10}$ decrease in viable counts was already achieved after 72 hours at 8 times the MIC (equivalent to 0.4 ug/ml which is approximately 2 times the concentration used by Dickinson and Mitchison (1966).

In further experiments by Dickinson and Mitchison (1987), the activity of the various rifamycins including RMP at 1.0 ug/ml and 0.2 ug/ml against *M. tuberculosis* H37Rv was investigated. Cultures were exposed to drugs for 9 days and viable counts were set up at intervals for 9 days. A 1 $\log_{10}$ unit reduction in viable counts was observed for 1 ug/ml of RMP after 3 days of exposure. In the present study a 1 $\log_{10}$ unit reduction in CFU/ml was already achieved for RMP at half the concentration (0.5 ug/ml, equivalent to 1 MIC of RMP) after 24 hours of exposure.

Gangadharam *et al* (1990) investigated the influence of exposure time, temperature and concentration and the state of microbial metabolism on the activity of EMB against *M. tuberculosis*. The authors exposed *M. tuberculosis* cultures continuously to EMB and viable counts were taken at intervals for 12 days. EMB was found to have bactericidal action at 10 ug/ml (peak concentration readily attainable with recommended weekly dosage of 50 mg/kg). As found in the present study, it only showed modest killing activity.

4.5.2 *Comparison with previous ex vivo studies*
We also compared our results with those obtained previously on the

EBA of anti-TB drugs in patients (see Table 4.3). During the first two days of treatment of TB patients, the EBA is directed against bacilli in sputum that are predominantly extracellular, that is, that arise from the very large population in cavity walls, distant from phagocytic cells. During this early treatment period, the bacilli are multiplying rapidly and are killed most rapidly by bactericidal drugs. It is their death that is measured by the EBA. The EBA thus estimates the activity of a drug against rapidly dividing, mainly extracellular bacilli and different killing kinetics studies with previous EBA findings are shown in Table 4.3 (Botha *et al*, 1996). In EBA *in vivo* situations, RMP and OFL have concentration-dependent effects (Sirgel *et al*, 1993; Botha *et al*, 1996) but this was not the case with INH and EMB (Table 4.3) in the present study.

In vitro experiments done by Gangadharam *et al* (1990) showed that the activity of EMB was dependent upon both the period of exposure and concentration. In the present study, there was evidence of exposure time effects on the activity of EMB however at the concentrations tested, this drug did not seem to be dose-dependent. In their EBA studies, Jindani *et al* (1980) also found that bactericidal activity was dose-related for RMP but not for INH (Table 4.3). Sirgel *et al* (1993) indicated that EBAs for EMB and INH were not dose related.

Yew *et al* (1994) treated multidrug resistant TB (MDR-TB) patients with OFL at 300mg and 800mg usually in combination with second line anti-TB agents and found culture conversion to be more rapid with the higher OFL dose. The EBA of 0.1-0.2 $\log_{10}$ decrease in CFUs for ciprofloxacin was also shown to be dose related (Sirgel *et al*, 1993).

The time-kill curve technique performed in the present study provided information on rates and magnitude of bacterial killing. It also provided information on the effects of concentration and exposure time on the activities of the six anti-TB drugs against *M. tuberculosis.* The technique is however, labour-intensive. Other potential problems with this technique include, inadequate dispersal of clumped bacilli which may interfere with viable counting and drug penetration, drug carry-over effects and the growth phase (late lag, exponential or early stationary) of inocula.

CHAPTER 5

RADIOMETRICALLY-DETERMINED REGROWTH OF *MYCOBACTERIUM TUBERCULOSIS* AFTER EXPOSURE TO SINGLE ANTI-MYCOBACTERIAL AGENTS

5.1 BACKGROUND

Conventional methods for the laboratory evaluation of candidate anti-tuberculosis (anti-TB) drugs are based on the determination of bacteriostatic and bactericidal activities and postantibiotic effects (PAEs). In the clinical setting, drugs may merely inhibit growth in the lesions or may kill a proportion of bacteria while the remainder may subsequently regrow at different rates, depending on the number of survivors and PAEs of the drugs or drug combinations involved. A model which measures regrowth of surviving *M. tuberculosis* cells following different drug-exposure periods and drug concentrations was designed. The background and design features of this model are given in Chapter 3.

5.2 OBJECTIVES

The objectives of the studies recorded in this chapter were (1) to evaluate the radiometric model which was designed to measure bacteriopausal effects and regrowth patterns using isoniazid (INH), rifampicin (RMP), ethambutol (EMB), streptomycin (SM), ofloxacin (OFL) and amikacin (AMK), and (2) to compare the activities of the six drugs in this model.

5.3   MATERIALS AND METHODS

Antimicrobial agents and test strains were the same as those used in Chapter 4. Aliquots that were used for assessing regrowth by the Bactec radiometric system, were taken from the same tubes and at the same time when samples were taken for colony forming unit (CFU) counting in the time-kill curve studies. A 0.1 ml sample was taken from each assay tube and was used undiluted to inoculate the respective drug-free standard 7H12 Bactec vials (4 ml, Becton Dickinson). The vials were incubated at 37°C and growth index (GI) readings were taken daily at 24-hour intervals, using a Bactec TB-460 instrument (Johnston Labs Inc.,Maryland) until a GI reading of 999 was reached or until the GI reached a maximum and started to decline. The cumulative GIs were plotted against time to describe the growth curves.

The time taken for the unexposed control and drug-exposed cultures to grow to a defined GI reading of 400, called T_{400} was calculated using linear interpolation. A GI of 400 was chosen because good discrimination between regrowth times following drug exposure is achieved at this level of metabolic activity, and it also represents approximate counts of 10^4 to 10^5 CFU/ml (Heifets, 1991a) which could relate to clinical relevance. Control cultures were determined by inoculating 0.1 ml of the continuously incubated unexposed cultures at the end of each exposure period (see Discussion). The difference between T_{400} readings of the exposed and the control cultures were then calculated as an index of post-exposure regrowth and was termed $(T-C)_{400}$. Delays in regrowth were expressed in terms of this value. Due to relatively few observations (as in the bactericidal activity studies) standard errors of the T_{400} means were calculated using the pooled estimate error mean square (s^2).

5.4   RESULTS

Post-exposure regrowth curves and curves of the unexposed cultures generated at the end of each exposure period are given in Figure 5.1 to Figure 5.6. The $(T-C)_{400}$ indices for the six anti-TB drugs at different concentrations, given in terms of minimal inhibitory concentrations (MICs), and exposure periods are set out in Table 5.1. The absolute T_{400} values with the pooled standard errors are given in Table 5.2.

5.4.1 *Isoniazid*

The radiometric regrowth patterns for INH at different exposure times are demonstrated in Figure 5.1. INH showed relatively long delays in regrowth, expressed as $(T-C)_{400}$ indices, and lag periods before regrowth started were already present after 6 hour exposure at the higher concentrations when, according to the bactericidal activity findings (see Figure 5.1), there was disproportionately less evidence of killing. This was especially noticeable at the 8 MIC/6h exposure where the $(T-C)_{400}$ index was > 14 days, providing strong evidence in favour of PAE. Delays in regrowth became progressively longer and growth rates slower with increasing exposure times of 6, 24, 48 and 72 hours, respectively.

Delays in regrowth also increased with increasing concentration and following 24 hour exposure, increased from approximately 5 days at 1 MIC to greater than 14 days at 3 MIC. At all exposure times with 8 MIC concentrations, there was no evidence of metabolic activity during the observation period of 14 days. The presence of sub-MICs in the 8 MIC Bactec vials most likely contributed to the long delay in regrowth, constituting a true subinhibitory PAE.

Table 5.1 T_{400} readings in days in Bactec vials of *M. tuberculosis* H37Rv regrowth following exposure to six anti-tuberculosis drugs in excess of time taken by the unexposed control cultures to reach a GI of 400.

Drug[a]	Conc (x MIC)[b]	T_{400} indices[c] in days after different exposure periods (hours)			
		6	24	48	72
INH	½	1.84	3.82	5.65	8.38
	1	2.35	5.31	6.45	9.19
	3	6.14	>14[d]	11.61	>14
	8	>14	>14	>14	>14
RMP	1	2.37	5.34	8.45	8.79
	3	6.29	>14	>14	>14
	8	>14	>14	>14	>14
EMB	1	3.82	4.29	4.79	4.78
	2	3.8	4.44	6.62	6.07
SM	1	0.52	1.05	0.16	0.65
	3	0.52	1.32	1.61	1.03
	8	1.19	1.64	2.55	2.66
	10	ND[e]	7.34	8.98	11.53
	20	ND	>14	>14	>14
OFL	1	1.44	1.57	2.12	1.97
	2	1.24	2.27	4.09	4.5
AMK	1	1.42	1.6	0.63	2.39
	3	1.34	2.83	1.58	3.15
	8	1.83	3.57	4.46	5.15
Control	0	3.38	2.35	2.17	2.02

[a] INH = isoniazid (MIC = 0.05 ug/ml), RMP = rifampicin (MIC = 0.5 ug/ml), EMB = ethambutol (MIC = 2.0 ug/ml), SM = streptomycin (MIC = 1.0 ug/ml), OFL = ofloxacin (MIC = 0.5 ug/ml), AMK = amikacin (MIC = 0.25 ug/ml). [b]Antimicrobial concentrations are shown as multiples of the MICs. [c]T_{400} indices = $(T-C)_{400}$, readings for the unexposed control cultures (C) subtracted from those of the drug exposed cultures (T) at each exposure time. [d]T_{400} readings of greater than 14 days where there was no metabolic activity even after 14 days of reading the Bactec 7H12 vials, and readings adjusted with the control. [e]ND = not done.

Table 5.2 Time taken for *M. tuberculosis* cultures to grow to a Bactec radiometric growth index of 400 (T_{400}) after exposure to six anti-tuberculosis drugs. Standard errors of the mean are given in parentheses.

Drug[a]	Conc[b] (x MIC)	T_{400} readings[c] at various exposure times (hours)			
		6	24	48	72
INH	½	5.217 (0.436)	6.164 (0.436)	7.814 (0.884)	10.397 (0.884)
	1	5.73 (0.884)	7.653 (0.884)	8.612 (0.884)	11.206 (0.884)
	3	9.518 (0.884)	>14 NM	13.772 (1.251)	>14 NM
	8	>14 NM[d]	>14 NM	>14 NM	>14 NM
RMP	1	5.752 (0.552)	7.699 (0.552)	10.625 (0.552)	10.809 (0.637)
	3	9.669 (0.552)	>14 NM	>14 NM	>14 NM
	8	>14 NM	>14 NM	>14 NM	>14 NM
EMB	1	7.205 (0.442)	6.642 (0.442)	6.962 (0.442)	6.793 (0.442)
	2	7.181 (0.626)	6.789 (0.626)	8.784 (0.442)	8.086 (0.626)
SM	1	3.904 (0.673)	3.394 (0.673)	2.321 (0.673)	2.663 (0.673)
	3	3.904 (0.673)	3.669 (0.673)	3.775 (0.673)	3.052 (0.673)
	8	4.568 (0.673)	3.988 (0.673)	4.717 (0.673)	4.673 (0.673)
	10	ND[e]	9.689 (0.952)	11.143 (0.952)	13.545 (1.436)
	20	ND	>14 NM	>14 NM	>14 NM
OFL	1	4.823 (0.445)	3.911 (0.445)	4.285 (0.445)	3.992 (0.445)
	2	4.621 (0.445)	4.619 (0.445)	6.254 ¡(0.445)	6.522 (0.445)
AMK	1	4.799 (0.6)	3.947 (0.6)	2.797 (0.6)	4.407 (0.6)
	3	4.724 (0.6)	5.177 (0.6)	3.747 (0.6)	5.171 (0.6)
	8	5.209 (0.6)	5.919 (0.6)	6.627 (0.6)	7.163 (0.6)
Control	0	3.381 (0.367)	2.345 (0.318)	2.166 (0.318)	2.018 (0.318)

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin. [b]Conc denotes concentration given in terms of multiples of the MICs. [c] No metabolic activity was registered and T_{400} readings of greater than 14 days were set to be missing. [d]NM = no metabolic activity was registered and standard errors of the mean were thus not calculated. [e]ND = not done.
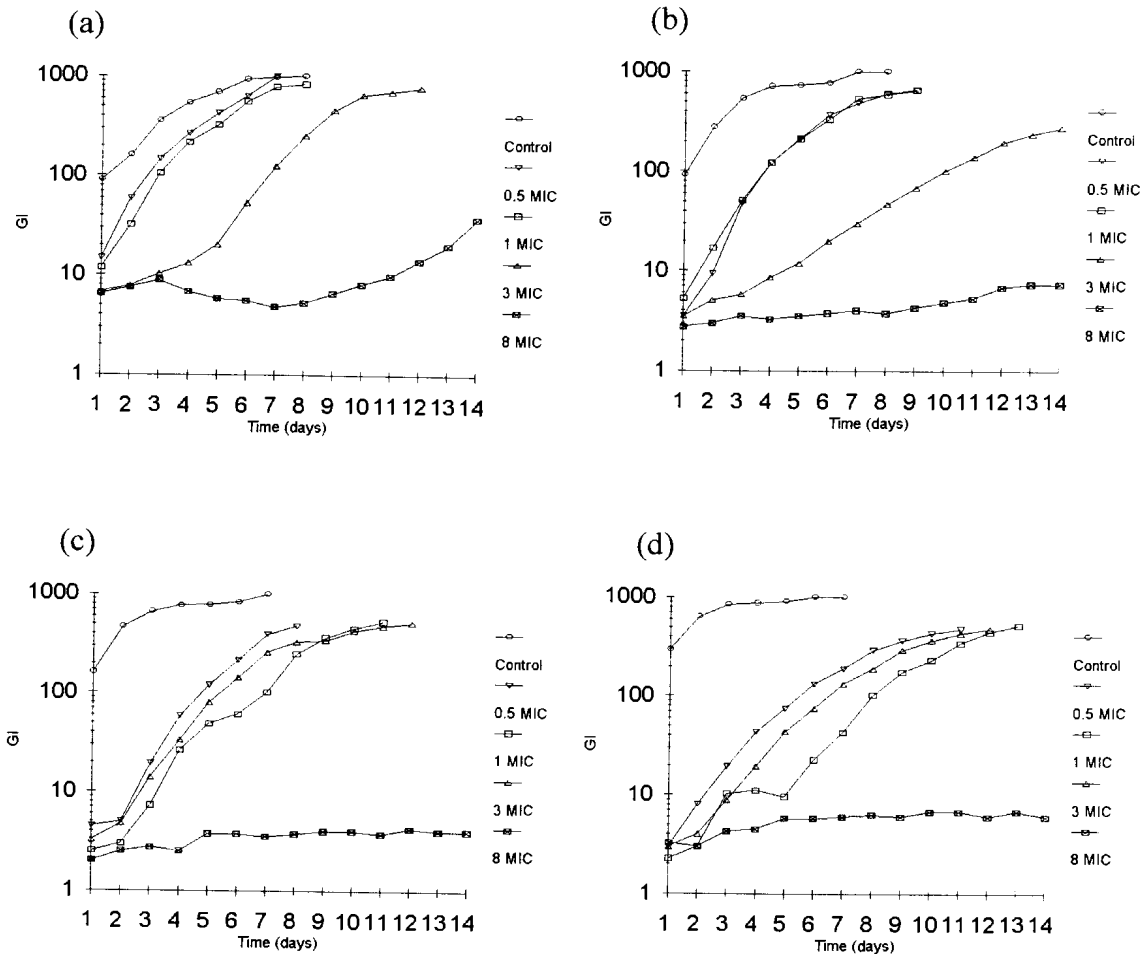
Figure 5.1 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rv after (a) 6, (b) 24, (c) 48 and (d) 72 hours of exposure to isoniazid (INH, MIC = 0.05 ug/ml). Antimicrobial concentrations are shown in terms of the MICs.

5.4.2 *Rifampicin*

RMP also showed delayed regrowth curves which were related to both concentration and duration of exposure (Figure 5.2). The delays in regrowth showed similar patterns to those of INH at 6, 24, 48 and 72 hour exposures. At 8 MIC/6h exposure, evidence of a subinhibitory PAE was again present.

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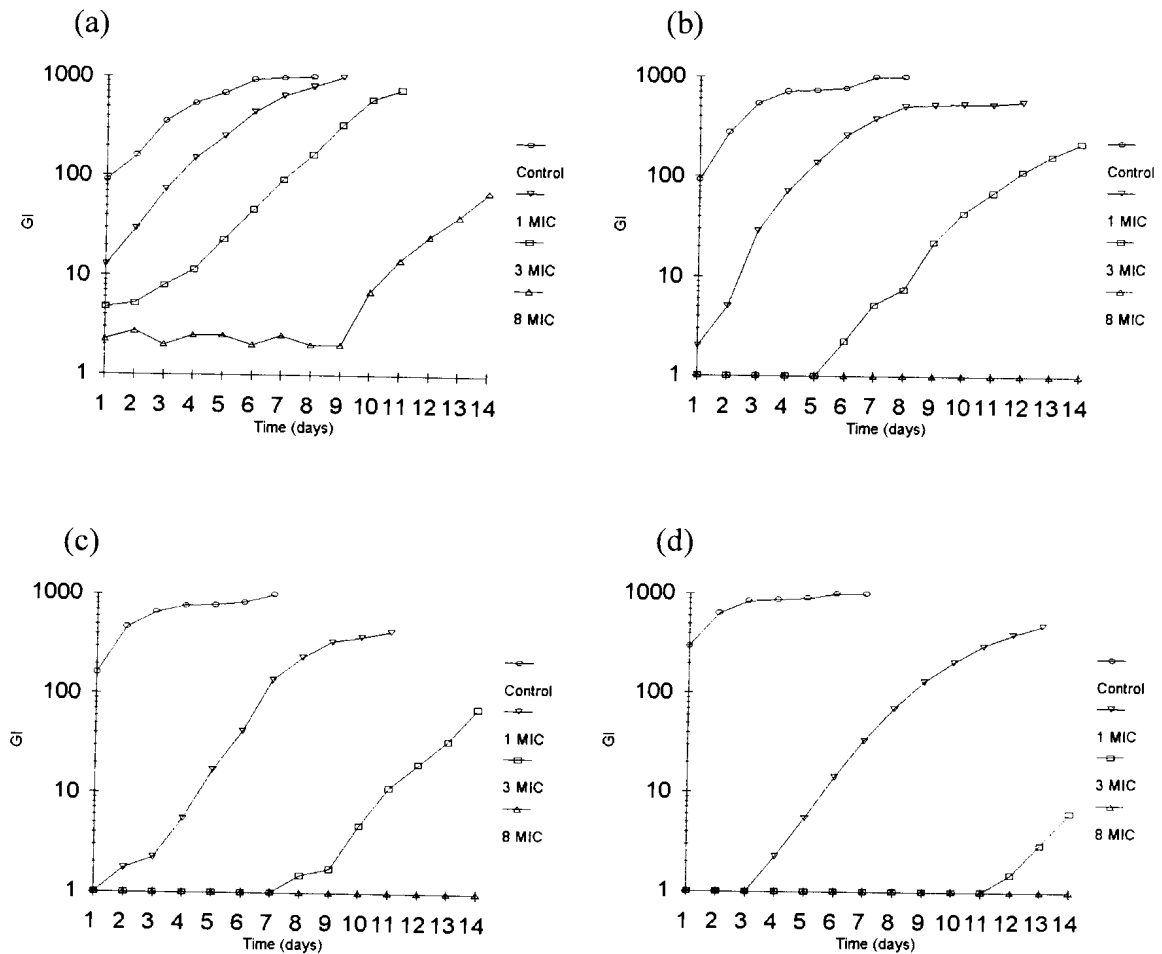
Figure 5.2 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rv after (a) 6, (b) 24, (c) 48 and (d) 72 hours of exposure to rifampicin (RMP, MIC = 0.5 ug/ml). Antimicrobial concentrations are shown in terms of the MICs.

5.4.3 *Ethambutol*

Because of the relatively narrow range of achievable blood/tissue levels of EMB compared with its MIC, exposure to only two concentrations, 1 MIC and 2 MIC, was employed to study regrowth. The regrowth curves following exposure to 1 MIC and 2 MIC were similar at all four exposure periods. Regrowth times increased slightly with increased drug exposure times (Figure 5.3).
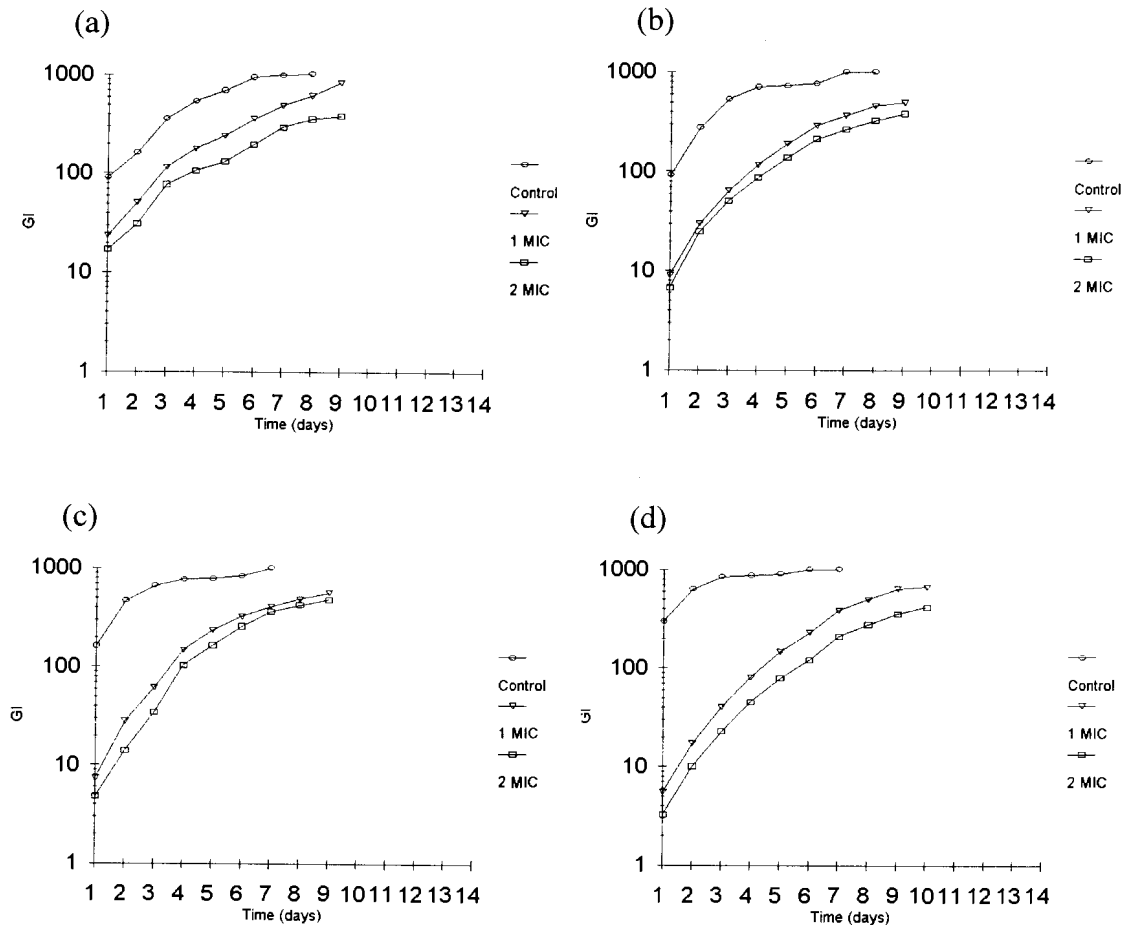
Figure 5.3 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rv after (a) 6, (b) 24, (c) 48 and (d) 72 hours of exposure to ethambutol (EMB, MIC = 2.0 *u*g/ml). Antimicrobial concentrations are shown in terms of the MICs.

5.4.4 *Streptomycin*

Delays in regrowth following SM exposure were short but increased with the use of sustainable blood/tissue levels (10 MIC and 20 MIC). Slower rates of regrowth occurred following both increased exposure times and increased concentration (Figure 5.4). These effects were clearly demonstrable despite the fact that in the present model, SM exerted a moderate bactericidal effect over the range of exposure periods (6 to 72 hours). The extent to which a

likely carry-over effect at the 10 MIC and especially 20 MIC played a role in prolonging regrowth (subinhibitory effect) is uncertain but was probably considerable in the case of 20 MICs.
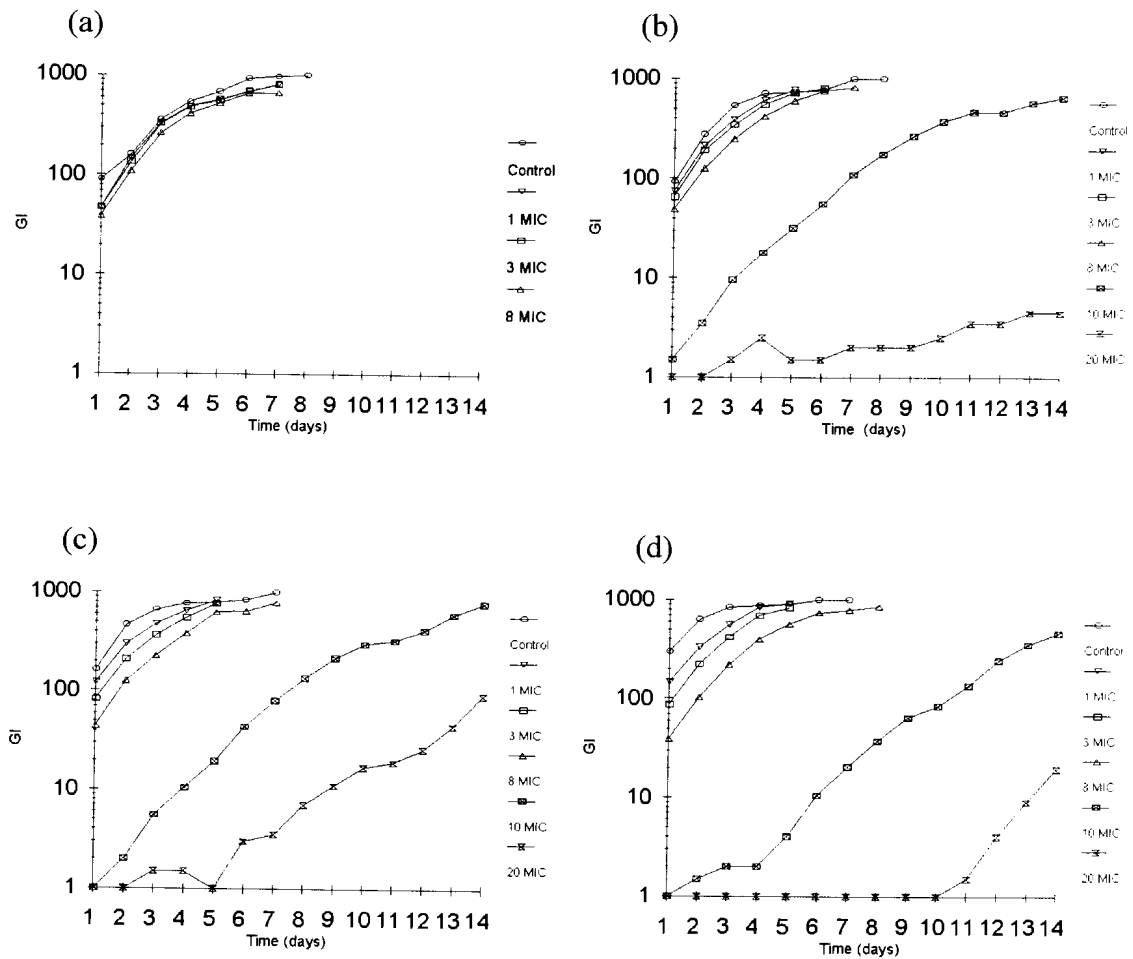
Figure 5.4 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rv after (a) 6, (b) 24, (c) 48 and (d) 72 hours of exposure to streptomycin (SM, MIC = 1.0 *u*g/ml). Antimicrobial concentrations are shown in terms of the MICs. At 6 hour exposure, the 10 and 20 MICs were not tested.

5.4.5 *Ofloxacin and amikacin*

Delays in regrowth following exposure to 1 MIC and 2 MIC of OFL were virtually the same at 6 hour exposures. The regrowth periods

were both concentration- and exposure time- dependent (Figure
5.5). The findings following AMK exposure (Figure 5.6) were similar
to those of OFL.

(a)

(b)

(c)

(d)
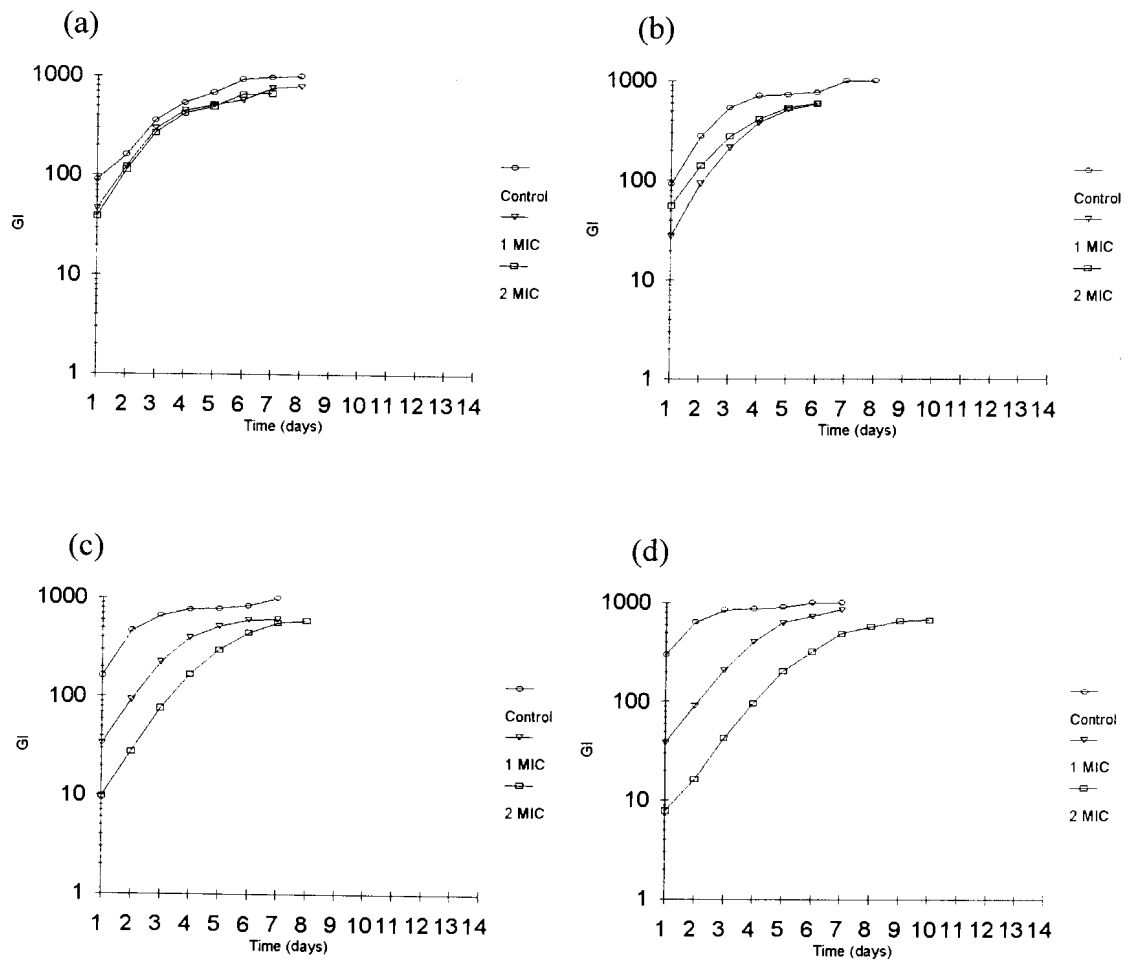
Figure 5.5 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rv after
(a) 6, (b) 24, (c) 48 and (d) 72 hours of exposure to ofloxacin (OFL, MIC = 0.5
μg/ml). Antimicrobial concentrations are shown in terms of the MICs.
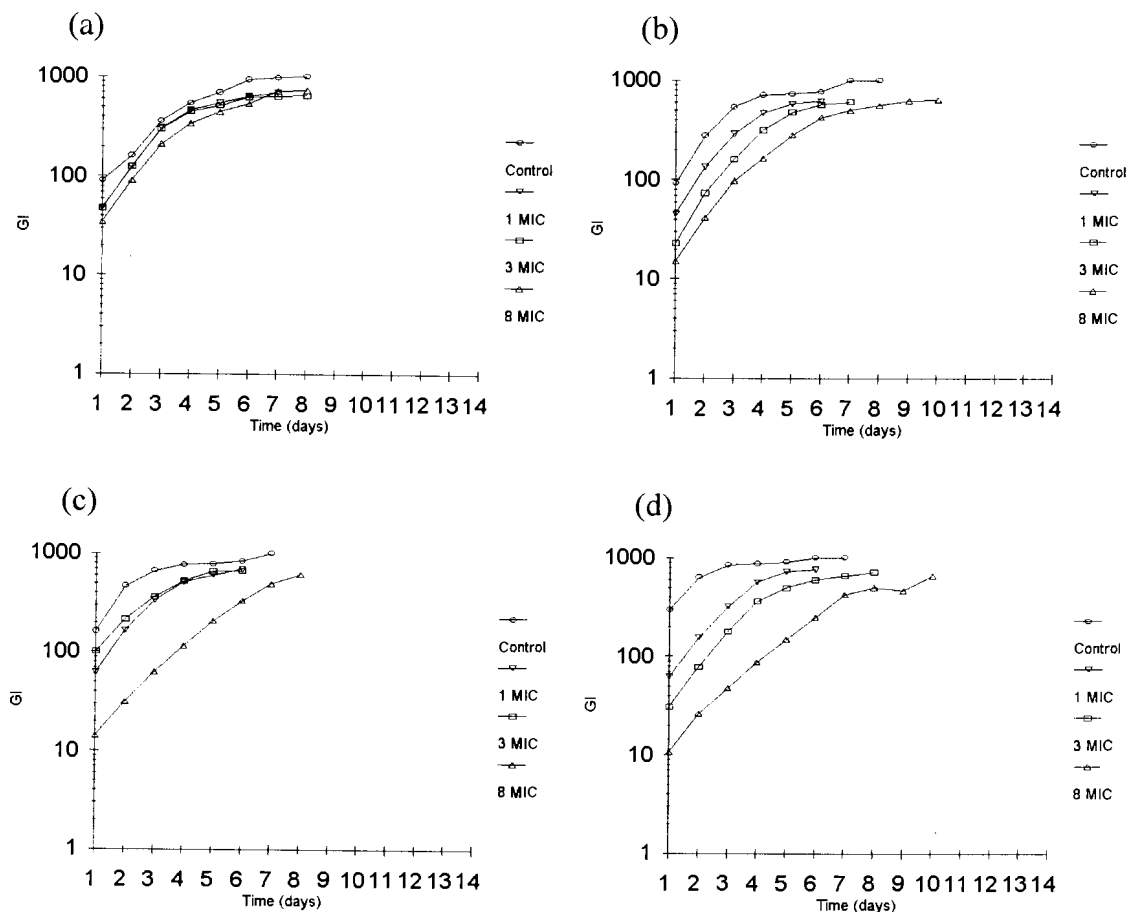
Figure 5.6 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rᵛ after (a) 6, (b) 24, (c) 48 and (d) 72 hours of exposure to amikacin (AMK, MIC = 0.25 ug/ml). Antimicrobial concentrations are shown in terms of the MICs.

5.4.6 *Variability of regrowth findings*

Variation in Bactec readings was expressed in terms of pooled standard errors of the mean (see Table 5.2) and by means of coefficients of variation (CVs). The CVs of the Bactec profiles were 15.2%, 7.9%, 6.0%, 3.0%, 8.9% and 7.6% for INH, RMP, EMB, SM, OFL and AMK, respectively. Both variability indices (standard errors of the means and CVs) were appreciably lower in the Bactec regrowth experiments compared with those of the colony counts.

5.4.7 *Effect of drug-exposure times on (T-C)$_{400}$ indices*

Table 5.1 gives the (T-C)$_{400}$ indices for all drug concentrations and exposure times as well as the control T$_{400}$findings. The absolute T$_{400}$ values for the various drugs and the control unexposed cultures as well as the standard errors of the mean for each reading are given in Table 5.2. The (T-C)$_{400}$indices of the six test drugs measured at 1 MIC of each drug as well as other selected MIC multiples, at exposure times of 24,48, and 72 hours are depicted in Figure 5.7 and Figure 5.8, respectively. The superiority of INH, RMP and EMB in causing prolonged post-exposure regrowth in this model, compared with the two aminoglycoside antibiotics as well as OFL is clearly indicated in Figures 5.7 and in Tables 5.1 and 5.2.
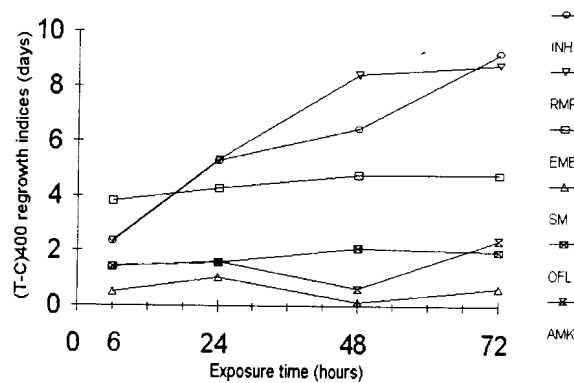
Figure 5.7 : Bactec radiometric regrowth time according to (T-C)$_{400}$ indices (in days) following exposure of *M. tuberculosis* H37Rv to 1 MICs of six anti-tuberculosis agents.

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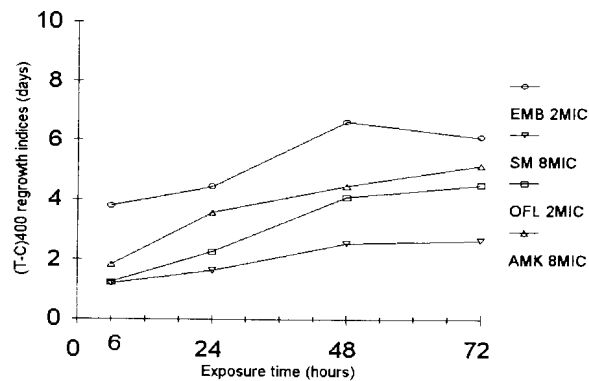
Figure 5.8 : Bactec radiometric regrowth time according to $(T-C)_{400}$ indices

(in days) following exposure of *M. tuberculosis* H37Rv to four anti-tuberculosis

drugs at achievable levels in terms of 2x and 8 x MICs.

5.5 DISCUSSION AND CONCLUSIONS

5.5.1 *Main findings and their interpretation*

In the Bactec system, the amount of radioactive CO_2 produced by

M. tuberculosis after metabolising the fatty acid substrate, palmitic

acid is measured. Thus, this system is based on mycobacterial

metabolism rather than a direct determination of bacterial numbers

and is expressed as GI. Several investigators (Fuursted, 1997;

Gottfredsson *et al*, 1991; Haug *et al*, 1998; Hoffner *et al*, 1987;

Löwdin *et al*, 1993) who employed similar systems that measure

microbial metabolism, have described various methods for the

quantification of regrowth after exposure to antimicrobial agents.

The methods can be interpreted in two ways (1) by measuring the

difference in the time required for the regrowth of unexposed and

exposed cultures to reach a defined reading, and (2) by calculating

cumulative readings obtained for the unexposed and exposed

cultures to reach a fixed regrowth time.

In some models in which regrowth is measured indirectly in liquid media, experiments can be designed to address the problem of a possible bactericidal effect of the drug. In such cases, a true PAE, comparable to that determined by colony counting, could be established (Hanberger *et al*, 1995; MacKenzie and Gould, 1993). In order to avoid confusion, Hanberger *et al* (1995) proposed the term control-related regrowth time (CERT) for determinations which do not compensate for the bactericidal effect of the drug.

Gottfredsson *et al* (1991) used the Bactec culture system that detects bacterial CO_2 by infrared spectroscopy. The authors described PAE, or CERT according to the definition of Hanberger *et al* (1995), as the time taken for regrowth of the exposed cultures compared with the control cultures to reach a growth value (GV) of 30. This value is equivalent to the pre-exposure standardized inoculum. The present study was not designed specifically to measure either PAE or CERT, but the same indicator system (Bactec) was employed. However, our experiments accommodated concentrations and exposure times to provide the bactericidal activity.

To facilitate analysis of the Bactec post-exposure regrowth findings, an arbitrary value, T_{400}, which represents the time taken for the unexposed and drug-exposed *M. tuberculosis* cultures to reach a defined GI reading of 400, was used to describe the delays in regrowth after drug exposure. This value represents approximate counts of 10^4 to 10^5 CFU/ml (Heifets, 1991a) which could relate to clinical relevance. The T_{400} readings were measured using linear interpolation for each Bactec profile.

The determination of the regrowth time was terminated at 14 days.

In retrospect, it would have been advantageous if the Bactec readings were extended beyond 14 days in the case of experiments in which no regrowth appeared within this period or the GIs were still low but rising slowly. It is possible that regrowth could have started beyond 14 days, although the presence of sub-MICs in Bactec 7H12 vials at the higher exposure concentrations could have been responsible for failure to regrow during this period.

INH and RMP were the most active drugs in terms of delays in regrowth in the Bactec radiometric model and EMB was third to INH and RMP. The highly bactericidal drugs, INH and RMP also showed the highest $(T-C)_{400}$ indices which, at least in part, are due to the smaller number of survivors that need more time to reach the target GI of 400 compared with larger numbers of survivors following exposure to agents that kill fewer organisms. PAE excluding bactericidal effects, probably also played a role in our model, especially at the higher concentration/short exposure times (8MIC/6h). The rates of bactericidal activity and PAE are discussed in some detail in Chapter 6.

Delays in regrowth as shown by $(T-C)_{400}$ indices were already observed at 6 hours exposure for INH and were the same as those of RMP. Although delays in regrowth were short, SM showed an increase in rates of regrowth following increasing exposure time, as was the case with the other anti-TB drugs tested (see Figure 5.8).

Following exposure to INH and RMP, delays in regrowth increased and growth rates decreased with increasing concentration and exposure time. There was little relationship between exposure time and concentration and the activity of EMB. SM (at 10 MIC and 20 MIC), OFL and AMK showed concentration dependent activity.

Compared with the findings in Chapter 4 relating to bactericidal activity, the Bactec radiometric system appears to be not only sensitive to changes in bacterial numbers as is the case with conventional CFU counts, but is also, in addition, sensitive to detecting reversible damage to bacterial cells. A more detailed analysis of these effects is recorded and discussed in Chapter 6.

The Bactec model is undoubtedly safer and more accurate than conventional CFU counting. Mycobacteria tend to clump together due to their lipophilic outer membrane (Haug *et al,* 1998). Different sized clumps contain bacteria in different physiological states and colony counting is gravely compromised by hydrophobic clumps. To obtain single-cell bacterial suspensions, Wayne (1994) recommends the use of detergent-containing medium for preparation of seed cultures and the use of detergent-containing diluent for serial 10-fold dilutions in the CFU counting method. Brief, moderate-energy sonication as well as vortexing using glass beads is also recommended for optimal dispersion of cell suspensions (Haug *et al,* 1998; Wayne, 1994).

Single-cell suspension is necessary both for accurate dilution and for accurate counting of CFUs. In the case of the radiometric quantification of growth, bacterial dilution is not necessary unless the bacterial concentration is suspected to be high ($>10^7$ mycobacteria, depending on the bacterial strain). Since in the Bactec radiometric system bacterial metabolism is measured, it is less likely affected by clumping (Haug *et al,* 1998).

When regrowth times in relation to the number of surviving bacteria are compared at 8 MICs as opposed to 1 MICs and 3 MICs, a definite subinhibitory concentration effect can be observed (see

Figure 5.1 to Figure 5.6). When the model was designed, such an effect was anticipated. In studies to further evaluate the Bactec model, this subinhibitory effect should be examined more fully and dilutions of post-exposure inocula, before injection into Bactec vials, should be employed as was done by Fuursted (1997)in a Bactec system for the determination of PAE against *M. avium* isolates.

5.5.2 *Comparison with other post-exposure regrowth models*

The radiometric regrowth findings after 24 hour exposure compare well with those found previously by other authors (Mitchison and Dickinson,1971) as illustrated in Table 5.3. Mitchison and Dickinson (1971) used the conventional colony counting method to assess regrowth after removing the drug by a filtration method. The authors calculated the difference in exposed and unexposed culture lag periods before regrowth starts to express PAE.

In the case of INH at 3 MIC (0.15 ug/ml) delays in regrowth of greater than 14 days were observed in this study compared with a period of 6-9 days observed at 1 ug/ml by other authors (Mitchison, 1998; Mitchison and Dickinson,1971). As can be seen, the delays in regrowth obtained for INH and RMP in the present study differed from those of Mitchison's group (see Table 5.3). Post-exposure delays of approximately 4.5 and 7.4 days were observed in the case of EMB and SM in our studies after exposure of 24 hours, while delays of 4-5 days and 8-10 days respectively were reported by other authors (Mitchison,1998; Mitchison and Dickinson,1971).

Experimental conditions in those instances however differed in several respects from those in the present model. Tween 80 was not used in this study, while the exposure concentrations were also

different.

Table 5.3 Delayed regrowth exerted by anti-tuberculosis drugs.

Drug[a]	Concentration in ug/ml (xMIC)[b]		Early lag period in days after 24 hour exposure	
			Literature[c]	Present study (adjusted)[d]
INH	1.0		6 - 9	
	0.15	(3 MIC)		> 14
RMP	0.2		2 - 3	
	0.5	(1 MIC)		5.34
EMB	10		4 - 5	
	4	(2 MIC)		4.44
SM	5		8 - 10	
	10	(10 MIC)		7.34
OFL	1.0	(2 MIC)	NA[e]	2.3
AMK	4	(8 MIC)	NA	3.6

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin, [b]Drug concentrations in multiples of the MICs, [c]Reference : Mitchison (1998), [d]Adjusted = in comparison with the control, [e]NA = not available, Multiples of MICs based on MICs determined in Bactec 7H12 medium.

The findings of the Bactec radiometric regrowth model are in accordance with clinical efficacy. INH, RMP and EMB showed the most marked depression of regrowth while less impressive findings were obtained with SM, OFL and AMK. The Bactec $(T-C)_{400}$ model again showed excellent discrimination between the anti-TB drugs, which is in broad agreement with conventional drug activity assessments. Although it was not specifically designed to measure PAE or CERT, the model showed similar trends in lag periods before regrowth starts compared with the findings of other authors. It was

also easy to use and showed reproducible results with a low variation between the findings. It should therefore prove to be useful in the evaluation of candidate drugs for the treatment of TB.

CHAPTER 6

RELATIONSHIP BETWEEN THE KILL-KINETICS AND RADIOMETRIC REGROWTH MODELS FOR THE ASSESSMENT OF ANTI-TUBERCULOSIS DRUGS

6.1 BACKGROUND

6.1.1 *Relationship between bactericidal activity of anti-tuberculosis drugs and radiometrically-determined post-exposure regrowth*

Heifets *et al* (1985b) in a study on the activity of ansamycin against *Mycobacterium avium* strains showed a clear correlation between growth curves obtained in the Bactec radiometric system and when measured by viable counts expressed as colony forming units (CFUs). Figure 6.1 is a reproduction of a figure illustrating this relationship published by the authors. During the exponential growth phase there was excellent correlation between the two systems while during the lag phase viable counting gave evidence of viable bacilli in the form of CFUs before increased metabolic activity was registered radiometrically. Also, during the stationary growth phase and the phase of decline, metabolic activity as shown by Bactec growth indices (GIs), declined more rapidly than the decrease in CFUs during these growth phases. It is also noteworthy that, except for the initial 3 days of growth, such correlation was also evident when the culture was exposed to sub-minimal inhibitory concentrations (sub-MICs) and a 1 MIC concentration of ansamycin (See Figure 6.1).
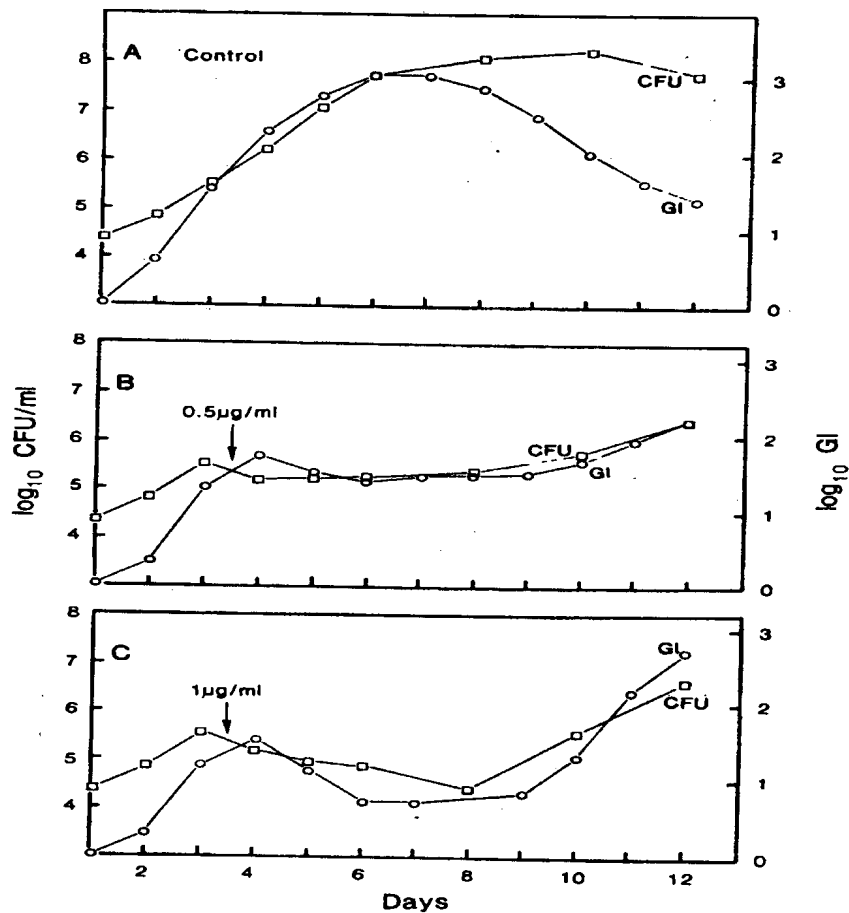
Figure 6.1 : Example of correlation between the growth curve (CFU per millilitre) and the daily GI readings in an experiment in which ansamycin was added after 3 days of cultivation. (A) Control; (B) with ansamycin at a low concentration (0.5 ug/ml); (C) with ansamycin at a concentration equal to the MIC (1.0 ug/ml) for this strain. Reproduced from reference Heifets *et al* (1985b).

Based on the correlation between cumulative GI radiometric readings and CFUs shown by Heifets *et al* (1985b), Inderlied *et al* in 1987 showed that at a GI of 100 a close correlation exists between the period of growth required to reach a cumulative index of GI 100 and the number of CFUs in the inoculum (Figure 6.2). These authors used this information to design a new method for susceptibility testing of *M. avium* against amikacin (AMK) (Inderlied,1987).
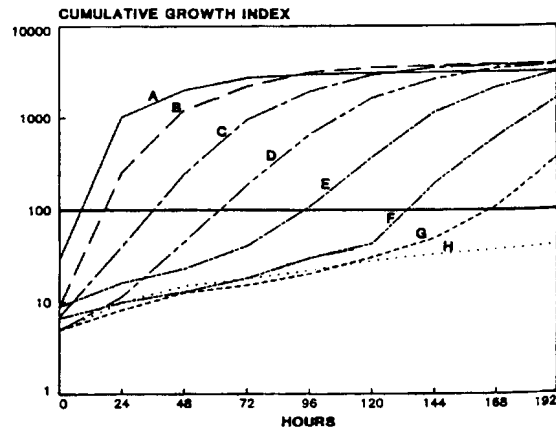
FIG.1. BACTEC containing 12B medium were inoculated with *M. avium* complex 101 at various initial viable cell counts. The cumulative release of $^{14}CO_2$, measured as the GI of 100 defines the T100 for each culture (point of intersection of the curve with the horizontal line at 100). The initial viable cell counts were as follows: A, 10^7 CFU/ml;B,10^6 CFU/ml; C, 10^5 CFU/ml; D, 10^4 CFU/ml; E, 10^3 CFU/ml; E, 10^3 CFU/ml; F, 10^2 CFU/ml; G, 10 CFU/ml.
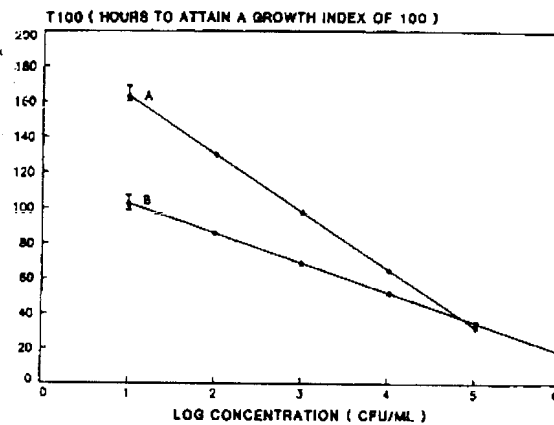
FIG. 2. The T100 values expressed as hours necessary to attain a cumulative GI of 100, for each culture, determined as for Fig. 1, are plotted as a function of the log concentration of the initial inoculum, *M. avium* complex 101 (A) and 100 (B).

Figure 6.2 : Graphs produced by Inderlied *et al* (1987) to show correlations between T_{100} values and colony counts.

More recently, Reddy *et al* (1994) reported the use of this correlation to measure growth of *M. avium* inside macrophages and in homogenates from infected organs by the Bactec method. These

authors again demonstrated a close correlation between Bactec GI readings and CFUs (Figure 6.3). In the same article, the authors reported the use of this approach to determine the effect of clofazimine and two of its analogues on *M. avium* infection in a beige mouse model.
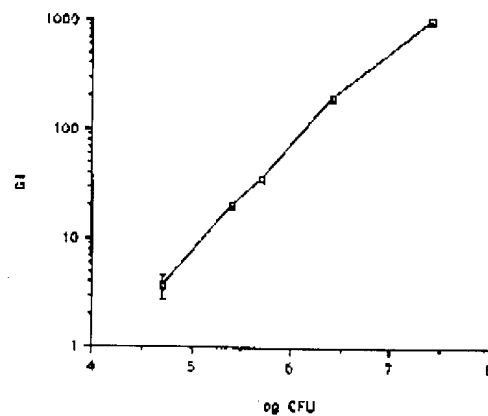
Fig. I - Relation between GI and concentration of MAC in 12B.

Figure 6.3 : Correlation between GI readings in Bactec 7H12 medium and CFUs of *M. avium* organisms in a mouse model reported by Reddy *et al* (1994).

In an extension of earlier studies on the use of Bactec to determine *M. avium* organism load in the determination of postantibiotic effect (PAE) (Zhanel *et al*, 1998) and in animal experiments (Reddy *et al,* 1994) Haug *et al* (1998), pointed out that the correlation between the rate of *M. avium* growth in Bactec medium and in CFUs only extends over a limited range of GI readings. They therefore proposed radiometric quantification of *M. avium* complex based on GI readings after 60h incubation. The graphs on which this approach was based are reproduced in Figure 6.4.

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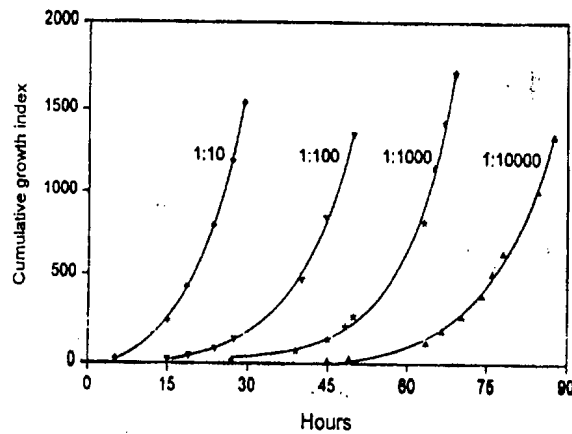
Figure 1 Cumulative growth index for four different dilutions of a
representative mycobacterial strain plotted against time.
Different symbols indicate different dilutions.
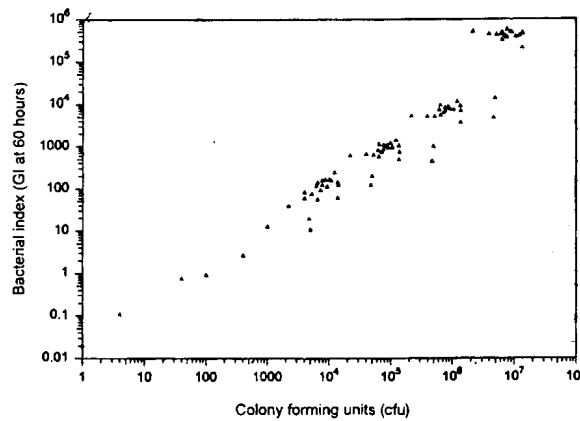
Figure 2 Relationship between calculated growth index at 60h (bacterial index) and a
number of colony forming unis of mycobacteria (cfu). Bacterial indexes from four
different dilutions of 20 strains of mycobacteria are plotted and show that there is
a high correlation between bacterial index and cfu (Spearman $R = 0.92$; $P < 0.001$)

Figure 6.4 : Correlation of GI readings in Bactec 7H12 medium after 60 hour incubation
and CFUs reported by Haug *et al* (1998).

All the evidence quoted thus far relates to the use of radiometric
technology to quantify *M. avium* organisms, which, unlike *M.
tuberculosis* do not tend to clump extensively on culture. However,
scrutiny of a graph produced in a paper by Rastogi *et al* (1989)
suggested that a similar relationship may exist between Bactec GI
readings showing the rates of growth of serial dilutions of a
suspension (See Figure 6.5).

The growth curves of *M. tuberculosis* inocula produced in this paper show a clear similarity with those recorded by Inderlied *et al* (1987) reproduced in Figure 6.2. When cumulative GI readings obtained after 5 days of growth (based on data provided in Figure 3 of the paper by Rastogi *et al*, 1989) are plotted against CFUs which produced the curves, an excellent approximation of a linear relationship is observed (correlation coefficient, $r = 0.9663$, $p < 0.01$) as can be seen in Figure 6.6. A similarly linear relationship can be demonstrated when the times taken for regrowth to reach a GI reading of 20 are plotted against CFUs ($r = 0.9809$, $p < 0.02$; see Figure 6.7).
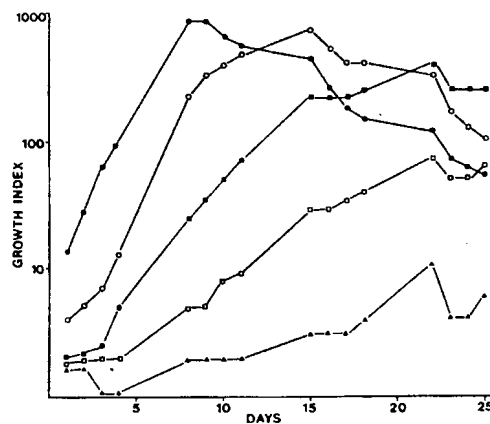
Figure 6.5 : Growth curves of inocula of *M. tuberculosis* with increasing numbers of bacilli as determined radiometrically in Bactec 7H12a medium by Rastogi *et al* (1989). Adapted from Figure 3 - Bactec GI values for 7H12a broth vials inoculated with 0.1 ml of serially diluted *M. tuberculosis* suspension. The GI was followed for 25 days. The initial inoculum added in each vial was quantified to know the exact CFU/ml. • = 3 X 10 CFU; o = 3, 000 CFU; ■ = 300 CFU; □ = 30 CFU; ▲ = 3 CFU.
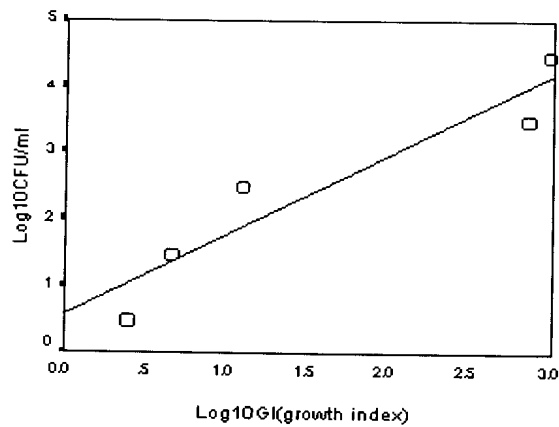
Figure 6.6 : Relationship between cumulative GI readings after 5 days of growth of *M. tuberculosis* in 7H12a broth and colony forming units CFUs) based on Figure of paper by Rastogi *et al* (1989).
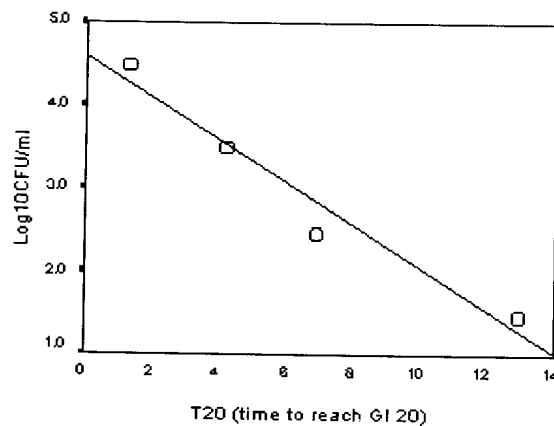
Figure 6.7 : Relationship between time taken for regrowth to reach a GI reading of 20 and the number of colony forming units (CFUs) of *M. tuberculosis* in 7H12a broth based on Figure 3 of paper by Rastogi *et al* (1989).

Observations relating to "non-clumping" *M. avium* as well as evidence from studies of Rastogi *et al* (1989) on *M. tuberculosis,* prompted us to examine the possibility of similar relationships between reduction in CFUs and regrowth rates following exposure of *M. tuberculosis* to anti-mycobacterial agents.

6.2   OBJECTIVES

It was decided to evaluate the following aspects of bactericidal-regrowth relationships :

(a) Relationship between unexposed inocula and regrowth rates. As the present studies were not specifically planned to address this issue, only information relating to control cultures used in regrowth experiments was available for analysis.

(b) Relationship between the number of post-exposure surviving bacteria and regrowth time.

(c) Relationship between bactericidal activity of anti-tuberculosis (anti-TB) drugs and regrowth times. It would be important to assess whether the Bactec radiometric regrowth system could predict likely bactericidal activity of drugs.

(d) To construct a mathematical model relating the number of post-exposure viable tubercle bacilli with regrowth patterns measured radiometrically in the Bactec system. Delays before effective regrowth commences are conventionally incorporated when times taken for drug-exposed cultures to regrow to a defined Bactec GI reading are determined. It was therefore decided to also examine separately lag periods and effective growth rates in a mathematical model.

Two approaches were used for the assessment of the ability of the radiometric (Bactec) system to predict bactericidal activity.

6.2.1 *Direct comparison of Bactec growth curves and colony counts*

This approach was based on studies of Rastogi *et al* (1989) discussed in Section 6.1. The time taken by the control cultures in the Bactec vials to reach the stationary growth phase or a GI reading of 999 was relatively short compared with those of the post-exposure cultures. Furthermore, with higher concentrations of anti-TB drugs and longer exposure times, cultures took much longer to reach even low GI readings (< GI 50). It was therefore decided to plot the periods required for unexposed control and post-exposure cultures to reach a GI of 400 (T_{400}) against corresponding colony counts. Correlation coefficients (r) were calculated by the Student t-test. To measure variability in the experimental findings, coefficients of variation (CVs) were calculated.

The aim of this exercise was to demonstrate a possible linear relationship between the post-exposure surviving bacteria and regrowth times. In the first instance, unexposed control cultures will be analysed and subsequently inocula of *M. tuberculosis* cultures exposed to the various anti-TB drugs.

6.2.2 *A statistical appraisal of a hypothetical logistic curve model*

The second approach employed a mathematical model in which multiple regression analysis was used to assess the relationship between the bactericidal activity of anti-TB drugs and the two main components of a typical growth curve (lag period and logarithmic growth phase) as exhibited in post-exposure regrowth curves. The application of this logistic model to our laboratory findings will feature in Chapter 7.

6.2.3 *Statistical methods for regression analysis and significance assessment of findings*

6.2.3.1 Relationship between T_{400} and $(T-C)_{400}$ regrowth findings and bactericidal activity

Simple linear regression analysis was employed to calculate the slope and the y-intercept of the best straight line fitted to the regrowth data versus $\log_{10}CFU/ml$ reflecting post-exposure surviving bacteria. The SPSS system was used. The r-values are determined according to the formula:

$$r = \sqrt{1 - \frac{\text{sum of squared deviations from regression line}}{\text{sum of squared deviations from mean}}}$$

The coefficient of determination (R^2) is derived from the formula above and is expressed as a percentage.

6.2.3.2 Statistical significance of regression analyses

In order to determine the significance of the regression analysis findings, the Student t-test value based on $t = \frac{r}{\sqrt{(1-r^2)(n-2)}}$ was calculated and the p-value read from the statistical tables, recording the significance limits of the student distribution. The F-value and its p-value were also obtained from the analysis of variance (ANOVA) table found by simple linear regression analysis.

6.3   RESULTS AND COMMENTS

6.3.1 *Relationship between colony counts of unexposed cultures and T_{400} growth times*

The concentrations of unexposed viable tubercle bacilli in terms of

CFU/ml were compared with the growth patterns of these unexposed cultures in the Bactec radiometric regrowth system. These cultures were derived from the control cultures used in the 6, 24, 48 and 72 hour drug-exposure experiments described in Chapter 4 and Chapter 5 (see Figure 5.1 to Figure 5.6 in Chapter 5). The concentrations of the cultures and their T_{400} readings are given in Table 6.1 while the respective growth curves are shown in Figure 6.8. The regression line relating $\log_{10}CFU/ml$ to T_{400} readings is depicted in Figure 6.9.

Table 6.1 Concentrations of control cultures compared with regrowth patterns in Bactec vials.

Assay time	$\log_{10}CFU/ml^1$	$T_{400}^{\ 2}$
6h	6.018	3.381
24h	6.162	2.345
48h	6.249	2.166
72h	6.215	2.018

[a]$\log_{10}$concentrations of bacterial suspensions in colony forming units/ml. [b]Times in days taken to regrow to growth index (GI) readings of 400.
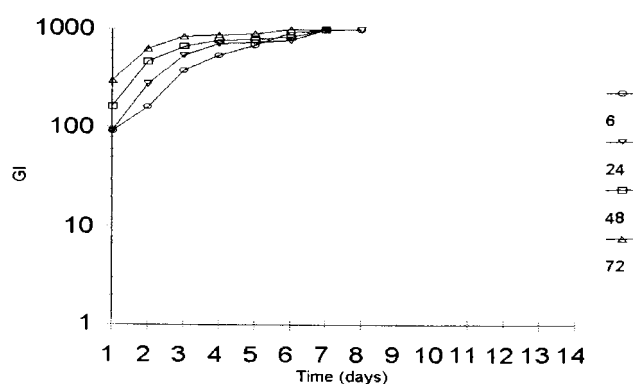
Figure 6.8 : Growth curves in Bactec 7H12 vials of control cultures at different exposure times (hours).
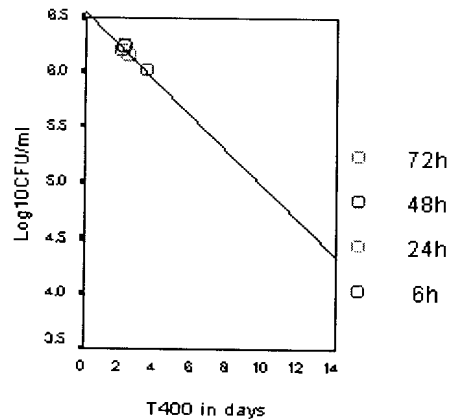
Figure 6.9 : $Log_{10}CFU/ml$ versus T_{400} readings of unexposed control cultures.

As can be seen from Figure 6.8, the growth curves of the control cultures are still in the logarithmic phase after 6h, 24h, 48h and 72h incubation periods. They reached the stationary phase progressively earlier as incubation times increased towards 72h.

Excellent correlation based on the means of the CFU/ml and regrowth data was shown between the time taken for growth to reach a GI 400 reading and the initial concentration of viable bacilli, with r = 0.9632 (p <0.05, see Figure 6.9). Enthusiasm for the evidence of correlation should be tempered by considerable variation experienced with colony counts (CV = 38.92%, see Section 6.3.2.3).

6.3.2 *Relationship between post-exposure viable bacilli and radiometrically-determined regrowth times*

Regression lines relating post-exposure $log_{10}CFU/ml$ counts to (a) T_{400} regrowth times and (b) $(T-C)_{400}$ regrowth indices are given in Table 6.2 and Figures 6.10(a) and 6.10(b) to Figures 6.15(a) and

6.15(b) respectively for isoniazid (INH), rifampicin (RMP), ethambutol (EMB), streptomycin (SM), ofloxacin (OFL) and AMK. In Figures 6.16(a) and 6.16(b), the relationship between viable counts and T_{400} regrowth readings, and decreases in viable counts versus $(T-C)_{400}$ times, respectively, is demonstrated collectively for all six drugs tested.

In keeping with a possible linear relationship between bactericidal activity and regrowth times, the regression lines of six anti-TB drugs, individually and as a group, showed a general tendency of concentration- and exposure time-related correlation between bactericidal activity and regrowth times (Figure 6.10 to Figure 6.16).

In several instances, findings relating to experiments performed at specific exposure times for individual drugs tended to yield results that deviated from the general pattern of points close to the regression lines, suggesting separate concentration-related linear relationships. Thus, with the 48h and 72h exposure experiments INH, EMB and OFL (see Figures 6.10, 6.12 and 6.14) showed proportionately greater bactericidal activity and regrowth times compared with the findings obtained with activity at the 24h exposure times. In the case of AMK, bactericidal activity after 24h and 48h exposure periods was considerably greater and out of step compared with the other exposure periods (see figure 6.15). These findings suggest that variable experimental conditions such as temperature effects and differences in clump dispersal may have affected the results of the exposure-related experiments.

Concentrations used and especially the duration of exposure to the test drugs tended to minimise true PAEs which require time for recovery and regrowth of damaged but still viable bacterial cells (that

is, excluding bactericidal effects). Even though the true PAEs were minimised, regrowth times following exposure to EMB, OFL and AMK for 6h, were distinctly delayed compared with those following more prolonged exposure times (see Figure 6.12, 6.14 and 6.15). When the 6h findings were excluded from regression analysis, the r-values for OFL improved from -0.5354 to 0.9393. Less marked trends were seen with EMB (r=-0.6605 to -0.7475; Table 6.2) and AMK (r=-0.7194 to 0.8034; Table 6.2).

It is interesting that r-values for these three drugs also improved when $(T-C)_{400}$ findings were plotted against bactericidal activity (see Table 6.2). The shorter T_{400} values for the control cultures at 48h and 72h exposures relative to the 6h and 24h periods, resulted in longer $(T-C)_{400}$ times at these exposure times. This had the effect of changing the slope of the $(T-C)_{400}$ regression lines, bringing the 6h points in closer alignment with the regression line. Whether this correction has a biological basis is unclear.

Following construction of a regression line involving all the drugs as a group, the six drugs were compared. These are shown in Figure 6.16(a) and Figure 6.16(b). OFL consistently exhibited proportionately greater bactericidal activity compared with regrowth times, while the reverse trend was seen with SM and EMB. These findings suggest that following OFL exposure, tubercle bacilli regrow faster than following SM and EMB exposures. In PAE experiments involving rapidly growing bacteria, more rapid regrowth has been demonstrated following exposure to β-lactam antibiotics (Gudmundsson et al, 1994).

The good r-value obtained for SM (Figure 6.13(a) and 6.13(b) may well be misleading. It is likely that the 10MIC and 20MIC exposure

experiments resulted in delayed regrowth due to a carry-over effect in the Bactec vials and that the relationship with the bactericidal activity was therefore distorted. The slopes of the SM regression lines were appreciably flatter than those of the other drugs (see Table 6.2). Furthermore, the points on the regression graph involving 1MIC and 3MIC because of poor activity, do not suggest a good correlation between the two variables. It is only when the 10MIC and 20MIC findings are involved that a good correlation is achieved. Possible reasons for the unexpectedly low SM activity found in our experiments and the need for further studies are discussed elsewhere.

(a1)

(a2)
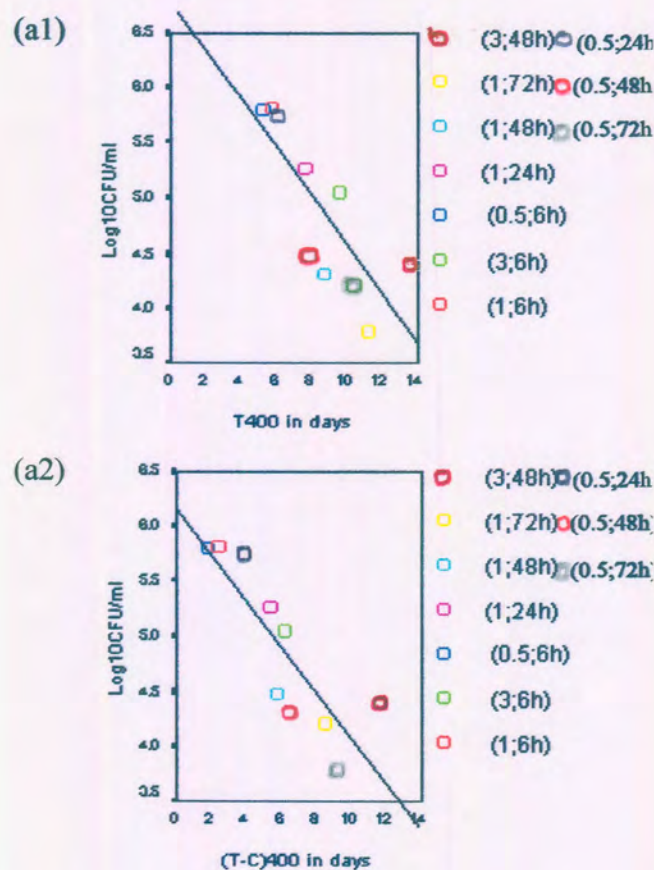
Figure 6.10 : Relationship between bacterial survivors in $log_{10}CFU/ml$ versus (a1) T_{400} regrowth times and (a2) $(T-C)_{400}$ indices in days following isoniazid (INH) exposure. Legends indicate MICs and exposure times in hours, respectively.
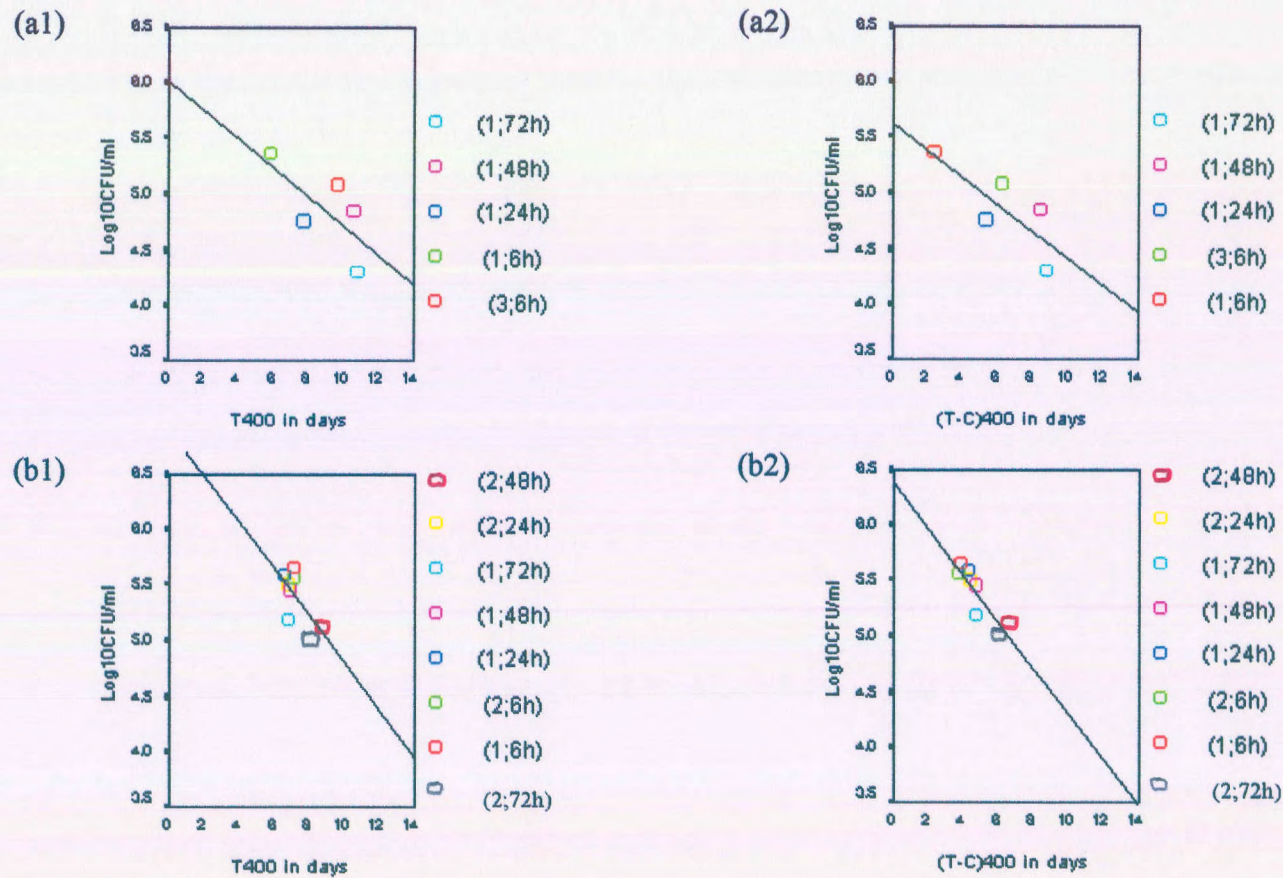
Figure 6.11 : Relationship between bacterial survivors in $\log_{10}$CFU/ml versus T_{400} regrowth times (a1, b1) and $(T\text{-}C)_{400}$ indices (a2,b2) in days following rifampicin (RMP; a1, a2) and ethambutol (EMB; b1, b2)) exposure. Legends indicate MICs and exposure times in hours, respectively.
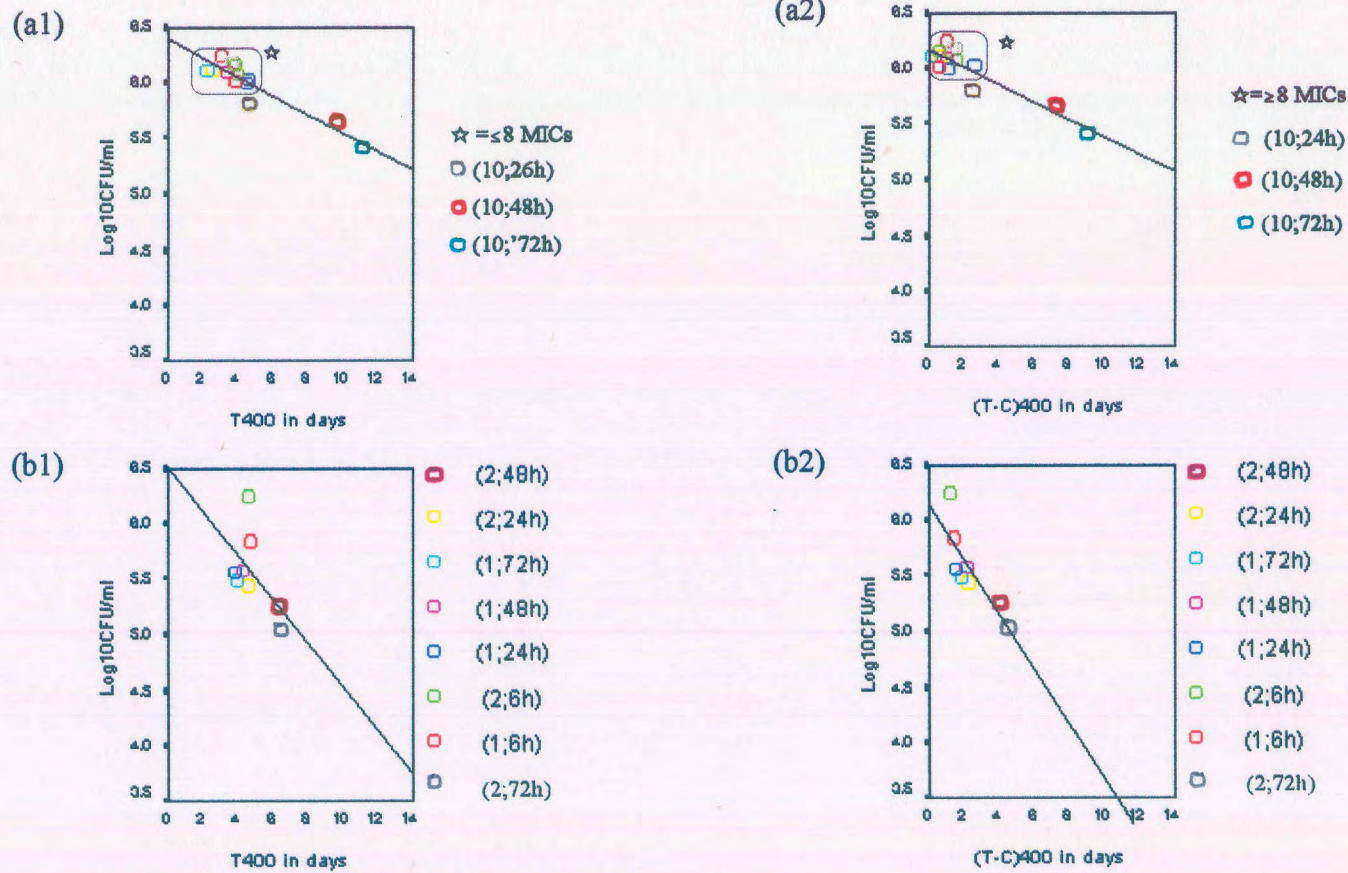
Figure 6.12 : Relationship between bacterial survivors in $\log_{10}$CFU/ml versus T_{400} regrowth times (a1, b1) and $(T-C)_{400}$ indices (a2,b2) in days following streptomycin (SM; a1, a2) and ofloxacin (OFL; b1, b2)) exposure. Legends indicate MICs and exposure times in hours, respectively.
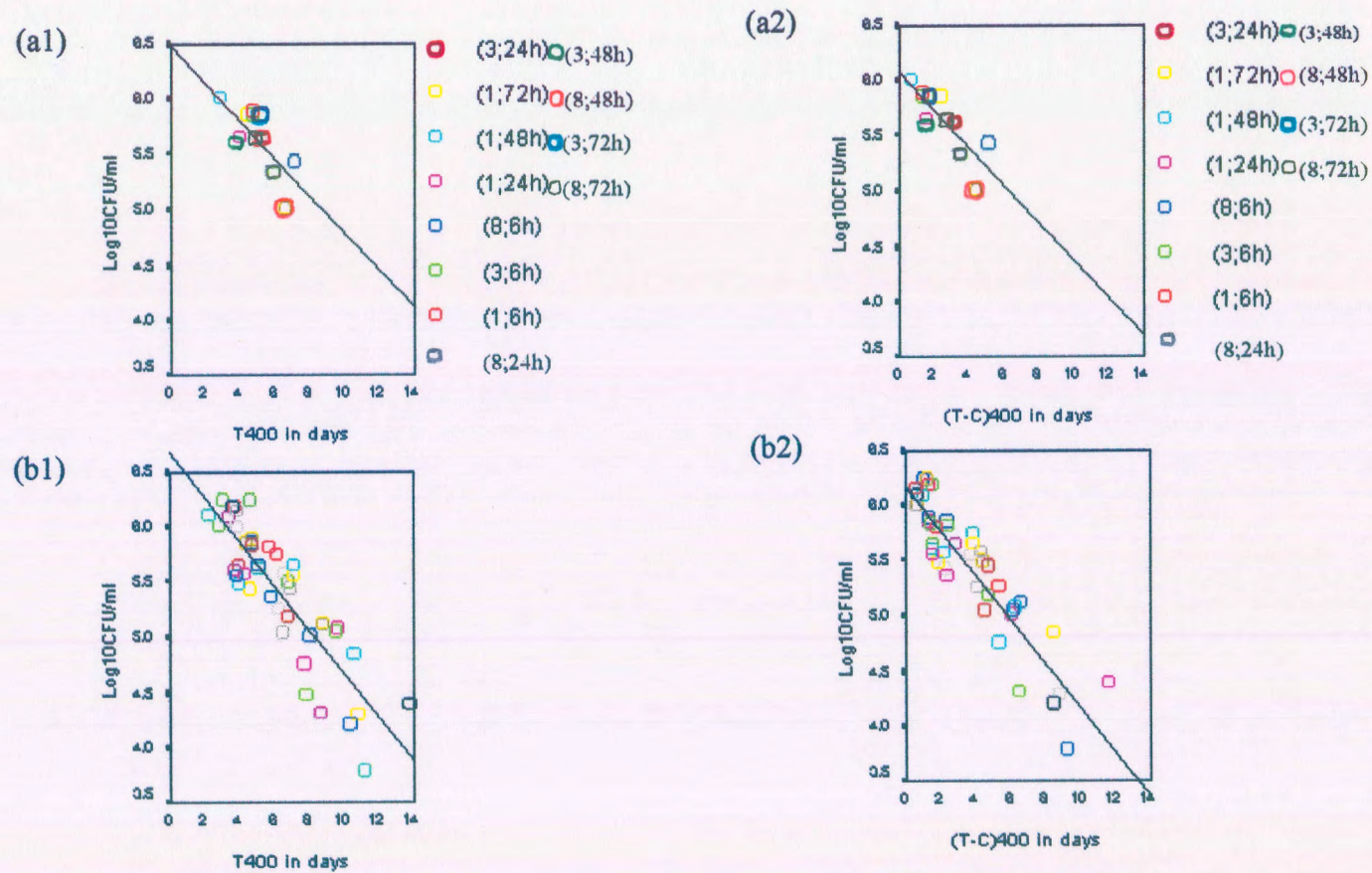
Figure 6.13 : Relationship between bacterial survivors in $\log_{10}$CFU/ml versus T_{400} regrowth times (a1, b1) and $(T-C)_{400}$ indices (a2,b2) in days following amikacin (AMK; a1, a2) and the six drugs (INH, RMP, EMB, SM, OFL, AMK; b1, b2) exposure. Legends for these graphs are shown in Appendix 4.

Table 6.2 Correlation between surviving bacteria after exposure to anti-mycobacterial drugs[a] and regrowth in a Bactec radiometric model

Drug/Control	CFU/ml[b] vs T_{400}[c]			CFU/ml vs $(T\text{-}C)_{400}$[d]		
	r[e]	p[f]	s[g]	r	p	s
Control	-0.963	<0.05	-0.159	NA[i]	NA	NA
INH	-0.852	<0.01	-0.223	-0.849	<0.005	-0.209
RMP	-0.707	<0.20	-0.129	-0.806	<0.1	-0.122
EMB_1[h]	-0.661	<0.10	-0.214	-0.881	<0.025	-0.209
EMB_2[h]	-0.748	<0.10	-0.199	-0.829	<0.05	-0.204
SM_1[i]	-0.956	<0.001	-0.085	-0.949	<0.001	-0.081
SM_2[j]	-0.959	<0.001	-0.085	-0.961	<0.001	-0.084
OFL_1	-0.535	<0.20	-0.197	-0.833	<0.02	-0.247
OFL_2	-0.939	<0.01	-0.163	-0.949	<0.005	-0.156
AMK_1	-0.719	<0.001	-0.168	-0.816	<0.001	-0.169
AMK_2	-0.803	<0.005	-0.166	-0.773	<0.02	-0.156
All drugs[k]	-0.896	<0.001	-0.194	-0.890	<0.001	-0.186

[a]Exposure times: 6h,24h,48h,72h; Concentrations: 1MIC, 2 or 3MIC, 8MIC (see l and text). [b]Control or post-exposure viable bacteria as colony forming units (CFU)/ml. [c]Regrowth time to reach a Bactec growth index (GI) of 400. [d]Control-related growth time to GI of 400. [e]r = correlation coefficient. [f]statistical probability. [g]s = slopes of regression lines. [h]The subscripts 1 and 2 denote inclusion and exclusion of 6h exposure findings, respectively. [i]10mIC and 20MIC findings are included. [j]10MIC and 20MIC are excluded. [k]6h exposures and 8MIC findings were excluded from calculations (see text). [l]NA = not available.

6.3.3 *Variability of experimental findings*

Variation among colony counts in the post-exposure experiments involving the six test drugs and corresponding variation encountered in the regrowth experiments were recorded in Chapter 4 and Chapter 5, respectively. These are reproduced in Table 6.3 together with the R^2-values used for assessing the degree of correlation between the two variables.

The CVs found for the two types of experiments appear to be realistic considering the nature of the procedures, and were in the same range as those recorded by Fuursted (1997) for similar studies involving *M. avium* isolates. Not surprisingly, greater variation was observed among CFU/ml counts compared with regrowth time experiments. Based on the ideal situation of one bacillus producing one colony, sampling differences due to unequal dispersion of organisms and clumping of tubercle bacilli are more likely to adversely affect colony counts than regrowth times. In the case of regrowth times however, organisms in small clumps are likely to show greater metabolic activity than a single bacillus in the regrowth medium.

The high CV of 38.72% for the control cultures is interesting and suggests that drug-exposed cultures, possibly due to antimicrobial action may show less clumping than the unexposed control cultures.

6.3.4 *Effect of experimental variation on correlation indices*

Correlation determinations presented in this chapter were based on means of CFU counts and regrowth times, respectively (Figures 6.10 to 6.15 and Table 6.2). Means and standard errors of the means were calculated from the pooled data and are given in Chapter 4 and Chapter 5. The R^2-values given in Table 6.4 were calculated by

fitting a simple linear regression. Most of the variation in colony counts is explained by its linear relationship with the regrowth findings. The inherent variation in the procedures also played a role. Poor correlation (100% denotes perfect correlation) was found in the case of OFL. Apart from the inherent variation associated with the procedures, additional factors which contributed to the poor R^2 findings in the case of OFL undoubtedly played a role. Exclusion of the 6h exposure times data which presumably resulted in true PAEs, improved dramatically the r-values (based on means of repeated experiments) of OFL from -0.5354 to -0.9393 and even further to -0.9498 when control-related $(T-C)_{400}$ data were used in the calculations. In addition R^2-values improved from 28.7% to 88.2%. Similar but less spectacular improvement was observed with EMB and AMK when 6h exposure data were excluded (Table 6.2). Probably more important was the greater bactericidal activity exhibited by AMK after 24h and 48h exposure periods (see Figure 6.15). As speculated earlier, these discrepant findings may be due to experimental conditions, including differences in clump dispersal and temperature variations between experimental steps. In general, the R^2 findings improved when control-related $(T-C)_{400}$ data was used.

Table 6.3 Variation amongst T_{400} readings and $\log_{10}CFU/ml$ following exposure to anti-tuberculosis drugs.

Drug/control[a]	$CV(\%)^b{}_{log10CFU/ml}$	$CV(\%)_{T400}$	$R^2(\%)^c$ $_{CFU/ml\ vs\ T_{400}}$	$R^2(\%)_{CFU/ml\ vs\ (T-C)400}$
control	38.72	4.16	92.8	NA^d
INH	21.22	15.24	72.6	72
RMP	12.53	7.85	49.9	64.9
$EMB_1{}^d$	12.13	5.98	43.6	77.6
$EMB_2{}^e$	NA	NA	55.8	68.8
SM_1	28.44	3.07	91.3	90
SM_2	NA	NA	92.1	92.3
OFL_1	18.23	8.89	28.7	69.4
OFL_2	NA	NA	88.2	90
AMK_1	24.16	7.57	51.8	66.5
AMK_2	NA	NA	64.6	59.7
All drugs[f]	NA	NA	82.1	78.8

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol SM = streptomycin, OFL = ofloxacin, AMK = amikacin, the subscripts 1 and 2 denote inclusion or exclusion of 6h exposure findings, respectively. [b]CV = coefficient of variation. $^cR^2(\%)_{log10CFU/ml\ vs\ T400}$ = coefficient of determination for the $\log_{10}CFU/ml$ vs T_{400} (regrowth time to reach a Bactec growth index (GI) of 400) relationship, $R^2(\%)_{log10CFU/ml\ vs\ (T-C)400}$ = coefficient of determination for the $\log_{10}CFU/ml$ vs $(T-C)_{400}$ (control-related regrowth time to GI of 400) relationship. [d]NA = not available.

6.3.5 *Predictive capability of the Bactec model*

Although only six anti-TB drugs were evaluated in the Bactec regrowth model, they varied sufficiently in their basic mechanisms of action and antimicrobial activities. As such it was possible to use them to assess the potential of the model to screen for the mycobactericidal activity of drugs. Evidence that the 6h exposure experiments may have introduced true PAEs which distorted correlation between regrowth time and bactericidal activity, as well as the likelihood that exposure concentrations of ≥8MIC may have

resulted in delayed regrowth due to subinhibitory concentrations in the regrowth vials, was presented earlier in this chapter. Because of this evidence, it was decided to exclude the 6h exposure times and $\geq$8MIC experiments from the prediction calculations. The $(T-C)_{400}$ times with their corresponding decreases in $\log_{10}$CFU/ml involving 1MIC and 3MIC in the case of INH, RMP, SM and AMK, and 2MIC for EMB and OFL after exposure times of 24h, 48h and 72h are given Appendix 3. The limits of the $(T-C)_{400}$ findings and those of the corresponding $\log_{10}$CFU/ml based on the pooled standard errors of the mean are indicated as ranges in the table.

Based on criteria which on scrutiny of the data (see Figure 6.16 b) suggest realistic predictions of levels of bactericidal activity of candidate drugs, outcomes resulting in true or false positive or negative predictions are set out in Table 6.4. The criteria chosen for bactericidal activity were decreases of equal to or in excess of $0.3\log_{10}$, $0.6\log_{10}$ and $1\log_{10}$ in CFU/ml (that is 2-fold, 4-fold and 10-fold decreases). A $(T-C)_{400}$ regrowth time of 2days was considered the minimum time likely to identify moderate to excellent activity of candidate drugs. The predictive values based on the abovementioned criteria are given in Table 6.5. Denominator and enumerator figures used in the calculations are given in brackets.

We attempted to cater for variability of results and the means were included in our calculations. The resulting predictive values therefore cover combinations of criteria at the extremes of the ranges determined by the standard errors from the means. Based on the means of the findings generated by the six drugs tested, good positive (lowest 89%) and negative (lowest 70%) predictions were obtained. Examination of Figure 6.16(b) indicates that better predictive values than those depicted in Table 6.5 could have been

achieved had different criteria been chosen, for example, $\geq 1.0\log_{10}$CFU/ml versus 5days for candidates with excellent activity. Criteria were good positive and negative capability.

According to the criteria selected reflecting degrees of bactericidal activity, all drugs except SM, could have been passed as candidates for the treatment of TB while EMB and OFL fell in the moderate to good activity range. INH and RMP met with the criteria predicting excellent activity. It is of concern that the Bactec system when used at 1MIC and 3MIC did not identify SM as a candidate for the treatment of TB. At 10MIC this drug would have qualified easily, although subinhibitory effects must have played a role at this concentration.

Table 6.4 Accuracy of predictions of bactericidal activity of the Bactec regrowth system based on decreases in $\log_{10}$CFU/ml counts and (T-C)$_{400}$ readings after 24h, 48h and 72h exposures at 1MICs and 3MICs.

Drug[b]	Prediction[c] assessment	Accuracy of (T-C)$^a_{400}$ predictions according to criteria of								
		≥2d vs ≥0.3$\log_{10}$CFU/ml[d]			≥3d vs ≥0.6$\log_{10}$CFU/ml			≥8d vs ≥1$\log_{10}$CFU/ml		
		Low[e]	ū[g]	High[f]	Low	ū	High	Low	ū	High
INH	TP	6	6	6	5	6	5	4	3	3
	FP	0	0	0	1	0	1	0	1	1
	TN	0	0	0	0	0	0	0	1	1
	FN	0	0	0	0	0	0	2	1	1
RMP	TP	6	6	6	6	6	6	3	5	5
	FP	0	0	0	0	0	0	0	0	0
	TN	0	0	0	0	0	0	0	0	0
	FN	0	0	0	0	0	0	3	1	1
EMB	TP	6	6	5	6	5	3	0	0	0
	FP	0	0	1	0	1	3	0	0	0
	TN	0	0	0	0	0	0	3	4	6
	FN	0	0	0	0	0	0	3	2	0
SM	TP	0	0	0	0	0	0	0	0	0
	FP	0	0	3	0	0	0	0	0	0
	TN	6	6	3	6	6	6	6	6	6
	FN	0	0	0	0	0	0	0	0	0
OFL	TP	2	5	4	2	2	3	0	0	0
	FP	0	0	2	0	0	0	0	0	0
	TN	0	0	0	0	2	1	3	5	6
	FN	4	1	0	4	2	2	3	1	0
AMK	TP	1	3	0	0	0	4	0	0	0
	FP	0	0	5	0	1	0	0	0	0
	TN	0	1	1	1	5	1	6	6	6
	FN	5	2	0	5	0	1	0	0	0

[a]Control-related regrowth time to Bactec growth index of 400. [b]INH =isoniazid, RMP =rifampicin, EMB =ethambutol, SM =streptomycin, OFL =ofloxacin, AMK =amikacin. [c]TP =true positive, FP =false positive, TN =true negative, FN =false negative. [d]CFU =colony forming units. [e]Low =Lowest limit of (T-C)$_{400}$ vs the highest limit decrease in $\log_{10}$CFU/ml based on the pooled standard errors of the means. [f]High =highest limit of (T-C)$_{400}$ vs lowest limit decrease in $\log_{10}$CFU/ml based on the pooled standard errors of the means. [g]ū =means of CFU/ml or radiometric regrowth findings .

Table 6.5 Predictive values of the radiometric (Bactec) model in relation to bactericidal activity following 24h, 48h and 72h exposures at 1MICs and 3MICsutilising $(T-C)_{400}$ regrowth times as indices

Predictive index	Predictive values in percentages based on								
	$\geq 2d$ vs $0.3log_{10}CFU/ml$[a]			$\geq 3d$ vs $0.6log_{10}CFU/ml$			$8d$ vs $1.0log_{10}CFU/ml$		
	Low[b]	$\bar{u}$[g]	High[c]	Low	$\bar{u}$	High	Low	$\bar{u}$	High
PPV[d]	100	100	66	95	90	84	100	89	89
$\left(\frac{TP}{TP+FP}\right)$[f]	$\left(\frac{21}{21}\right)$	$\left(\frac{26}{26}\right)$	$\left(\frac{21}{32}\right)$	$\left(\frac{19}{20}\right)$	$\left(\frac{19}{21}\right)$	$\left(\frac{21}{25}\right)$	$\left(\frac{7}{7}\right)$	$\left(\frac{8}{9}\right)$	$\left(\frac{8}{9}\right)$
NPV[e]	40	70	100	44	87	73	62	81	93
$\left(\frac{TN}{TN+FN}\right)$	$\left(\frac{6}{15}\right)$	$\left(\frac{7}{10}\right)$	$\left(\frac{4}{4}\right)$	$\left(\frac{7}{16}\right)$	$\left(\frac{13}{15}\right)$	$\left(\frac{8}{13}\right)$	$\left(\frac{18}{29}\right)$	$\left(\frac{22}{27}\right)$	$\left(\frac{25}{27}\right)$
Sensitivity	70	90	100	68	90	88	25	62	82
$\left(\frac{TP}{TP+FN}\right)$	$\left(\frac{21}{30}\right)$	$\left(\frac{26}{29}\right)$	$\left(\frac{21}{21}\right)$	$\left(\frac{19}{26}\right)$	$\left(\frac{19}{21}\right)$	$\left(\frac{21}{24}\right)$	$\left(\frac{7}{28}\right)$	$\left(\frac{8}{13}\right)$	$\left(\frac{9}{11}\right)$
Specificity	100	100	27	88	87	67	100	96	96
$\left(\frac{TN}{TN+FP}\right)$	$\left(\frac{6}{6}\right)$	$\left(\frac{7}{7}\right)$	$\left(\frac{4}{15}\right)$	$\left(\frac{7}{8}\right)$	$\left(\frac{13}{15}\right)$	$\left(\frac{8}{12}\right)$	$\left(\frac{8}{18}\right)$	$\left(\frac{22}{23}\right)$	$\left(\frac{24}{25}\right)$

[a]CFU = colony forming units. [b]Low = Lowest limit of $(T-C)_{400}$ vs higher limit decrease in $log_{10}CFU/ml$ based on pooled standard errors of the means. [c]High = Highest limit of $(T-C)_{400}$ vs lowest limit of decrease in $log_{10}CFU/ml$ based on pooled standard errors of the means. [d]PPV = positive predictive value. [e]NPV = negative predictive value. [f]TP = true positive, FP = false positive, TN = true negative, FN = false negative. [g]$\bar{u}$ = means of findings.

In order to cater for drugs such as SM, the Bactec model will require modification to include 10MIC concentrations diluted sufficiently to minimize or exclude subinhibitory effects due to excess carry-over of drug into regrowth vials.

Based on the evaluation of the radiometric regrowth model evaluated in this chapter, the following scheme may prove to be useful for the screening of anti-TB drugs:

Initial screening

Exposure to 10MIC for 24h, 48h and 72h followed by regrowth in Bactec 7H12 vials as described in our model. The likely effect on regrowth time due to subinhibitory concentrations will render the model more sensitive than a modified system which excludes subinhibitory concentrations. This approach will have the potential advantage of including an element of drug action (delays in regrowth due to subinhibitory concentrations) which must be operative in the *in vivo* situation. Further studies using control antibiotics with little or modest activity against *M. tuberculosis* such as the macrolides or coamoxiclav as well as the secondary drugs such as ethionamide and cycloserine, are however required before this screening proposal can be offered with sufficient confidence.

Subsequent screening

Exposure to 1MIC and 3MIC (or 2MIC depending on the pharmacokinetics, if available) for 24h, 48h and 72h. The criteria based on the predictive values obtained with our test drugs could then be applied to categorize candidate drugs into candidates for further evaluation, good prospect drugs and excellent candidates. The relative simplicity and safety of the Bactec model adds to its attractiveness as a screening procedure for candidate anti-TB drugs.

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Its good correlation with *ex vivo* early bactericidal activity (EBA) of drugs as discussed in Chapter 5 (EBA ratings : INH 3 + , RMP 2 + , EMB 2 + , ciprofloxacin 2 + , SM poor) also augurs well for its use as a surrogate or screening marker of EBA (Mitchison, 1992; Sirgel *et al*, 1997).

6.4 CONCLUDING REMARKS

The findings discussed in this chapter provide good evidence that under defined conditions, regrowth time exhibits a linear relationship with bactericidal activity, as was shown earlier with *M. avium* (Reddy *et al*, 1994; Haug *et al*, 1998) and as suggested for *M. tuberculosis* by findings published by Rastogi *et al* (1989). Conditions that may compromise the linear relationship in the Bactec model are PAE brought on by short drug exposure (6h) and a carry-over effect due to subinhibitorry concentrations (≥ 8MICs) in regrowth vials.

For the prediction of bactericidal activity, interpretation of the model needs to accommodate the abovementioned restrictions. In addition, modification to include 10MIC exposures, preferable with suitable dilution to minimise a carry-over effect, would improve its usefulness. The inclusion in the model of regrowth under subinhibitory concentrations can be justified on scientific grounds and further study and possible modification of the model on this aspect of drug evaluation could be rewarding. Further studies to include determination of true PAEs would be relatively easy to accomplish, for example, by using an exposure time of 2h and MICs related to achievable peak serum levels (Fuursted, 1997), and may be worth pursuing.

CHAPTER 7

THE RELATIONSHIP BETWEEN BACTERICIDAL ACTIVITY AND RADIOMETRIC REGROWTH ACCORDING TO A LOGISTIC GROWTH CURVE MODEL

7.1   BACKGROUND

Evidence in favor of a linear relationship between the bactericidal activity of six anti-tuberculosis (anti-TB) drugs and $(T-C)_{400}$ regrowth times in a radiometric Bactec system was presented in Chapter 6. It was decided earlier to study such a relationship in a mathematical model based on the premise that post-exposure regrowth would follow a sigmoid curve.

Anti-TB agents are known to affect *Mycobacterium tuberculosis* by (a) inducing a lag period due to a postantibiotic effect (PAE) followed by (b) a period when the organisms would multiply logarithmically at a drug-dependent growth rate, described by the slope of the growth curve, and finally (c) by a stationary growth phase when multiplication of the organism levels off. Post-exposure radiometric regrowth curves can therefore be expected to follow a sigmoid slope which can be described mathematically, according to the components of the curve. Experimental data recorded in Chapter 4 and Chapter 5 were accordingly applied to such a model, anticipating that the propensity of the various anti-TB drugs to cause delays in regrowth (lag periods) could be assessed separately from and in conjunction with changes in growth rates.

In Chapter 6 we showed that the 6h exposure experiments affected the relationship between bactericidal activity and regrowth time, mainly in the case of ethambutol (EMB) and ofloxacin (OFL), while there was also distinct evidence that concentrations of ≥8MIC led to subinhibitory effects which distorted the relationship between the two parameters. We therefore excluded all 6h exposure and ≥8MIC data from the evaluation of the logistic model.

7.2   MATHEMATICAL BASIS OF A LOGISTIC CURVE MODEL

7.2.1 *Structure of the mathematical formula*

Multiple regression was used to determine whether the $\log_{10}$ colony forming units (CFU) counts were related to either the rate of cumulative metabolic activity by Bactec, or the lag period before regrowth starts to increase or both. The increase in growth index (GI) readings was assumed to follow a logistic growth curve over time with the equation :

$$GI = \frac{c}{1 + e^{-b(x-m)}} \quad \text{where}$$

x = time

b = measure of growth rate

m = measure of lag time

c approaches 1000 which is the upper limit of GI readings in the experimental model.

Post-exposure regrowth was determined radiometrically after different exposure times and drug concentrations at multiples of the minimal inhibitory concentrations (MICs) of the drugs. Radiometric (Bactec)

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readings were taken on consecutive days over a maximum period of 14 days and continued until a GI reading of 999 was reached or after readings levelled off at an earlier stage.

It was assumed that the GI profiles ideally exhibited a logistic type growth curve with asymptotes at 0 and 999. The curves were separately fitted to each experiment in order to obtain the effect of exposure on the lag period (m), and the rate of growth (b) and both parameters.

Bactericidal activity was assumed to be characterized by a longer lag period and/or slower growth rate. When the growth profile did not show a logistic-type growth, for example, when there was very little evidence of metabolic activity towards the end of the 14-day observation period, the parameters were still calculated by artificially imposing logistic curve-based GI readings up to 999, over a predicted period of 400 days. Such instances were checked individually for possible inclusion in the final analysis. Analysis of variance involving dependent variables was performed using the SAS system.

7.2.2 *Application of the model*

For an assessment of unexposed cultures, the numbers of viable bacteria of cultures used as controls in the four drug-exposure experiments were compared with their respective growth curves while in the case of the drug-exposed bacteria, the relationship between the numbers of surviving bacteria and their regrowth curves was analysed. The relative roles of the lag periods, growth rates and the two parameters combined were assessed for their relationship with CFU/ml using the following formula: $\breve{y}$ (CFU/ml) = Intercept + b_1 - b_2, where

b_1 is the estimated Bactec growth rate and b_2 the estimated lag period. The formula dictates that colony counts would increase with increased growth rates ($+b_1$), while there would be a negative correlation between CFU/ml and lag periods ($-b_2$).

Standard errors of the intercept estimates, growth rates and lag periods, were calculated and the validity of the model, taking both growth rates and lag periods into consideration, was assessed for each drug and the control cultures. Before fitting the colony count and regrowth data to the model, the means of the three parameters were calculated for each combination of exposure time and drug concentration in the various experiments.

For the assessment of the model, analysis of variance (ANOVA) using multiple regression was used to determine the F-values incorporating degrees of freedom (dfs), sum of squares (SSs) of deviations from the mean and mean squares (SS ÷ df). Two dfs were used for regression analysis comparing CFU/ml with growth rates and lag periods directly, while the numbers of direct comparisons of data sets were used to derive residual dfs for ANOVA. P-values were based on the F-distribution, where 2 dfs constituted the numerator, and numbers of dfs involved in the residual comparisons, denominator.

7.2.3 *Correlation between bactericidal activity and regrowth according to coefficient of determination assessment*

Coefficients of determination (R^2s) were calculated to assess correlation between the two variables, colony counts and regrowth curves. The means of individual experiments were used in sets of data that were being compared with each other. R^2 which is a square of the

correlation coefficient (r), is based on the formula

$$r = \frac{\Sigma \text{ sum of squared deviations from regression curve}}{\Sigma \text{ squared deviations from the mean of the dependent variable (regrowth curve data)}}$$

and is expressed as a percentage. An R^2 of 100% denotes perfect correlation. To illustrate the statistical approach used, of the relationship between colony counts and the corresponding growth curves for the unexposed control cultures was analyzed as follows:

(a) *Appropriateness of the logistic model*

Source	df	SS	MS	F	p-value
Regression	2	0.3960	0.1980	6.55	0.0207
Residual	8	0.2418	0.0302		
Total	10	0.6378			

R^2 (adjusted) = 0.5261 (52.6%)

(b) *Relative role of regrowth rate and lag period in the Bactec system*

Variable	Estimate	Standard error	t-interval	p-value
Intercept	4.472	0.484	9.237	0.0001
Bactec rate	0.7203	0.1992	3.615	0.0068
Bactec lag	0.2429	0.0857	2.834	0.0220

7.3 RESULTS AND COMMENTS

7.3.1 *Drug-exposure findings*

Analysis of the relative contributions of lag periods and growth rates,

as well as an overall assessment of the validity of the model to predict bactericidal activity, including R^2 values for the individual drugs and the control unexposed control cultures, are given in Table 7.1.

Excellent overall correlation of the observed data compared with the logistic model was achieved in the case of isoniazid (INH) and rifampicin (RMP) exposure experiments. Correlation in the case of the other four drugs failed to yield statistically significant p-values (EMB = 0.066, OFL = 0.36, amikacin (AMK) = 0.70). The relationship between colony counts and, growth rates and lag periods showed positive and negative correlations respectively. Good correlation between bactericidal activity and lag periods was achieved in the case of RMP (p = 0.0007) and INH (p = 0.023). It is noteworthy that the lag periods consist of two components (a) a true PAE due to damage to still viable bacteria and (b) the relative insensitivity of the Bactec system to detect metabolic activity of small numbers of surviving bacteria. RMP findings also showed good correlation between regrowth rates and the number of surviving tubercle bacilli (p = 0.0074). Trends towards bactericidal activity correlating with growth rate were seen with streptomycin (SM) and OFL.

The R^2 values were as expected higher with INH and RMP comparisons which fitted best with the logistic model (36.06% and 60.12%, respectively), as well as EMB (34.12%) and low for SM, OFL and AMK (24.22%, 14.67%, 5.28%).

7.3.2 Unexposed control culture findings

The overall findings correlated well with the model (p = 0.02), growth rate (p = 0.01) as well as the lag period (p = 0.02). Despite the good

correlation between the regrowth curves and CFU/ml shown by the control cultures, the lag periods exhibited a positive (rather than the expected negative) correlation. The lack of a negative relationship between lag periods and the number of viable bacteria can be explained by the fact that the four growth curves were generated by subcultures from the same original seed culture over a period of 72h. During this period, the subcultures progressed from short but clear lag periods to no lag periods at all, as the numbers of bacteria increased, suggesting that subcultures from the original seed culture adapted more quickly to the 7H12 medium in the Bactec regrowth vial as growth in the seed culture proceeded. Heifets *et al* (1985b) showed that in the case of an *M. avium* culture in a Bactec 7H12 medium, early growth is associated with lower GI readings compared with CFU/ml, resulting in a GI lag period before the GI readings and CFU/ml increase at the same rate. A similar effect is seen during the stationary phase and the phase of decline (see Figure 6.8 in Chapter 6). The R^2 value for experiments relating to the unexposed cultures was found to be equal to 52.6%.

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Table 7.1 Assessment of growth rate, lag period and total regrowth model as predictors of bactericidal activity after excluding the
6h and ≥8MIC data.

Drug/Control[g]	Intercept				Growth rate				Lag period				Validity of model					
	Estimate	SE[a]	T[b]	p	Estimate	SE	T	p	Estimate	SE	T	p	df[c]	SS[d]	SE	F[e]	p	R^2 (%)[f]
Control	4.47	0.48	9.25	0.001	0.721	0.19	3.62	0.01	0.24	0.09	2.83	0.02	10	0.64	0.24	6.55	0.02	52.6
INH	5.87	0.41	14.5	0.0001	0.00	0.00	0.46	0.65	-0.12	0.04	-2.45	0.02	23	15.88	0.69	5.92	0.009	36.1
RMP	5.94	0.34	17.64	0.0001	0.00	0.00	3.17	0.007	-0.14	0.03	-4.39	0.0007	15	5.37	0.41	9.79	0.003	60.12
EMB	5.33	0.52	10.2	0.01	0.001	0.001	1.61	0.13	-0.07	0.04	-1.47	0.17	15	1.93	0.31	3.38	0.066	34.18
SM	5.76	0.31	18.43	0.0001	0.00	0.00	1.24	0.27	-0.02	0.44	-0.43	0.68	7	0.05	0.09	0.79	0.499	24.22
OFL	4.49	0.86	5.22	0.0002	0.002	0.001	1.49	0.159	-0.03	0.12	-0.26	0.79	15	3.56	0.48	1.12	0.36	14.67
AMK	5.06	0.72	7.05	0.0001	0.001	0.001	0.54	.059	0.04	0.12	0.35	0.73	15	2.74	0.45	0.36	0.70	5.28

[a]SE = standard error. [b]T = Value for t-test statistic. [c]df = degrees of freedom including 2 for regression evaluation. [d]SS = sum of squares, Mean square, not given in table = SS ÷ df. [e]F = value of F-test statistic. [f]R^2 = coefficient of determination.[g]Control = based on CFU/ml of unexposed cultures used as control (see Chapter 5 and 6), INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin.

7.4   DISCUSSION

7.4.1 *Appropriateness of the logistic model*

The excellent fit of the logistic model when applied to the unexposed cultures, as evidenced by a p-value of 0.02 and an R^2 value of 52.6% (the reverse correlation with lag periods was explained earlier and was attributed to the different growth phases of the control cultures at the time of regrowth in Bactec vials), strongly suggests that the model adequately reflects regrowth of undamaged viable tubercle bacilli.

The reasons why the model appeared to perform poorly in the case of at least three anti-TB drugs (SM, OFL and AMK) are likely to relate to specific properties of the drugs or to the experimental design features or both, while the high variability inherent to some experimental procedures notably in the case of colony counting may have further contributed to the apparent failure of the model with these drugs. It should be emphasized that the logistic model was designed to evaluate regrowth patterns as a marker of bactericidal activity. It is therefore logical that regrowth patterns of poorly bactericidal drugs would more likely be related to other properties of the drugs such as PAE and effects of subinhibitory concentrations.

The good correlation achieved with SM when 8MIC, 10MIC and 20MIC were included in the simple regression analysis (Chapter 6) is misleading as it is likely that subinhibitory effects played a role. It is however possible that, if our experiments were designed to minimize the effects of subinhibitory effects by for example diluting the drug-exposed cultures before regrowth in Bactec 7H12 vials, the logistic model would have fitted better with the bactericidal activity of SM.

Higher concentrations of EMB and OFL may also have resulted in improved correlations.

7.4.2 *Possible role of specific properties of drugs*

Delayed post-exposure regrowth (more recently, depending on the experimental model and definition, labeled as PAE) had been well described for INH, RMP, SM (Mitchison and Dickinson, 1971; Mitchison, 1992) following exposure periods of 24h or longer. Such delays which are likely to differ between different drugs may have played a role in our experiments. Table 7.2 adapted from Mitchison and Dickinson (1971) illustrates delays in regrowth generated by INH, RMP, EMB and SM.

Table 7.2 Lag periods after pulsed exposures to anti-tuberculosis drugs

Drug	Concentration of drug (*u*g/ml)	Lag period (days) after exposure for	
		6h	≥24h
INH	1.0	0	6-9
RMP	0.2	2-3	2-3
EMB	10	0	4-5
SM	5.0	8-10	8-10

Adapted from (Mitchison and Dickinson, 1971).

These studies suggest that the drugs investigated previously by other authors, probably also OFL and AMK (no PAE data relating to *M. tuberculosis* available), may have increased lag periods, in addition to detectable regrowth attributable to the direct bactericidal activity of the drugs. Lag periods correlated well with bactericidal activity in the case

of INH (p = 0.02) and RMP (p = 0.0007). Poor correlations were found in the case of EMB (p = 0.17), SM (p = 0.68), OFL (p = 0.79) and AMK (p = 0.73). It is important to note that although the anti-TB drugs may have shown some degree of lag before regrowth started, these delays with the exception of INH and RMP, did not correlate with bactericidal activity. It is noteworthy that Li *et al* (1997) demonstrated statistically significant correlation between PAE and bactericidal activity in the case of five antimicrobial agents with different mechanisms of action. However, in the case of disinfectants, Fuursted (1997) failed to show such a correlation. Correlation between PAE as defined internationally, and bactericidal activity, as was postulated by Li *et al* (1997), is likely to occur at a different level than that between lag periods and bactericidal activity in the present study. In the latter instance, lag periods comprised true PAE as well as delays in registering GI readings due to a reduction in the number of post-exposure tubercle bacilli requiring multiplication to a level registrable as GI readings in the Bactec system

7.4.3 *Design features compromising the logistic growth curve model*

The short 6h exposure time which was shown to have had marked PAE, especially noticeable in the case of EMB and OFL (see Chapter 6), and (b) the effect of subinhibitory concentrations also discussed in Chapter 6 were excluded in the logistic model. Good correlation with a logistic model was demonstrated in the case of the highly bactericidal agents, INH and RMP. There is a distinct possibility that if higher concentrations were used in the case of the less bactericidal agents (EMB, SM, OFL, AMK), much better correlations would have been achieved. The design of such experiments should ensure that carry-over effects and therefore subinhibitory concentrations be excluded.

7.4.4 *Summary and conclusions*

The logistic model appeared to be appropriate for unexposed cultures as well as for INH and RMP. In the case of the control unexposed cultures, however, lag periods correlated positively (instead of the expected negative correlation) with CFU/ml. Such good correlations were however not found in the case of EMB, SM, OFL and AMK. The validity of the model as well as the relative roles of lag periods and regrowth rates as assessed in the present experiments, should be viewed with caution because of the variability inherent in the techniques used (for example, colony counting) as well as the design of the radiometric regrowth model which did not cater sufficiently for bactericidal effects in the case of the less bactericidal anti-TB agents.

The significance of the good fit of the model in the case of INH and RMP which also exhibited the greatest bactericidal activity probably relates to a marked reduction in organisms which overshadowed other effects including experimental variation to the techniques used. The distinct possibility that higher concentrations of EMB, SM, OFL and AMK, after elimination of subinhibitory effects in the experimental design, may show a better correlation between regrowth patterns and bactericidal activity, needs to be explored further. Better organism dispersal and other measures to improve technical aspects of future studies may also result in a more reliable evaluation of the model.

CHAPTER 8

PERFORMANCE CHARACTERISTICS OF THE RADIOMETRIC POST-EXPOSURE REGROWTH MODEL

8.1 Drug screening

The post-exposure regrowth model using T_{400} regrowth times and $(T-C)_{400}$ indices showed good discrimination between the activities of anti-tuberculosis (anti-TB) drugs and the findings are generally in accordance with clinical efficacy. The model incorporates both the postantibiotic effect (PAE) and bactericidal activity of the drugs. Good agreement was found between surviving tubercle bacilli and regrowth times (T_{400}) and even better correlation was achieved when survivors were plotted against $(T-C)_{400}$. The shorter the T_{400} values for the control cultures at the higher exposure times (48h and 72h) relative to the 6h and 24h periods, resulted in longer $(T-C)_{400}$ times at these exposure times. This brought the 6h points in closer alignment with the regression line. Whether this correction has a biological basis is unclear.

We suggest the use of this model for early screening of new drugs for anti-TB activity. Based on criteria used for prediction of bactericidal activity, all but streptomycin (SM) could have passed as candidates for the treatment of TB. We suggest a scheme involving two stages for the screening of candidate anti-TB drugs, namely (a) initial screening where high drug concentrations are used. This approach is based on the fact that the regrowth time due to subinhibitory concentrations which should not exceed 0.2 minimal inhibitory concentrations (MICs), will render the model more sensitive than a modified system which excludes the subinhibitory

concentrations; (b) subsequent screening where drugs can be categorized into candidates for further evaluation, good prospect drugs and excellent candidates. Further studies using control antibiotics with little or modest activity against *Mycobacterium tuberculosis* are required before this screening proposal can be offered with confidence. The relative simplicity and safety of the Bactec radiometric model adds to its attractiveness as a screening procedure for candidate anti-TB drugs. Furthermore, its good correlation with *ex vivo* early bactericidal activity (EBA) of drugs as found in Chapter 5 and Chapter 7, also augurs well for its use in this capacity.

8.2   Drug carry-over

Drug carry-over is minimised in this model as drug solutions are diluted below the inhibitory concentrations (1:40 dilution) when 0.1 ml sample portions of the unexposed and exposed culture are inoculated into the Bactec 7H12 vials. Exposure concentrations of 8 MIC will therefore result in 0.2 MIC in regrowth vials.

When very active drugs such as isoniazid (INH) and rifampicin (RMP) are used at high concentrations, growth index (GI)-derived curves did not reach the GI of 400 threshold within the 14-day observation period. In such instances the delays in regrowth are likely to be prolonged by the effects of drug carry-over and more importantly by subinhibitory concentrations (sub-MICs).

Sub-MICs are defined as concentrations of drugs that would not inhibit growth completely but would slow it down during periods of regrowth (Mitchison, 1998). *In vitro,* sub-MICs have been shown to inhibit the rate of bacterial growth (Rolinson, 1977) and experiments

by Odenholt-Tornqvist *et al* (1991) showed that the pharmacodynamic effects of sub-MICs for some antibiotic-bacterial combinations may be more important for the inhibition of regrowth between doses than the PAE.

8.3 The inoculum

The initial inoculum for the radiometric post-exposure regrowth studies was prepared in 7H9 broth with magnetic stirring. An alternative would be to prepare the seed culture in Bactec 7H12 vials. This medium allows homogenous growth of bacilli and hence minimal clumping of *M. tuberculosis.* Cultures in this case could be allowed to grow until a GI reading of 400-500 is reached. They will then be in the mid-logarithmic growth phase and the initial bacterial concentration will be the same for all the experiments.

In cases where the 7H9 broth dilution method is preferred due to cost implications, better clump dispersal methods such as sonication and other forms of mechanical shaking can be used when growing cultures. To obtain single-cell growth of mycobacterial suspensions in 7H9 medium, a combination of the use of detergent, sonication, vortexing with glass beads is necessary for optimal dispersal of clumps. Sonication, vortexing, serial 10-fold dilutions, and growth on open petri dishes however, pose infectious hazards while the use of a detergent may affect drug-organism interaction (Naik *et al,*1989).

8.4 Extensions of the radiometric post-exposure regrowth model

8.4.1 *Susceptibility testing*
The model can be used for susceptibility testing of wild strains of *M.*

tuberculosis. This approach has been used previously (Inderlied *et al,* 1987) for *M. avium.* However, in this case, the authors used a defined value termed T100 for data analysis. In their studies, a modified proportion method for MIC determination was used. A 99% and 99.9% proportions were used to define the MICs, that is, the drug-free control cultures were diluted 1:100 and 1:1000, respectively. The MIC 99 was the concentration of drug necessary to increase the T100 value to the T100 value of the 1:100 control and the MIC 99.9 was the drug concentration necessary to increase the T100 value to the T100 value of the 1:1000 control. The MIC 99 and MIC 99.9 were extrapolated from the dose-response curves. The same principles can be applied for susceptibility testing against *M. tuberculosis* strains but using the radiometric post-exposure regrowth model used in the present study.

8.4.2 *Effects of drugs during the postantibiotic phase*

The present study was designed to evaluate regrowth patterns after exposure of actively replicating *M. tuberculosis* to anti-TB drugs. Further studies can be done on metabolically inactive bacteria in the stationary phase or phase of decline in non-exposed cultures or in drug-exposed bacteria, such as those obtained in the radiometric regrowth postantibiotic phase (PA). Such studies would provide insight into the sterilizing activity of the drugs *in vitro.* A PA phase has been previously defined as a period of persistent suppression of growth before metabolic activity starts. The Bactec manufacturer recommends that GIs for susceptibility testing inocula be equal to or over 300 (Siddiqi, 1988). Based on the GI-derived growth curves obtained in the present study, it is possible that post-exposure GI readings of below 100 or 200 would be reflective of the PA phase. However, correlation with actual counts on agar media needs to be established in a form of a growth dynamics experiment before such

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studies could be initiated.

8.4.3 *Comparison between the model and viable counts*

Post-exposure regrowth studies incorporating washing which removes the bactericidal activity of drugs, can be designed for the Bactec radiometric system followed by sampling and plating on agar media. Comparisons of effects of drugs between the Bactec radiometric methods and the conventional colony forming unit (CFU) counts method can thus be made in terms of bactericidal activity and PAE. However, because such methods involve sampling and plating, they would be laborious and time consuming, and pose increased safety risks.

The regrowth method performed in the present study did not involve any washing to remove the drugs. As such it was easy to perform and quick. Furthermore, the Bactec system employed obviates the use of colony counting to assess regrowth. Moreover, the use of this closed system minimised the risk of contamination and optimised safety.

8.5   Significance of the findings

Little is known about how killing and regrowth observed *in vitro* relate to antimicrobial activities of drugs *in vivo* (Gerber and Feller-Segessemann,1985). If a drug produced prolonged PAEs and exhibited major concentration-dependent killing, the goal would be to maximize drug concentrations. Administration of larger doses in such cases would increase peak drug concentrations and enhance bactericidal activity. Longer dosing intervals should also be possible as the PAE would prevent bacterial regrowth when serum and tissue concentrations fall below the MIC (Craig and Ebert, 1991).

If a drug produced more time-dependent killing and exhibited short or no PAEs, the goal of the dosing regimen would be to maximize antimicrobial exposure. High concentrations of the drug would not kill bacteria any faster than would be observed at the lower concentrations. Furthermore, since regrowth would start as soon as the levels fall below the MIC, one would want to provide bactericidal concentration throughout the dosing interval. Thus, more continuous dosing of these drugs would appear to be desirable for maximum efficacy (Craig and Erbert, 1991).

With regard to sub-MICs, it has been previously suggested that the effects of the subinhibitory levels on bacteria may be an additional explanation for the success of intermittent dosage schedules. The authors further suggested that it is the combined effect of the PAE and sub-MICs that will prevent bacterial regrowth between doses in some antibiotic-bacterial combinations (Odenholt-Tornqvist, 1991).

8.6   Conclusions

The rapidity, safety and simplicity of the Bactec system cannot be overstressed. This was important in the present study where six anti-TB drugs were tested at a range of concentrations and exposure times. The speed that this system provides would be particularly useful in laboratories where a large number of candidate drugs are routinely screened for anti-TB activity. Another potential advantage of this model is that it allows a continuation of reading after reincubation unlike the conventional CFU counts methods where readings have to be taken as a final process. As such growth of cultures unexposed or exposed to drugs can be monitored daily. This is important because potential problems which can be encountered in an experiment such as contamination can be observed earlier than

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in the conventional CFU counts method. Furthermore, the Bactec radiometric system for quantification of mycobacteria minimizes the risk of laboratory-acquired infection. It is quick, safer and more accurate than the colony counting method.

CHAPTER 9

KINETICS OF KILLING OF DRUG COMBINATIONS AND RADIOMETRIC POST-EXPOSURE REGROWTH OF *MYCOBACTERIUM TUBERCULOSIS*

9.1 BACKGROUND

Combinations of anti-tuberculosis (anti-TB) drugs have been widely used since soon after the introduction of isoniazid (INH) in 1952 (Fox, 1990). They are most often used for the following reasons: (1) to minimize the probability of emergence of drug resistance, (2) to increase the activity (particularly bactericidal activity) of the agents which have only marginal activity when used singly, or in immunocompromised patients when the bactericidal effect is essential, (3) to reduce a potentially toxic effect by employing lower dosages than would be required in monotherapy, (4) to provide broad coverage of infections caused by unidentified organisms, and, (5) for treatment of polymicrobial infections (Heifets, 1991b; Krogstad and Moellering, 1986; Young, 1980). Another indication not generally mentioned which pertains specifically to TB concerns the fact that conditions in TB lesions may favour one drug over another, examples include situations where some drugs would be more active at a high pH (aminoglycosides) and others, for example, pyrazinamide (PZA), under acid conditions. This has been discussed extensively in the introductory chapter of this dissertation (Chapter 1, Section 1.2.1).

The main reason for the introduction of combination TB therapy was the prevention of drug resistance. Current use of drug combinations in the chemotherapy of TB is aimed at designing the most efficient

short course regimen. The drugs in a combination are aimed at the heterogenous bacterial population which persists in different environments and in different states of metabolism (Heifets, 1991b; Moellering, 1979b).

Antimicrobial agents in combination can produce synergistic, additive, indifferent or antagonistic effects. Various definitions have been used to describe these phenomena. However, there are no uniformly accepted definitions of antagonism or synergy, instead, interpretation of results is linked to the specific methods used (Heifets, 1991b).

The best known and most common *in vitro* techniques for the assessment of drug combinations are the checkerboard and the time-kill curve methods (Heifets, 1991b; Li *et al*, 1993; Moellering, 1979a; Young, 1980; Norden *et al*, 1979).

9.1.1 *The time-kill curve technique*

The principles behind the time-kill curve technique have been discussed in Chapter 4. In drug combination studies using this technique, synergy has been described in many ways. Some authors have assumed that synergy is present if the effect of the combination simply exceeds the algebraic sum of the effects of its constituents (Jawetz, 1967). Others require that the survival level before growth resumes after drug-combination exposure, be less than that with the most active drug by a factor 1 $\log_{10}$ (Schoeneknecht, *et al*, 1985) or 2 $\log_{10}$ (Yajko *et al*, 1987).

Most recent publications in the scientific literature adapt the criteria used by Krogstad and Moellering (1986) and Heifets (1991b). Their interpretation of results is based on comparison of the effect

produced by the drug combination and that of the most active single drug alone. Synergy is considered to occur when there is a 100-fold or greater increase in killing, in comparison with the most active drug alone and this takes place within a specified exposure time. Antagonism on the other hand is defined as a 100-fold or greater decrease in killing under the same terms. Indifference is seen when the difference between the killing by the drug combination and killing in the presence of the most active single drug is less than 10-fold (Krogstad and Moellering, 1986; Heifets, 1991b).

9.1.2 *The checkerboard titration method*

This technique has been widely used for studying drug combinations in *M. avium* strains (Heifets, 1991b; Bergman and Woods, 1998). In this method organisms are exposed to serial dilutions of no more than three drugs in broth or agar media. The organisms are exposed to all possible combinations of drug concentrations within a range of dilutions used. The endpoint is usually based on the minimal inhibitory concentration (MIC) (Moellering, 1979b; Young, 1980). Unlike the time-kill curve method, this technique yields only inhibitory data unless sampling of the culture broth containing antimicrobial agents is done to determine microbicidal activity.

Another disadvantage to the use of the checkerboard method is that results are examined only at one point in time. Thus the technique provides static, rather than a dynamic view of an antimicrobial interaction. However, despite these limitations, the checkerboard method is simple to perform and remains the most widely used technique to assess antimicrobial combinations (Krogstad and Moellering, 1986).

9.2 OBJECTIVES

Experiments covered in this chapter were designed specifically to measure possible synergy between two drugs when used in combination against *M. tuberculosis* and, to use the conventional time-kill curve technique and the Bactec regrowth model for this purpose.

9.3 MATERIALS AND METHODS

Antimicrobial agents

INH, rifampicin (RMP), ethambutol (EMB), streptomycin (SM), ofloxacin (OFL), and amikacin (AMK) were prepared as in Chapter 2, Section 2.2.1.

Test strain

The international *M. tuberculosis* H37Rv ATCC 27294 was used.

Procedure

(i)   The conventional time-kill curve technique

Time-kill curve assays of combined antimicrobial activity were performed at least three times with the various two-drug combinations tested. The inoculum was prepared in the same way as in the time-kill curve assays using single drugs (see Chapter 4, Section 4.3).

The assay mixture in this case contained 4.5 ml of the bacterial suspension and 0.5 ml of working solutions of drugs in combination (0.25 ml of each drug). The MIC was used when drugs were tested singly and in combination, respectively, for INH, RMP and EMB. In

the case of SM, OFL, and AMK, 2 times the MIC was used for single drugs and in combination. Multiples of the MICs chosen for combination studies were based on slopes of the kill curves and Bactec regrowth curves performed on single drugs (Chapter 4 and Chapter 5). A culture with a final volume of 5 ml in which no drug was added was used as a control.

After addition of drugs cultures were incubated at 37°C. Sampling to determine the number of survivors was performed after 24 hours of exposure. The exposure time was standardized to assess the *in vitro* early bactericidal activity (EBA) of the drugs. (The classic *ex vivo* EBA is performed on sputum samples after exposure of 48 hours).

As in the time-kill curve method using drugs singly (Chapter 4, Section 4.3), the conventional colony forming unit (CFU) counting method was used to determine the number of bacterial survivors while post-exposure regrowth was determined in Bactec vials as in Chapter 5, Section 5.3. Possible drug carry-over effects were accounted for as in Section 4.3. However, in this case, drug combinations as opposed to single drugs were evaluated.

Synergy in the time-kill curve model was defined as a $2 \log_{10}$ CFU/ml (100-fold) or greater reduction of a drug combination compared with the most active single drug. Antagonism on the other hand was a $2 \log_{10}$ CFU/ml or greater increase under the same terms. Indifference was a less than $1 \log_{10}$ CFU/ml difference between the combination and the most active single drug. In order to describe trends, a $0.5 \log_{10}$ CFU/ml or greater decrease following drug-combination exposure, as compared with the most active drug, was used to define synergistic trends. Antagonistic trends were a $0.5 \log_{10}$

CFU/ml or greater increase under the same terms. Indifference comprised post-exposure counts of drug combinations ranging from a > -0.50 $\log_{10}$ decrease to a 0.50 $\log_{10}$ increase in CFU/ml.

(ii)   The Bactec radiometric regrowth model

The same radiometric regrowth model was used as for the single drug studies (Chapter 5). The drug concentrations in terms of multiples of the MICs and exposure times were the same as those employed in the time-kill curve model mentioned above. Results of the combination studies were analysed by calculating the time taken for growth of the unexposed control and drug- or drug combination-exposed cultures to reach a growth index (GI) reading of 400 (T_{400}) using linear interpolation. $(T-C)_{400}$ indices (T_{400} for drug- or drug-combination exposed cultures (T), minus those of the unexposed control (C) cultures) were calculated. Standard errors of the T_{400} readings were calculated for each Bactec T_{400} profile and because the number of observations (one concentrations and one exposure time for each drug or drug combination) were few, standard errors of the mean were derived from the pooled error square estimate (s^2). This assumes that variation of GI readings between drugs and drug combinations is constant. For each drug combination and single drug set, results obtained from the radiometric regrowth model were then compared with those obtained by the conventional colony counting technique.

Definitions of synergy, antagonism additivity and indifference were based on a paper by Gudmundsson *et al* (1991). Synergy was defined as a $(T-C)_{400}$ index of a drug combination being at least 2 days longer than the sum of the $(T-C)_{400}$ indices for the individual drugs, and an additive effect as the combination $(T-C)_{400}$ index being roughly similar to the mathematical sum of the individual $(T-C)_{400}$

indices. Indifference was defined as the combination $(T-C)_{400}$ index being no different (less than 1 day) from the longest of the individual indices and, antagonism as the combination $(T-C)_{400}$ index being at least 2 days less than the longest of the individual $(T-C)_{400}$ indices.

9.4 RESULTS

9.4.1 *The conventional time-kill curve model*

9.4.1.1 Synergy based on the 2 log_{10}CFU/ml definition

Results of exposing cultures to single drugs and two-drug combinations of INH, RMP, EMB, SM, OFL and AMK are set out in Table 9.1. None of the combinations tested in the present study showed synergy or antagonism since the effect of the combinations did not result in a 2 log_{10} CFU/ml or greater, increase or decrease in killing, respectively, in comparison with the most active drug alone.

Although combinations of RMP plus EMB, RMP plus SM, RMP plus AMK and, INH plus EMB did not result in a 2 log_{10}CFU/ml decrease, as compared to RMP or INH, the most active drugs in the combinations, definite trends of increased activity were observed with these combinations. Indifference was found for all the combinations studied since the difference between the killing by the drug combination and killing in the presence of the most active single drug was less than 1 log_{10}CFU/ml.

Addition of OFL, SM or AMK to INH decreased the colony counts of the combination, but the decrease was not greater than that of INH alone. Similarly, addition of INH or OFL to RMP, decreased colony counts but the decrease was not greater than that of RMP when

used alone.

9.4.1.2 Trends based on the 0.5 $\log_{10}$CFU/ml criterion

The decreases in viable counts for the drug combinations were compared with viable counts obtained for the most active single drug (Table 9.1). Standard errors of the mean are given in Table 9.2. Synergistic trends were arbitrarily defined as a 0.5 $\log_{10}$ CFU/ml or greater decrease of the drug combination compared with the most active drug, and antagonism as a 0.5 $\log_{10}$CFU/ml or greater decrease under the same terms. Drug combinations tested in the present study showed neither synergy nor antagonism. All combinations tested were indifferent. Indifferent trends were found when the difference between the drug combination and the most active drug was less than -0.50 $\log_{10}$ CFU/ml to 0.5 $\log_{10}$ CFU/ml. Examples of indifferent trends are graphically illustrated in Figure 9.1.

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(a)

(b)
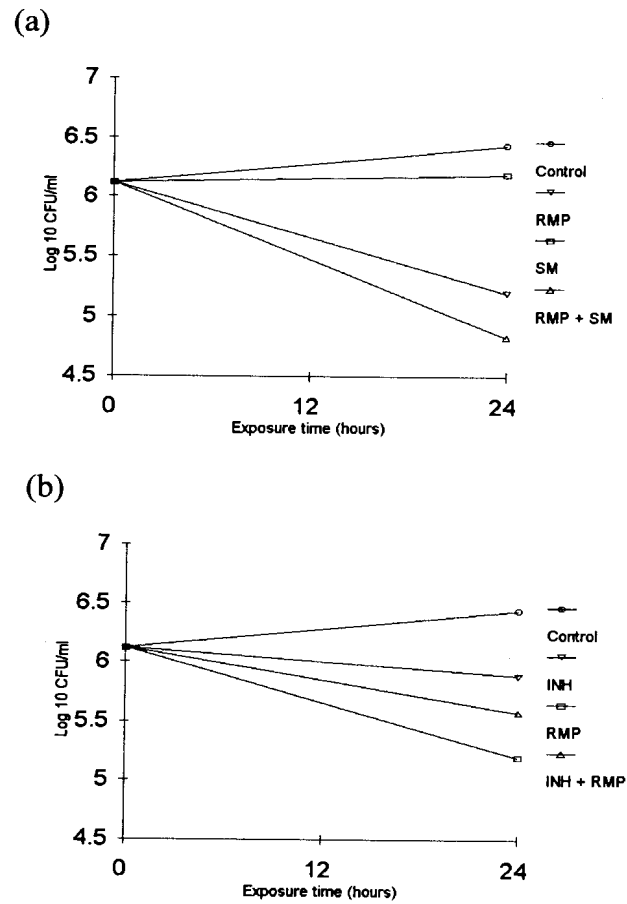
Figure 9.1 : Bactericidal activity in $\log_{10}$ CFU/ml of (a) rifampicin (RMP) plus streptomycin (SM), and (b) isoniazid (INH) plus RMP. An indifferent trend was found when the difference between the drug combination and the most active drug was less than - 0.5 $\log_{10}$CFU/ml to 0.5 $\log_{10}$CFU/ml.

Table 9.1 Bactericidal activity of single drugs and drug combinations against *M. tuberculosis* H37Rv after 24 hour exposure, expressed in colony forming units (CFUs)/ml.

Single drugs[a] (x MIC)	Mean decrease[b] in viable counts ($\log_{10}$ CFU/ml) (A)	Drug combinations	Mean decrease[b] in viable counts by combination ($\log_{10}$ CFU/ml) (B)	Decrease with most active drug in combination ($\log_{10}$ CFU/ml) (C)	Index of drug combination ($\log_{10}$ CFU/ml) (B-C)	Trends exhibited by combinations[c]
INH_1	0.299	$INH_1 + RMP_1$	0.603	0.985	-0.382	Ind
RMP_1	0.985	$INH_1 + EMB_1$	0.569	0.299	0.270	Ind
EMB_1	0.035	$RMP_1 + EMB_1$	1.223	0.985	0.238	Ind
SM_2	-0.01	$INH_1 + SM_2$	0.218	0.299	-0.081	Ind
OFL_2	0.15	$INH_1 + OFL_2$	0.089	0.299	-0.210	Ind
AMK_2	0.014	$RMP_1 + OFL_2$	0.812	0.985	-0.173	Ind
Control	6.274	$INH_1 + AMK_2$	0.512	0.299	0.213	Ind
		$RMP_1 + SM_2$	1.352	0.985	0.367	Ind
		$RMP_1 + AMK_2$	1.033	0.985	0.048	Ind

[a] INH = isoniazid (1 MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1 MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC), subscript numbers denote multiples of the MICs which were equal to 0.05 *u*g/ml, 0.5 *u*g/ml, 2.0 *u*g/ml, 1.0 *u*g/ml, 0.5 *u*g/ml and 0.25 *u*g/ml for INH, RMP, EMB, SM, OFL and AMK, respectively. [b] The decrease in viable counts was calculated from the initial bacterial concentration of $6.178 \log_{10}$ colony forming units (CFU)/ml with a standard error = 0.172), None of the combinations meet with the definition of synergy (decrease of combination $\geq 2 \log_{10}$ CFU/ml vs the most active drug). [c] On arbitrary grounds, B - C $\geq$ 0.50 suggest a synergistic trend (Syn), B - C $\leq$ -0.5 $\log_{10}$ CFU/ml an antagonistic trend (Ant), B - C >-0.50 to 0.50 an indifferent trend (Ind).

Table 9.2 Viable counts for *M. tuberculosis* H37Rv after 24 hour exposure to six anti-tuberculosis drugs singly and in two-drug combinations.

Drug/drug[a] combination	Viable counts in $\log_{10}$ CFU/ml	
	Mean	Standard error[b]
INH_1	5.879	0.172
RMP_1	5.193	0.172
EMB_1	6.143	0.172
SM_2	6.189	0.172
OFL_2	5.988	0.172
AMK_2	6.164	0.172
$INH_1 + RMP_1$	5.575	0.172
$INH_1 + EMB_1$	5.609	0.172
$INH_1 + SM_2$	5.96	0.172
$INH_1 + OFL_2$	6.089	0.172
$INH_1 + AMK_2$	5.666	0.172
$RMP_1 + EMB_1$	4.955	0.172
$RMP_1 + SM_2$	4.825	0.172
$RMP_1 + OFL_2$	5.356	0.172
$RMP_1 + AMK_2$	5.145	0.172
Control	6.274	0.172

[a] INH = isoniazid (1 MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1 MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC). MICs were equal to 0.05 *u*g/ml, 0.5 *u*g/ml, 2.0 *u*g/ml, 1.0 *u*g/ml, 0.5 *u*g/ml and 0.25 *u*g/ml, for INH, RMP, EMB, SM, OFL and AMK, respectively. [b] Standard errors were calculated from the pooled error estimate (s^2, see Materials and methods).

9.4.2 The radiometric post-exposure regrowth model

GI-derived growth curves for the single drugs as well as the drug combinations are shown in Figures 9.2 to 9.4. As in the single drug studies (Chapter 5), T_{400} (time taken for unexposed control and drug-exposed cultures to reach GI 400 readings were calculated from the GI-derived growth curves and $(T-C)_{400}$ indices (T_{400} for the test drug (T) minus that for the unexposed control (C) cultures) were used to facilitate analysis of results obtained in the drug interaction studies.

Table 9.3 summarizes the T_{400} findings in terms of $(T-C)_{400}$ indices and standard errors of the means for the T_{400} readings are given in Table 9.4. Definitions used for synergy, additivity, indifference and antagonism were based on Gudmundsson *et al* (1991), see Materials and methods). Synergy was found for INH plus RMP and RMP plus EMB combinations (Figure 9.2). Combinations of INH plus EMB, INH plus OFL and RMP plus OFL were indifferent (Figure 9.3), while those of INH plus SM, INH plus AMK, RMP plus SM and RMP plus AMK were additive (Figure 9.4). In all these instances GI-derived growth curves for the drug combinations lay below those obtained for the single drugs when used alone. The combination of INH plus SM was more active than those of INH plus EMB, INH plus AMK, and INH plus OFL. No antagonistic effects were found.
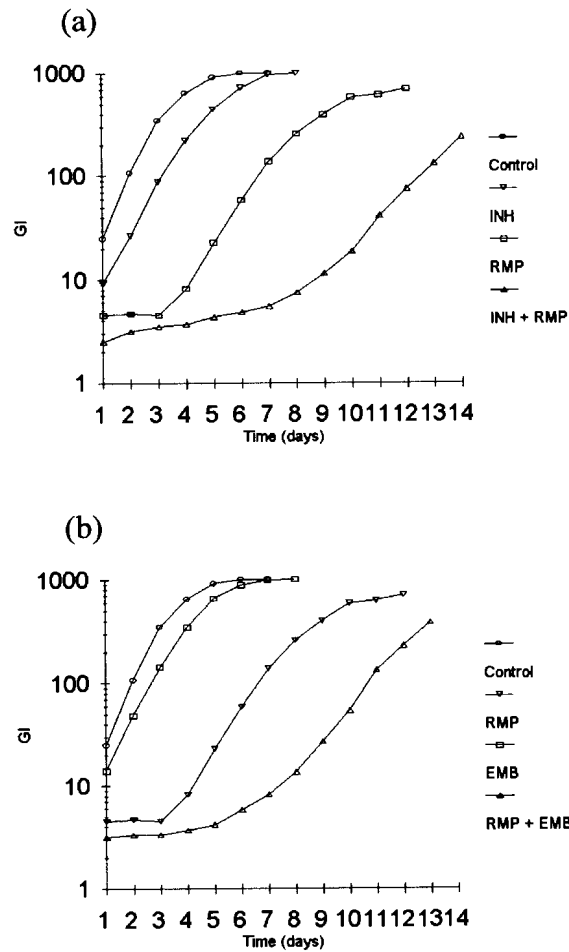
Figure 9.2 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rv showing synergistic interactions after exposure to single drugs and combinations of (a) isoniazid (INH, 1 MIC) plus rifampicin (RMP, 1 MIC) and (b) RMP (1 MIC) plus ethambutol (EMB, 1 MIC). Synergy was found when the $(T-C)_{400}$ indices for the drug combinations were greater than or equal to 2 days as compared with the those obtained for the most active single drugs.

(a)

(b)

(c)
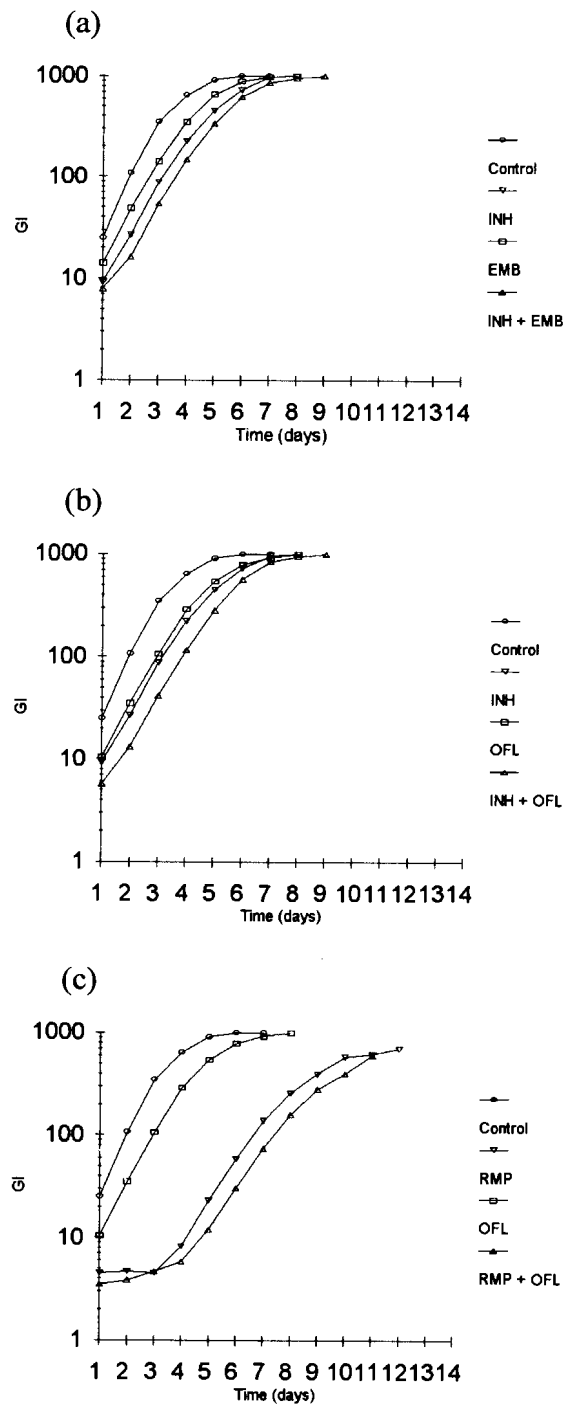
Figure 9.3 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rv showing indifferent drug interactions after exposure to single drugs and combinations of (a) isoniazid (INH, 1 MIC) and ethambutol (EMB, 1 MIC), (b) INH (1 MIC) and ofloxacin (OFL, 2 MIC) and (c) rifampicin (RMP, 1 MIC) and OFL (2 MIC). Indifference was found when the combination $(T-C)_{400}$ index was < 1 day as compared with the most active drug.
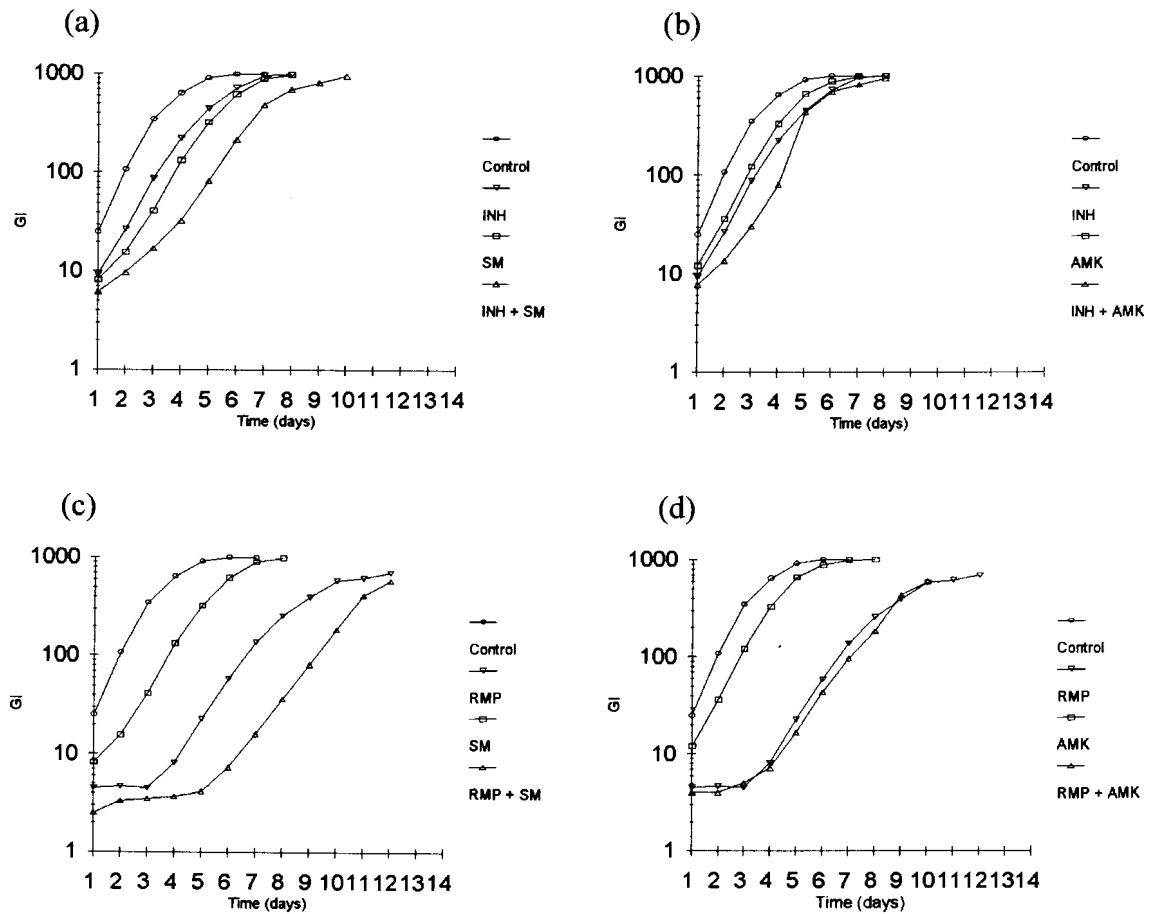
Figure 9.4 : Bactec radiometric regrowth curves for *M. tuberculosis* H37Rv showing additive drug interactions after exposure to single drugs and combinations of (a) isoniazid (INH, 1 MIC) plus streptomycin (SM, 2 MIC), (b) INH (1 MIC) plus amikacin (AMK, 2 MIC) and (c) rifampicin (RMP, 1 MIC) plus SM (2 MIC), and (d) RMP (1 MIC) plus AMK (2 MIC). Additivity was found when the combination $(T-C)_{400}$ index was similar (within 0.5 day) to the $(T-C)_{400}$ indices for the most active single drug.

Table 9.3 Evaluation of two-drug combinations in radiometrically determined regrowth model.

Drug[a] combination	S_1, S_2[b] (A)	$\sum T_{400} (t - c)$[c] (B)	$C\ T_{400} (t - c)$[d] (C)	combination surplus/deficit[e] (D)	$T_{400} (C - S)$[f] (E)	Outcome[g]
INH$_1$ + RMP$_1$	2.1, 6.01	8.11	10.52	2.41	4.51	Syn
INH$_1$ + EMB$_1$	2.1, 1.1	3.2	2.32	-0.88	0.22	Ind
INH$_1$ + SM$_2$	2.1, 2.33	4.43	4.09	-0.34	1.99	Add
INH$_1$ + OFL$_2$	2.1, 1.82	3.92	2.45	-1.47	0.35	Ind
INH$_1$ + AMK$_2$	2.1, 1.03	3.13	2.97	-0.16	0.87	Add
RMP$_1$ + EMB$_1$	6.01, 1.1	7.11	9.89	2.78	3.88	Syn
RMP$_1$ + SM$_2$	6.01, 2.33	8.34	8.01	-0.33	2.0	Add
RMP$_1$ + OFL$_2$	6.01, 1.82	7.83	6.83	-1.0	0.82	Ind
RMP$_1$ + AMK$_2$	6.01, 1.03	7.04	7.45	0.41	1.44	Add
Control	3.022					

[a]INH = isoniazid (1 MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1 MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC), subscript numbers indicate multiples of the MICs equal to 0.05 ug/ml, 0.5 ug/ml, 2.0 ug/ml, 1.0 ug/ml, 0.5 ug/ml and 0.25 ug/ml for INH, RMP, EMB, SM, OFL, and AMK, respectively. [b]S_1 and S_2 denote $(T-C)_{400}$ indices (T_{400} for test drug, T minus that for the control, C , of the single drugs. [c]Sum of $(T-C)_{400}$ indices of drugs in each combination. [d]$(T-C)_{400}$ indices for drug combination. [e]Difference between columns (C) and (B). [f]$(T-C)_{400}$ index for the drug combination minus that of the most active single drug, column (C) minus column (A). [g]Outcome : the outcome is derived from columns (D) and (E) and is based on a calculation used by Gudmundsson *et al* (1991), Syn = synergy(D $\geq$ 2 days), Ant = antagonism (E $\leq$ 2 days), Add = additivity (D = <-0.5 to 0.5 days), Ind = indifference (C no different (< 1 days) from the most active drug in column A.

Table 9.4 T_{400} readings in days for *M. tuberculosis* after 24 hour exposure to six anti-tuberculosis drugs singly and in two-drug combinations.

drug/drug[a] combination	T_{400} readings in days	
	Mean	Standard error[b]
INH$_1$	5.125	0.283
RMP$_1$	9.033	0.283
EMB$_1$	4.131	0.283
SM$_2$	5.35	0.283
OFL$_2$	4.838	0.283
AMK$_2$	4.056	0.283
INH$_1$ + RMP$_1$	13.539	0.283
INH$_1$ + EMB$_1$	5.339	0.49[c]
INH$_1$ + SM$_2$	7.112	0.283
INH$_1$ + OFL$_2$	5.474	0.283
INH$_1$ + AMK$_2$	5.989	0.283
RMP$_1$ + EMB$_1$	12.909	0.283
RMP$_1$ + SM$_1$	9.852	0.283
RMP$_1$ + OFL$_2$	10.473	0.31[c]
RMP$_1$ + AMK$_2$	11.027	0.283
Control	3.022	0.283

[a]INH = isoniazid (1 MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC). MICs were equal to 0.05 ug/ml, 0.5 ug/ml, 2.0 ug/ml, 1.0 ug/ml, 0.5 ug/ml and 0.25 ug/ml for INH, RMP, EMB, SM, OFL and AMK, respectively. [b]Standard errors were derived from the pooled error estimate (s^2, see Materials and methods. [c]Standard errors were slightly different from the others since Bactec regrowth profiles some experiments did not reach the GI reading of 400 and were thus set as missing in the analysis.

9.5   DISCUSSION AND CONCLUSIONS

9.5.1 The conventional time-kill curve technique

9.5.1.1   *Comparison with in vitro studies*

There have been very few reports on the interaction *in vitro* of anti-TB drugs against mycobacteria (Heifets, 1982). One of the first reports on the interaction of drugs against *M. tuberculosis in vitro* was published in 1970 (Hobby and Lenert, 1972). In these 28-year old studies, referred to by some authors in the field as classical, the terms synergy and antagonism were not used. It is therefore difficult to compare data obtained in these "classical" experiments on drug interactions with results obtained in the present study. In his report on drug combinations, Heifets (1991b) emphasized that details from these "classical" experiments be analysed in the light of modern standards for the evaluation of drug interactions.

The actual bactericidal interaction of anti-TB drugs *in vitro* against *M. tuberculosis* was first addressed in 1977 (Urbanczik,1980). In these studies the terms synergy and antagonism were used. Interpretation of synergy and antagonism was based on comparison of the effect produced by the drug combination (the average daily decrease in the number of CFU/ml) with the sums of effects produced by each drug singly in the same concentration. This approach was sharply criticized by Berenbaum (1977, 1978) who labelled it a fallacy and showed that by comparing a combined effect with the sum of effects of the individual agents (instead of comparing the concentrations of drugs singly and in combination that produce the same effect) a synergistic effect can be detected in a combination of two portions of the same drug, for example, just by increasing the total concentration.

Heifets (1982) compared data obtained by different authors on the interactions of drugs *in vitro*. Contradictions of data were obtained when these experiments were compared. In a later report, Heifets (1991b) suggested that the differences in data may be explained by the different criteria and methods used by the authors. He then concluded that it is important to use approaches and criteria for synergy studies in the field of mycobacteriology that yield statistically reliable data.

In the present study definitions used to describe synergy, additivity, indifference and antagonism were based on the generally accepted proportion of a 100-fold reduction or increase (2 log_{10}CFU/ml decrease or increase of the drug combination as compared to the most active drug in a combination). In addition, in order to describe possible trends, synergistic trends were defined as a 0.5 log_{10} CFU/ml increase or decrease of the drug combination compared with the most active single drug. This arbitrary value was regarded as realistic, taking into account the limitations of the experimental conditions prevailing in our studies as evidenced by a pooled standard error of 0.172 log_{10}CFU/ml (see Table 9.2).

As in the present study, early experiments on drug combinations (Dickinson and Mitchison, 1976; Dickinson *et al*, 1977) were performed in liquid medium by the sampling and plating technique. Dickinson and Mitchison (1976) found no synergy between INH and RMP. The absence of synergy between these drugs was derived from the fact that the curve for INH plus RMP lay between those of INH and RMP alone. Although modern standards for interpreting effects of drug combinations were not used in their study, their results correspond with those obtained in the present study as no synergy but indifference was found between INH and RMP when

using the 2 $\log_{10}$ CFU/ml and the 0.5 $\log_{10}$ CFU/ml definitions. Dickinson and colleagues (1977) found indifference in a combination of INH and RMP. The same results were obtained in this study by using the 2 $\log_{10}$ CFU/ml and the 0.5 $\log_{10}$ CFU/ml increase or decrease definition.

Using post-exposure regrowth as a model for studying drug interactions (see Section 9.5.2) the combinations INH plus SM and RMP plus SM showed addition (or borderline synergy) and an additive effect, respectively. Indifference was found for all drug combinations tested (INH plus RMP, EMB, SM, OFL, or AMK, and RMP plus EMB, SM, OFL and SM) when using the 2 $\log_{10}$ CFU/ml and 0.5 $\log_{10}$ CFU/ml definitions.

The difference in the findings can be explained by the different criteria used in interpreting synergy, antagonism, additivity and indifference. Dickinson and colleagues (1977) used a method criticized by Berenbaum (1977, 1978) where the effect of the combination is compared with the sum of effects of the individual agents. In order to define synergy or synergistic trends in the present study, the effect of the drug combination was compared with that of the most active single drug in a combination.

9.5.1.2 *Comparison with ex vivo studies*

Experiments done by Jindani *et al* (1980) in patients showed that during the first two days of treatment, the mean fall in colony counts for 2-drug regimens containing INH with other conventional TB drugs, RMP, EMB, SM and pyrazinamide (PZA), was not greater than that for the regimen of INH (the most active drug) used alone, that is, no synergy was found with these 2-drug regimens. The addition of INH to each of the conventional drugs mentioned above

increased their EBA, but not above the EBA achieved by INH alone. When using the 2 $\log_{10}$ CFU/ml and 0.5 $\log_{10}$ CFU/ml definitions, the same results, except for the activities of INH and RMP alone were obtained in the present study. In patients INH was found to be the most active drug during the first two days of treatment (with the highest mean fall in counts). In the present study in which *in vitro* methods were used, RMP (with a mean decrease in counts of 0.985 $\log_{10}$ CFU/ml) was found to be the most active single drug in the combination studies. In the EBA studies however, the effects of the combination were compared with INH instead of RMP.

The possible explanations to the difference in the activity of RMP *in vitro* and in patients during the first two days of treatment, that is, the lower bactericidal activity of RMP in patients during the early days of treatment, has been discussed in Section 4.5.2, in Chapter 4), that is, the microbiological unavailability of the 85% of RMP that is loosely bound to plasma proteins (Boman and Ringberger, 1974) and possible interference of membrane lipids due to the lipid solubility of RMP (Hand *et al*, 1985).

The experiment was done at least three times and results obtained were reproducible and colony counts to determine the number of surviving bacteria were done in triplicate. Concentrations used in the drug combination studies showed neither synergy nor antagonism when using the 100-fold reduction criterion. These concentrations were multiples of the MIC and were based on the slopes of the time-kill curves and the Bactec regrowth curves performed on single drugs. Computations of different concentrations could possibly have given different drug combination effects. However, the time-kill curve technique is tedious and thus limits the number of feasible antimicrobial concentrations as well as drug combinations that can

be tested with any one bacterial isolate.

The time-kill curve assay was done in liquid medium by the sampling and plating technique. Logarithmic phase cultures were exposed to drugs singly and in combination for 24 hours. Early experiments (Dickinson and Mitchison, 1976; Dickinson *et al*, 1977) on interactions of drugs were also performed in liquid medium and by the sampling and plating technique. In these experiments cultures were exposed to drugs for longer periods of time (sampling and plating were done on days 4 to 5, 10 to 11, 21 and 28) (Dickinson and Mitchison, 1976) as opposed to 24 hours used in this study.

The following are reasons for choosing one exposure time in the present study : (1) to standardize for a realistic *in vitro* EBA, (2) the time-kill curve technique is labour intensive, time-consuming and costly and (3) anti-TB drugs act differently on cultures *in vitro*, for example, RMP acts rapidly on the bacterial population whereas INH requires approximately 24 hours to be active (Mitchison and Dickinson, 1978). Therefore, 24 hours was chosen as a reasonable time of exposure to accommodate all the anti-TB drugs.

The bactericidal activities of the drug combinations tested by the time-kill curve technique did not show synergy or antagonism when using the 2 $\log_{10}$ CFU/ml and the 0.5 $\log_{10}$ CFU/ml definitions. Comparison of our findings with previous studies on drug interactions requires consideration of criteria used to define synergy as well as the experimental conditions but, as discussed, they are in general agreement with the literature. The results are also in accordance with previous *ex vivo* studies.

9.5.2 Bactec post-exposure regrowth model

Definitions used to describe synergy, additivity, antagonism and indifference were based on the paper by Gudmundsson *et al,* (1991) and as in the single drug studies (Chapter 5), $(T-C)_{400}$ indices were used to facilitate analysis of drug combination findings. Combinations of INH or RMP with each of the aminoglycosides (SM and AMK) were additive. The interaction of RMP with the highly bactericidal drug (INH), or the moderately bactericidal drug (EMB) was synergistic. When RMP or INH was combined with OFL, the effects were indifferent. Another drug combination which was also found to be indifferent was INH plus EMB.

Values of 0.5, 1.0 or 2 days were arbitrarily chosen to facilitate analysis of the findings. These times partly, were based on the 0.5, 1.0 and 2.0 hour values proposed by Gudmundsson *et al* (1991) for rapidly growing bacteria whose mean generation times are approximately 36-72 times faster than those of *M. tuberculosis* (20-30 minutes versus 18-24 hours). In terms of growth kinetics, 1 hour in the case of rapidly growing bacteria would therefore be equivalent to 36-72 hours for *M. tuberculosis*. Another important criterion used in the choice of the 2 days (48 hours) is that such a delay appeared to be realistic in terms of the lag periods and growth kinetics observed.

Comparison with the conventional drug combination time-kill curve findings showed that effects obtained by the two methods were not the same. For example, the INH plus RMP was synergistic in the post-exposure radiometric model but indifferent in the time-kill curve bactericidal model (using both definitions, the 0.5 $\log_{10}$ CFU/ml and 2 $\log_{10}$ CFU/ml).

An alternative approach to that used in this study (Gudmundsson *et al*, 1991) to define drug interactions in Bactec regrowth experiments was employed by Hoffner *et al* (1987). These authors used Bactec GI readings at 4 days, of single drugs and drug combinations, for their assessment of drug combination activity against *M. avium* isolates. For their definition of synergy they employed the formula $^C/_{S_1} < {}^1/_Z$ where C is the GI reading of the combination, S_1 that of the most active single agent of the combination and Z the number of drugs in the combination ($^1/_Z$ equals 0.5 in the case of two-drug combinations). In our experiments it was more convenient to use the time taken for growth to reach a GI reading of 400 for the assessment of synergy. We modified the formula proposed by Hoffner *et al* (1987) in which $^C/_{S_1} > 2.0$ signifies synergy, a reading of > 1.5 would indicate a positive additive effect, < 0.5 antagonism and < 0.75 a negative effect not yet consistent with antagonism, while readings between 0.75 and 1.75 would be interpreted as indifferent.

In Table 9.5 the four approaches to assess the activity of drug combinations are compared. With the Hoffner *et al* (1987) approach, all drug combinations showed an additive effect while no antagonistic effect was found with the Gudmundsson *et al* (1991) approach. There were no major discrepancies between the time-kill curve findings and those of the regrowth experiments. Indifferent trends were seen for all drug combinations using the time-kill curve technique. No antagonism was found in the radiometric regrowth experiments while synergistic trends were observed for combinations of INH plus RMP, and RMP plus EMB.

Table 9.5 Comparison of four approaches to assess the activity of two-drug combinations in the time-kill curve and Bactec regrowth experiments.

Drug combinations[a]	Drug interactions and activity indices in the case of							
	Time-kill curve experiments			Bactec regrowth experiments				
	Decrease in CFU/ml[b]		(Index)[c]	Interaction according to				
	$\geq 2 \log_{10}$	$\geq 0.5 \log_{10}$		Gudmundsson et al (1991)	(Index a)[d]	(Index b)[d]	Hoffner et al (1987)	(Index)[e]
$INH_1 + RMP_1$	Ind[f]	Ind	(-0.382)	Syn[f]	(4.51)	(2.41)	Add	(1.75)
$INH_1 + EMB_1$	Ind	Ind	(0.270)	Ind	(-0.88)	(0.22)	Add	(1.10)
$INH_1 + SM_2$	Ind	Ind	(-0.081)	Add[f]	(-0.34)	(1.99)	Add	(1.76)
$INH_1 + OFL_2$	Ind	Ind	(-0.210)	Ind	(-1.47)	(0.35)	Add	(1.17)
$INH_1 + AMK_2$	Ind	Ind	(0.213)	Add	(-0.16)	(0.87)	Add	(1.41)
$RMP_1 + EMB_1$	Ind	Ind	(0.238)	Syn	(2.78)	(3.88)	Add	(1.65)
$RMP_1 + SM_2$	Ind	Ind	(0.367)	Add	(-0.33)	(2.0)	Add	(1.33)
$RMP_1 + OFL_2$	Ind	Ind	(-0.173)	Ind	(-1.0)	(0.82)	Add	(1.14)
$RMP_1 + AMK_2$	Ind	Ind	(0.048)	Add	(0.4)	(1.44)	Add	(1.24)

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin. Subscript numbers indicate the drug concentrations in multiples of the MICs. [b]Drug interactions based on $\geq 2 \log_{10}$ or $\geq 0.5 \log_{10}$ decrease in CFU/ml of combination (C) compared with the most active single drug (S_1) . [c]Index = observed difference between C and S_1 ,(C-S_1). in $\log_{10}$CFU/ml. Ant = $\leq$-0.5 $\log_{10}$decrease in CFU/lm with C vs S_1, Syn = $\geq$ 0.5 $\log_{10}$ decrease in CFU/ml with C vs S_1, Ind = $\geq$ - 0.5 $\log_{10}$ to 0.5 $\log_{10}$in CFU/ml with C vs S_1. [d](Index a) = (T-C)$_{400}$ (C-S_1) with <-2 days and <1 day signify Ant and Ind, respectively, (Index b) = (T-C)$_{400}$ (C- $\sum S_1$ and S_2) >2 days and with a margin of 0.5 days signifies Syn and Add (Gudmundsson et al , 1991). [e](Index) = (T-C)$_{400}$^{C}/$_{S1}$ with > 2 days signifying Syn, <0.5 day Ant, equal to 1.5 to 2.0 a positive Add effect, equal to 0.5 to 0.75 a negative Add effect and equal to 0.75 to 1.5 Ind (Hoffner et al , 1987). [f]Syn = synergy, Ant = antagonism, Add = additive effect, Ind = indifference.

Studies on drug interactions using the Bactec system can be done by the sampling and plating method, that is, the minimal bactericidal concentration (MBC) method using the Bactec system discussed in Chapter 2 can be modified to study drug interactions. In such a model, modern standards for evaluating drug combinations as explained by Heifets (1991b) can be used. It is also suggested that when using the T_{400} method for assessment of drug combination effects, further analysis should be done using CFU counts particularly for combinations whose curves lay below or above those of the most active single drug, possibly suggesting synergistic or antagonistic trends, respectively. Such experiments would give information on whether the interactions were synergistic, antagonistic, additive or indifferent based on the generally accepted definition of 100-fold reduction (2 $\log_{10}$ CFU/ml decrease of the drug combination as compared to the most active drug in a combination).

Results obtained with the Bactec radiometric T_{400} method are not directly comparable with clinical experiments on drug interaction such as those previously performed by Jindani *et al* (1980) because in their experiments bacterial killing is measured whereas in the present study regrowth after post-exposure suppression which incorporates both the bactericidal activity and PAE of a drug, is measured. However, as regrowth in the radiometric model is likely to be largely dependent on bacterial cell damage and cell loss due to bactericidal activity (Li *et al*, 1997), a certain degree of correspondence between the two models is to be expected (see Chapters 6 and 7).

As mentioned in Section 9.5.1.1, the combination effects of INH plus SM and RMP plus SM, measured by regrowth times correlated better with the *in vitro* findings of bactericidal activity reported by

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Dickinson and her colleagues (1977) than with our kill kinetics data (see Table 9.5). The discrepant findings may be related to the different techniques employed and especially the Tween 80 used by Dickinson *et al* (1977) in their experiments. The extent of correlation found between the kill kinetics and regrowth times models to assess the activity of drug combinations against the H37Rv strain is discussed further in Chapter 9 where the findings are compared with those demonstrated with two resistant *M. tuberculosis* isolates. It is however clear that good correlation was shown between the conventional assessment of bactericidal synergy and the criteria proposed by Hoffner *et al* (1987) and to a lesser extent those of Gudmundsson *et al* (1991) in radiometric regrowth experiments. The exceptions were INH plus RMP, INH plus SM and RMP plus EMB combinations, which all showed drug enhancing effects in the regrowth experiments (see Table 9.5).

CHAPTER 10

IN VITRO **ACTIVITY OF ANTIMICROBIAL COMBINATIONS AGAINST RESISTANT CLINICAL ISOLATES OF *MYCOBATERIUM TUBERCULOSIS***

10.1 BACKGROUND

The effectiveness of newly developed or alternative anti-tuberculosis (anti-TB) drugs against clinical isolates of *Mycobacterium tuberculosis* has become more important in the past few years predominantly due to the emergence of multidrug resistant TB (MDR-TB) (Friedman *et al*, 1993). Reasons to using combination therapy against TB or any disease have been discussed in the previous chapter. Since TB is always treated with two or more drugs and because of the emergence of resistant TB, *in vitro* testing of drug combination against resistant clinical isolates of *M. tuberculosis* is an important component of drug evaluation. Most *in vitro* drug combination investigations against mycobacteria have involved isolates of *M. avium* complex (Heifets *et al*, 1988a; Hoffner *et al*, 1989; Yajko *et al* 1988). Bergmann and Woods in 1998 investigated the *in vitro* activity of drug combinations against susceptible and resistant isolates of *M. tubeculosis*. Anti-TB agents tested in their study included the primary drug, isoniazid (INH) and alternative drugs such as the fluoroquinolone, sparfloxacin and the rifamycin, rifabutin. In the present study, the efficacy of two-drug combinations each containing a primary and an alternative drug, was investigated against resistant isolates of *M. tuberculosis.* Two-drug combinations consisting only of standard anti-TB drugs were also tested.

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The susceptibility patterns of two isolates tested in the present chapter were determined in Chapter 2. One was found to have a relatively low-level of resistance to INH (minimal inhibitory concentration (MIC) = 2.5 ug/ml) and the other isolate had a relatively low-level of resistance to rifampicin (RMP, MIC = 2.0 ug/ml). These concentrations are attainable in patients at least during peak levels and for varying periods. Both isolates were also found to be resistant to other standard anti-TB drugs (see Materials and methods).

The approach used for the assessment of drug combination activity against resistant clinical isolates of *M. tuberculosis* was the same as that used for the international H37Rv strain (Chapter 9). The following were exceptions :

(1) Concentrations used were selected based on levels of drugs achievable in tissues, and the previously determined MICs of the drugs were also taken into account.

(2) The exposure times used also depended on the drug combination. Bactericidal kinetics studies on single drugs (Chapter 4) showed that INH killing was more dependent on exposure time. Therefore, exposure times used for drug combinations consisting of INH were selected based on the time taken for this drug to achieve significant killing. The same principle was used for RMP-containing combinations.

10.2 OBJECTIVES

The objectives covered in this chapter were (1) to investigate possible synergistic effects of drug combinations consisting of INH or RMP (to which *M. tuberculosis* isolates showed low-level resistance) plus amikacin (AMK) or ofloxacin (OFL) against two *M. tuberculosis* isolates and (2) to determine whether the post-exposure radiometric model would predict the bactericidal activity of the drug combinations.

10.3 MATERIALS AND METHODS

Antimicrobial agents

Stock and working solutions of INH, RMP, ethambutol (EMB), streptomycin (SM), OFL and AMK were prepared as in the earlier chapters.

Test strains

Two *M. tuberculosis* clinical isolates (TB 0368/93 and MR 84452) resistant to at least one standard anti-TB drug were used. The MICs for these isolates were determined in Chapter 2 and are given in Table 10.1.

Procedure

The procedure was the same as that used for drug combination studies on *M. tuberculosis* H37Rv (Chapter 9). Concentrations used were 1 x MIC at 2.5 ug/ml (TB 0368/93) and ½ x MIC at 5 ug/ml (MR 84452) for INH, 1 x MIC for EMB (2.0 and 4.0 ug/ml) and RMP (8.0 and 2.0 ug/ml) and, 2 x MIC, for SM (16 and 8.0 ug/ml), OFL (1.0 ug/ml each) and AMK (0.5 ug/ml each) singly and in combination. Exposure times of 24 and 48 hours were used for TB

0368/93 and MR 84452, respectively.

Synergy in the bactericidal kinetics studies was defined as for *M. tuberculosis* H37Rv (Chapter 9), that is, as a 2 $\log_{10}$ CFU/ml or greater decrease of the drug combination as compared with the most active single drug. In addition, in order to describe possible trends, a 0.5 $\log_{10}$ CFU/ml increase or decrease criteria were used. Standard errors of the mean were derived from the pooled estimate as in the previous chapters (see Materials and methods in Chapter 4 and Chapter 5) since the number of observations were relatively few (one concentration and exposure time was used for each strain).

Table 10.1 MICs performed in 7H12 medium of *M. tuberculosis* H37Rv and two drug-resistant isolates   .

Drug[a]	MICs in *ug*/ml of *M. tuberculosis* strains		
	H37Rv	TB 0368/93	MR 84452
INH	0.05	*2.5*[b]	*10*[b]
RMP	0.5	*16*[b]	*2.0*[b]
EMB	2.0	0.5	*4.0*[b]
SM	1.0	*>8.0*[b]	*4.0*[b]
OFL	0.5	0.5	<0.5
AMK	0.25	<0.25	<0.25

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin.[b]MICs in italic indicate resistance.

The radiometric post-exposure regrowth findings were also analysed as in the earlier chapters. T_{400} (time to reach a growth index (GI) reading of 400) were calculated from the GI-derived growth curves by using linear interpolation, and $(T-C)_{400}$ indices (T_{400} readings for the drug or drug combination (T) cultures minus those of the control

(C) unexposed cultures) were calculated. Standard errors were derived from the pooled error estimate. Definitions of synergy were based on papers by Gudmundsson *et al* (1991) and Hoffner *et al* (1987).

10.4 RESULTS

10.4.1   *The conventional time-kill curve model*

Results of exposing cultures of *M. tuberculosis* TB 0368/93 and MR 84452 clinical isolates are set out in Table 10.2 and Table 10.3. Standard errors of the viable counts expressed in $\log_{10}$ colony forming units (CFU)/ml are given in Table 10.4. All the drug combinations tested against the two *M. tuberculosis* isolates showed an increase in CFUs/ml as compared to the most active single drug.

10.4.1.1   Synergy based on the 2 $\log_{10}$ CFU/ml definition

None of the combinations tested against the isolates meet with the definition of 2 $\log_{10}$ CFU/ml increase or decrease in killing as compared to the most active drug. Combinations tested against the isolates were indifferent since a less than 1 $\log_{10}$ CFU/ml in killing by the combination as compared with the most active single drug, was obtained.

10.4.1.2   Trends based on the 0.5 $\log_{10}$ CFU/ml criterion

Synergistic trends were defined as a 0.5 $\log_{10}$ CFU/ml or greater decrease in killing of the combination as compared with the most active drug alone. Such trends were not found in the combinations tested. Antagonistic trends were observed for a combinations of INH with OFL against TB 0368/93, an isolate with low-level resistance to INH. These trends comprised of a 0.5 $\log_{10}$ CFU/ml or

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greater increase of the drug combination compared with the most active drug alone (Table 10.2).

Other combinations tested in the present study against the two resistant isolates resulted in indifferent trends. An indifferent trend was a >-0.5 to 0.5 $\log_{10}$ CFU/ml of the combination versus the most active single drug.

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Table 10.2 Bactericidal activity of single drugs and drug combinations against low-level isoniazid (INH)-resistant *M. tuberculosis* TB 0368/93 after 48 hour exposure, expressed in colony forming units (CFUs)/ml.

Single drugs[a] (x MIC)	Mean decrease[b] in viable counts ($\log_{10}$ CFU/ml) (A)	Drug combinations	Mean decrease[b] in viable counts ($\log_{10}$ CFU/ml) (B)	Decrease with most active drug in combination ($\log_{10}$ CFU/ml) (C)	Activity index of drug combinations (B-C)	Activity of drug combinations (trends)[c]
INH_1	0.49					
RMP_1	0.156	$INH_1 + RMP_1$	0.286	0.49	-0.204	Ind
EMB_1	0.623	$INH_1 + EMB_1$	0.575	0.623	-0.048	Ind
SM_2	0.616	$INH_1 + SM_2$	0.34	0.616	-0.267	Ind
OFL_2	0.817	$INH_1 + OFL_2$	0.233	0.817	-0.584	Ant
AMK_1	0.982	$INH_1 + AMK_2$	0.521	0.982	-0.461	Ind
Control	-0.96					

[a]INH = isoniazid (1 MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1 MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC), subscript numbers denote multiples of the MICs. [b]The decrease in viable counts was calculated from the initial bacterial concentration (6.384 $\log_{10}$ colony forming units (CFU)/ml, standard error = 0.135), None of the combinations tested meet with the definition of synergy (>2 $\log_{10}$ CFU/ml vs the most active drug). [c]On arbitrary grounds, B-C $\geq$0.50 $\log_{10}$CFU/ml suggests a synergistic trend (Syn), B-C $\leq$-0.50 $\log_{10}$ CFU/ml an antagonistic trend (Ant), B-C > -0.50 to 0.50 $\log_{10}$CFU/ml an indifferent trend (Ind).

Table 10.3 Bactericidal activity of single drugs and drug combinations against low-level rifampicin (RMP)-resistant *M. tuberculosis* MR 84452 after 24 hour exposure, expressed in colony forming units (CFUs)/ml.

Single drugs[a] (x MIC)	Mean decrease[b] in viable counts ($\log_{10}$ CFU/ml) (A)	Drug combinations	Mean decrease[b] in viable counts ($\log_{10}$ CFU/ml) (B)	Decrease with most active drug in combination ($\log_{10}$ CFU/ml) (C)	Activity index of drug combinations (B-C)	Activity of combinations (trends)[c]
$INH_{1/2}$	0.838					
RMP_1	0.738	$RMP_1 + INH_{1/2}$	0.573	0.838	-0.265	Ind
EMB_1	0.616	$RMP_1 + EMB_1$	0.46	0.738	-0.278	Ind
SM_2	0.605	$RMP_1 + SM_2$	1.108	0.738	0.37	Ind
OFL_2	1.043	$RMP_1 + OFL_2$	1.279	1.043	0.236	Ind
AMK_2	1.052	$RMP_1 + AMK_2$	1.415	1.052	0.363	Ind
Control	-0.008					

[a]INH = isoniazid (½ MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1 MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC), subscript numbers denote multiples of the MICs. [b]The decrease in viable counts was calculated from the initial bacterial concentration (6.384 $\log_{10}$ colony forming units (CFU)/ml, standard error = 0.135). None of the combinations meet with the definition of synergy ($\geq$ 2 $\log_{10}$ CFU/ml vs the most active drug). [c]On arbitrary grounds, B-C $\geq$ 0.50 $\log_{10}$ CFU/ml suggests a synergistic trend (Syn), B-C $\leq$ - 0.5 $\log_{10}$ CFU/ml an antagonistic trend (Ant), B-C > -0.50 to 0.50 $\log_{10}$ CFU/ml an indifferent trend (Ind).

Table 10.4 Viable counts for *M. tuberculosis* clinical isolates after exposure to single drugs and drug combinations.

Drug/drug combination[a]	Viable counts in $\log_{10}$ CFU/ml			
	TB 0368/93[b]		MR 84452[b]	
	Mean	Standard error[c]	Mean	Standard error[c]
$INH_{1\,(\frac{1}{2})}{}^{d}$	5.965	0.135	5.546	0.386
RMP_1	6.228	0.135	5.647	0.386
EMB_1	5.762	0.135	5.768	0.386
SM_2	5.768	0.135	5.779	0.386
OFL_2	5.567	0.135	5.431	0.386
AMK_2	5.403	0.135	5.332	0.386
$INH_{1(\frac{1}{2})}{}^{c} + RMP_1$	6.116	0.135	5.811	0.386
$INH_1 (RMP_1)^{d} + EMB_1$	5.809	0.135	5.924	0.386
$INH_1 (RMP_1) + SM_2$	6.043	0.135	5.276	0.386
$INH_1 (RMP_1) + OFL_2$	6.151	0.135	5.105	0.386
$INH_1 (RMP_1) + AMK_2$	5.863	0.135	4.969	0.386
Control	6.48	0.135	6.392	0.386

[a]INH = isoniazid (1 MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1 MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC). [b]Exposure times of 48 and 24 hours were used for TB 0368/93 and MR 84452, respectively. [c]Standard errors were calculated from the pooled error estimate (See Materials and methods). [d]Subscript numbers denote drug concentrations at multiples of the MICs and INH subscript number in parentheses indicate INH concentrations used against *M. tuberculosis* MR 84452 isolate. [d](RMP_1) = RMP concentration used against MR 84452.

10.4.2 *The radiometric post-exposure regrowth model*

Radiometric regrowth findings of single drugs and drug combinations against the two *M. tuberculosis* isolates are graphically illustrated in Figure 10.1 and Figure 10.2. Tables 10.5 and 10.6 summarize the findings in terms of $(T-C)_{400}$ indices and standard errors of the T_{400} means are depicted in Table 10.7. Definitions of synergy were based on a paper by Gudmundsson *et al* (1991, see Chapter 9). No synergistic effects (a combination $(T-C)_{400}$ index of 2 days or greater compared with the most active drug) or antagonism (a combination $(T-C)_{400}$ index of 2 days or less as compared with the most active drug) were obtained for all the drug combinations tested against the two isolates. The effects were indifferent, that is a combination $(T-C)_{400}$ index being less than 1 day compared with the most active single drug. Examples of indifferent trends are illustrated in Figures 10.1 and 10.2.

Table 10.5 Evaluation of two-drug combinations against low-level isoniazid (INH)-resistant *M. tuberculosis* TB 0368/93 after 48 hour exposure in the radiometric regrowth model.

Drug[a] combination	S_1, S_2[b] (A)	$\sum T_{400} (t - c)$[c] (B)	$C\ T_{400} (t - c)$[d] (C)	Combination surplus/deficit[e] (D)	$T_{400} (C - S)$[f]	Outcome[g]
INH_1 + RMP_1	2.49, 0.6	3.09	1.29	-1.8	-1.2	Ind
INH_1 + EMB_1	2.49, 2.51	5	2.49	-2.51	-0.02	Ind
INH_1 + SM_2	2.49, 0.77	3.26	1.89	-1.37	-0.6	Ind
INH_1 + OFL_2	2.49, 0.44	2.93	1.55	-1.38	-0.83	Ind
INH_1 + AMK_2	2.49, 1.69	4.18	2.11	-2.07	-0.38	Ind
Control	2.539					

[a]INH = isoniazid (1 MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1 MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC), subscript numbers indicate multiples of the MICs. [b]S_1 and S_2 denote T_{400} indices (T_{400} for test drug, t minus that for the control, c) for the single drugs. [c]Sum of $(T-C)_{400}$ indices of individual drugs in each combination. [d]$(T-C)_{400}$ indices for drug combination. [e]Difference between columns (C) and (B). [f]$(T-C)_{400}$ indices for drug combination minus that of the most active single drug in combination, column (C) minus (A). [g]Outcome : the outcome is derived from columns (D) and (E) and is based on the calculation used by Gudmundsson *et al* (1991), Syn = synergy (D $\geq$ 2 days), Ant = antagonism (E $\leq$ -2 days), Add = additivity (D = -0.5 to 0.5 days), Ind = indifference (C < 1 day).

Table 10.6 Evaluation of two-drug combinations against low-level rifampicin (RMP)-resistant *M. tuberculosis* MR 84452 after 24 hour exposure in the radiometric regrowth model.

Drug[a] combination	S_1, S_2[b] (A)	$\sum T_{400} (t - c)$[c] (B)	$C\ T_{400} (t - c)$[d] (C)	Combination deficit[e]	$T_{400} (C - S)$[f]	Outcome[g]
$RMP_1 + INH_{\frac{1}{2}}$	1.49, 1.01	2.5	1.25	-1.25	-0.24	Ind
$RMP_1 + EMB_1$	1.49, 1.01	2.5	1.64	-0.86	0.15	Ind
$RMP_1 + SM_2$	1.49, 2.02	3.51	1.4	-2.11	-0.62	Ind
$RMP_1 + OFL_2$	1.49, 1.94	3.43	1.69	-1.74	-0.25	Ind
$RMP_1 + AMK_2$	1.49, 2.95	4.44	2.06	-2.38	-0.89	Ind
Control	4.481					

[a]INH = isoniazid (1 MIC), RMP = rifampicin (1 MIC), EMB = ethambutol (1 MIC), SM = streptomycin (2 MIC), OFL = ofloxacin (2 MIC), AMK = amikacin (2 MIC), subscript numbers indicate multiples of the MICs. [b]S_1 and S_2 denote $(T-C)_{400}$ indices (T_{400} for test drug, T minus that for the control, C) for the single drugs. [c]Sum of $(T-C)_{400}$ indices of individual drugs in each combination. [d]$(T-C)_{400}$ indices for drug combination. [e]Difference between columns (C) and (B). [f]$(T-C)_{400}$ indices for the drug combination minus that of the most active single drug in combination, column (C) minus (A). [g]Outcome : the outcome is derived from columns (D) and (E) and is based on the calculation used by Gudmundsson *et al* (1991), Syn = synergy (D $\geq$ 2 days), Ant = antagonism (E $\leq$ -2 days), Add = additivity (D = -0.5 to 0.5 days), Ind = indifference (C < 1 day).

Table 10.7 T_{400} readings in days for resistant *M. tuberculosis* isolates after exposure to single drugs and drug combinations.

Drug/drug combination[a]	T_{400} readings in days			
	TB 0386/93[b]		MR 84452[b]	
	Mean	Standard error[c]	Mean	Standard error[c]
$INH_{1\,(\frac{1}{2})}$[d]	5.034	0.406	5.486	0.408
RMP_1	3.139	0.406	5.978	0.408
EMB_1	5.054	0.406	5.492	0.408
SM_2	3.312	0.406	6.503	0.408
OFL_2	2.975	0.406	6.419	0.408
AMK_2	4.228	0.406	7.430	0.408
$INH_{1(\frac{1}{2})}$[d] + RMP_1	3.832	0.406	5.726	0.408
INH_1 (RMP_1)[e] + EMB_1	5.032	0.406	6.121	0.408
INH_1 (RMP_1) + SM_2	4.481	0.406	5.893	0.408
INH_1 (RMP_1) + OFL_2	4.086	0.406	6.180	0.408
INH_1 (RMP_1) + AMK_2	4.700	0.406	6.543	0.408
Control	2.539	0.406	4.481	0.408

[a] INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin, subscript numbers indicate drug or drug combination concentrations in multiples of the MICs. [b] Exposure times of 48 and 24 hours were used for TB 0368/93 and MR 84452, respectively. [c] Standard errors were derived from the pooled estimate (see Materials and methods). [d] $INH_{(\frac{1}{2})}$, the subscript in parentheses indicate the INH concentration used against the MR 85442 clinical isolate. [e] (RMP_1), RMP at 1 MIC used against MR 84452 strain.

(a)

(b)

(c)
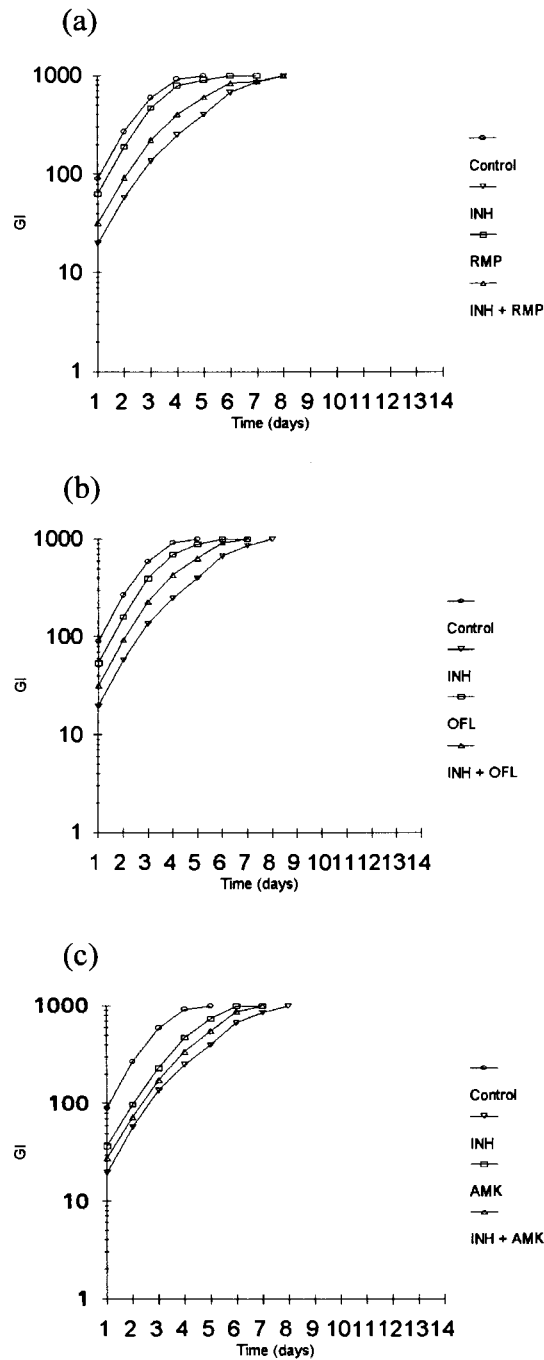
Figure 10.1 : Bactec radiometric regrowth curves for *M. tuberculosis* TB 0368/93 showing indifferent trends after 48 hour exposure to single drugs and drug combinations of (a) isoniazid (INH, 1 MIC) plus rifampicin (RMP, 1 MIC), (b) INH (1 MIC) plus ofloxacin (OFL, 2 MIC) and (c) INH (1 MIC) plus amikacin (AMK, 2 MIC). Indifference was a T_{400} index <1 day with combination vs most active single drug.

(a)

(b)

(c)
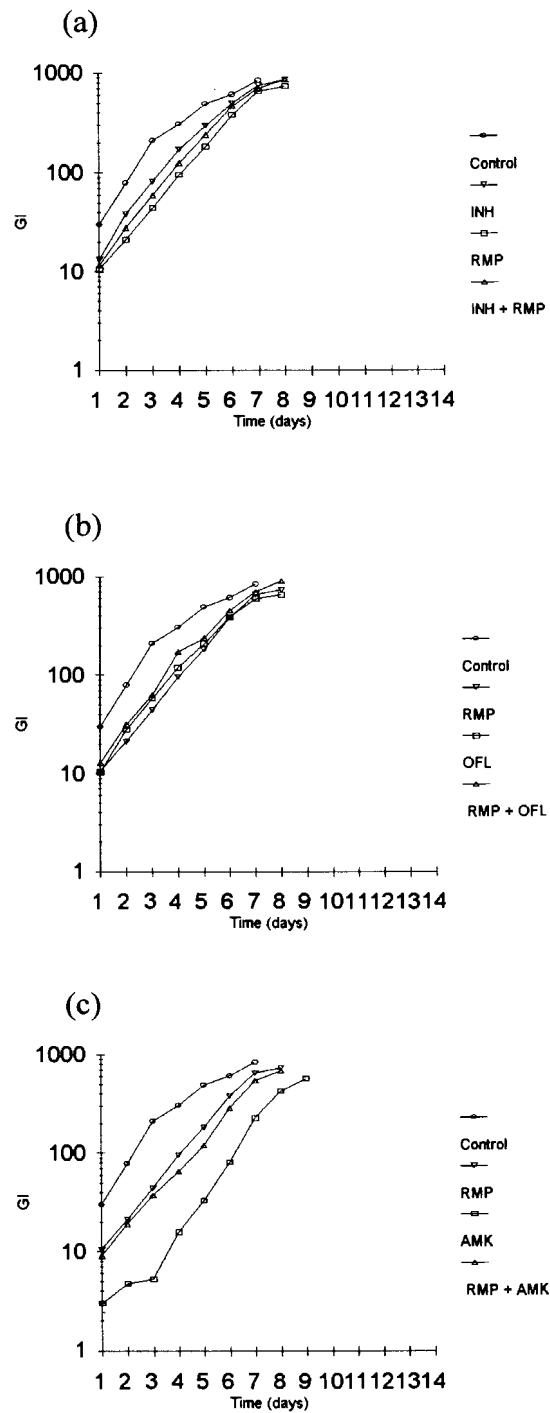
Figure 10.2 : Bactec radiometric regrowth curves for *M. tuberculosis* MR 84452 showing indifferent trends after 24 hour exposure to single drugs and drug combinations of (a) isoniazid (INH, ½ MIC) plus rifampicin (RMP, 1 MIC), (b) RMP (1 MIC) plus ofloxacin (OFL, 2 MIC), and (c) RMP (1 MIC) plus AMK (2 MIC). Indifference was T_{400} index < 1 day with combination vs most active single drug.

10.4.3   *Comparison between bactericidal and regrowth models*
in assessing drug interactions

An analysis of the activity of drug interactions involving different criteria for synergy and antagonism, and various degrees of interaction between these extremes (indifference and additivity) is set out in Table 10.8. In order to facilitate comparison between drug-interactive activities, a simple calculation in which points were assigned to different degrees of similarity was designed. The findings are displayed in Table 10.9. A score of 4 was assigned for full identity, 3 for one-degree discrepancy, and 2, 1 and zero for two- and three-degree discrepancy, respectively, and 0 for completely opposite findings.

The regrowth findings using the Gudmundsson *et al* (1991) criteria (89%) and Hoffner *et al* (1987) criteria (93%) correlated very well with the conventional 2 $\log_{10}$ decrease in CFU/ml definition. Comparison of bactericidal trends using 0.5 $\log_{10}$ decreases in CFU/ml criteria, with the regrowth findings using the two approaches, also resulted in good correlation, 85% and 93% with Gudmundsson *et al* (1991) and Hoffner *et al* (1987), respectively (see Table 10.9).

Table 10.8 Assessment of the activities of drug combinations against three *M. tuberculosis* strains using four different criteria.

Drug combinations	Drug interactions against *M. tuberculosis* strains														
	H37Rv					TB 0368/93					MR 84452				
	KK_1[a]	KK_2[a]	G[b]	H[c]	H index[d]	KK_1	KK_2	G	H	H index	KK_1	KK_2	G	H	H index
INH+RMP	Ind[e]	Ind	Syn[e]	Add+[c]	1.75	Ind	Ind	Ind	Add-[c]	0.52	Ind	Ind	Ind	Ind	0.84
INH+EMB	Ind	Ind	Ind	Ind	1.1	Ind	Ind	Ind	Ind	1.0	ND[f]	ND	ND	ND	ND
INH+SM	Ind	Ind	Add[e]	Add+	1.75	Ind	Ind	Ind	Ind	0.76	ND	ND	ND	ND	ND
INH+OFL	Ind	Ind	Ind	Ind	1.17	Ind	Ant[e]	Ind	Add-	0.62	ND	ND	ND	ND	ND
INH+AMK	Ind	Ind	Add	Ind	1.41	Ind	`Ind	Ind	Ind	0.85	ND	ND	ND	ND	ND
RMP+EMB	Ind	Ind	Syn	Add+	1.65	ND	ND	ND	ND	ND	Ind	Ind	Ind	Ind	1.10
RMP+SM	Ind	Ind	Add	Ind	1.33	ND	ND	ND	ND	ND	Ind	Ind	Ind	Ind	0.94
RMP+OFL	Ind	Ind	Ind	Ind	1.14	ND	ND	ND	ND	ND	Ind	Ind	Ind	Ind	1.13
RMP+AMK	Ind	Ind	Add	Ind	1.24	ND	ND	ND	ND	ND	Ind	Ind	Ind	Ind	1.38

[a]KK_1 and KK_2 denote kill kinetics with 2 $\log_{10}$ and 0.5 $\log_{10}$ differences in CFU/ml used to assess bactericidal interaction. [b]G = Gudmundsson *et al* (1991) approach to assess drug interactions in regrowth experiments. [c]H = Hoffner *et al* (1987) approach to assess drug interactions in regrowth experiments. [d]H index-drug interaction based on combination effect divided by effect of most active drug: >2.0 denotes synergy (Syn), 1.5 - 2.0 a positive additive effect(Add+), 0.5-0.75 a negative additive effect (Add-), 0.75-1.5 signifying indifference (Ind) and <0.5 antagonism (Ant). [e]Ind = indifference, Add = additive, Ant = antagonism, Syn = synergy. [f]ND = not done.

Table 10.9 Comparison between two methods of determining synergy in drug combinations against *M. tuberculosis* strains.

Bactericidal vs regrowth methods[a]	*M. tuberculosis* strains tested							
	H37Rv		TB 0368/93		MR85442		Overall	
	Maximum points[b]	Index[b]	Maximum points	Index	Maximum points	Index	Maximum points	Index
$2\log_{10}$ vs Gudmundsson *et al* (1991)[a]	36	0.78	20	1.0	20	1.0	76	0.89
$0.5\log_{10}$ vs Gudmundsson *et al* (1991)	36	0.78	20	0.85	20	1.0	76	0.85
$2\log_{10}$ vs Hoffner *et al* (1987)[a]	36	0.92	20	0.9	20	1.0	76	0.93
$0.5\log_{10}$ vs Hoffner *et al* (1987)	36	0.92	20	0.9	20	1.0	76	0.93

[a]Kill-kinetics expressed in terms of $\log_{10}$ decreases in colony forming units (CFU)/ml following exposure to single drugs and drug combinations and regrowth times to reach a growth index (GI) reading of 400 according to the interpretations based on Gudmundsson *et al* (1991) and Hoffner *et al* (1987), respectively. [b] Maximum points and index refer to a calculation according to which 4 points were assigned to full identity, 3 to one-degree discrepancy, 2 for a two-degree discrepancy and 1 for a three-degree discrepancy and 0 for a completely opposite finding. Degrees of drug interaction were synergy, positive additivity, indifference, negative additivity and antagonism. The scores were expressed as fractions of unity.

10.5 DISCUSSION AND CONCLUSIONS

10.5.1   *The conventional time-kill curve technique*

As found with drug combination studies on the fully susceptible *M. tuberculosis* H37Rv strain (Chapter 9), none of the combinations tested against the resistant clinical isolates meet with the definition of a 2 $\log_{10}$CFU/ml decrease or increase in killing of the drug combination compared with the most active single drug. Combination effects were indifferent since a less than 1 $\log_{10}$ difference in CFU/ml between the drug combination and the most active drug alone was found (Table 10.2 and Table 10.3).

With the exception of the INH plus OFL combination (against TB 0368/93) indifference was also found for all drug combinations tested when the 0.5 $\log_{10}$CFU/ml criterion was used to analyse the effects of drug combinations against the two resistant *M. tuberculosis* clinical isolates, TB 0368/93 and MR 84452. No synergistic trends were observed with the combinations studied.

There were no major differences relating to the drug combination activities between the strains when using the 0.5 $\log_{10}$ CFU/ml decrease or increase criteria (Table 10.8). The presence of uniformity observed with the combination activity patterns between strains including the fully susceptible international strain, suggests that the activity of drug combinations against *M. tuberculosis* strains can be predicted. However this warrants further investigations. TB 0368/93 with low-level resistance to INH was fully resistant to RMP and SM. MR 84452 with low-level resistance to RMP was moderately resistant to EMB and SM and fully resistant to INH. It is reassuring that despite the fact that synergistic trends were not demonstrated, all combinations showed at least some evidence of

killing. It should also be noted that the concentrations used were achievable in patients treated with conventional doses of the drugs.

10.5.2   *Radiometric post-exposure regrowth model*

Based on the approach employed by Gudmundsson *et al* (1991), no synergistic or antagonistic trends were observed. Indifference was suggested for all drug combinations tested against the two *M. tuberculosis* isolates using the radiometric post-exposure regrowth model. An alternative approach to analysing radiometric regrowth findings, based on Hoffner *et al* (1987) was discussed in detail in Chapter 9. As with the Gudmundsson *et al* (1991) approach, no major differences were found when the activity patterns of the drug combinations were compared between strains. In addition, neither synergy nor antagonism were obtained.

Comparisons of drug combination effects obtained using the four approaches against *M. tuberculosis* isolates are summarized in Table 10.8. There were no major discrepancies between the time-kill curve and the radiometric regrowth models. Although differences observed were not significant, results show that combination studies using a single technique cannot be relied upon to predict clinical efficacy of anti-TB drugs in combination. The use of the radiometric regrowth model together with the conventional colony counting method is therefore recommended for drug combination studies against *M. tuberculosis* isolates. It is also important to use standardized criteria of synergy that yield statistically reliable data. The *in vivo* implications of these *in vitro* data are unknown and warrant further investigation with a number of completely susceptible as well as resistant *M. tuberculosis* strains.

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CHAPTER 11

CONCLUSIONS

The *in vitro* bacteriostatic and bactericidal activities of the standard anti-tuberculosis (anti-TB) drugs are well known. The present studies confirmed previous findings on susceptibility testing using breakpoint as well as a wide range of concentrations (minimal inhibitory concentrations, MICs), and the minimal bactericidal concentrations (MBCs), by other investigators. *Mycobacterium tuberculosis* clinical isolates with different susceptibility patterns to the standard anti-TB drugs, isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and streptomycin (SM) were completely susceptible to the alternative drugs, ofloxacin (OFL) and amikacin (AMK). Bactec 7H12 determined MICs were lower than those obtained on 7H10 agar media.

INH, RMP, SM, OFL and AMK were bactericidal, with the standard drugs being more bactericidal than the alternative drugs. EMB on the other hand showed moderate activity. The technique for MBC determination is laborious and is therefore mainly suited for the testing of candidate anti-TB drugs or for research purposes and not for routine testing of wild strains from patients. MBC experiments described by Heifets (1991a) have the added advantage in that they can show the kinetics of killing of anti-TB drugs which can be illustrated graphically. The MIC and MBC experiments were also performed as preliminary studies to subsequent investigations.

The findings of the Bactec post-exposure radiometric model are in accordance with clinical efficacy. INH, RMP and EMB showed the most marked depression of regrowth while less impressive findings

were obtained with SM, OFL and AMK. Regrowth patterns in the radiometric model are related to both the bactericidal activity and the postantibiotic effects (PAEs) of the anti-TB drugs. The highly bactericidal drugs, INH and RMP, as shown by the conventional time-kill curve model, showed the highest $(T-C)_{400}$ indices. This in part, is due to the smaller number of surviving tubercle bacilli that needed more time to reach the target growth index (GI) of 400 compared with larger numbers of survivors following exposure to agents that kill fewer organisms. It is also possible that the presence of residual sub-MICs after exposure to the higher concentrations of the more potent drugs, INH and RMP, may contribute to the long delays in regrowth. Recovery from non-lethal reversible damage of tubercle bacilli may also contribute to the delays in regrowth.

The T_{400} model showed excellent discrimination between the standard activities of the drugs which compare well with those found previously by other authors. Linear trends between colony forming units (CFUs) and the radiometric regrowth times (T_{400}), were observed. The correlation became statistically significant when CFUs were related to $(T-C)_{400}$. Whether this correction with the control cultures has a biological basis is unclear. Conditions that may compromise the linear relationship in the Bactec regrowth model are PAE brought on by the short drug exposure (6h) and a carry over effect due to the subinhibitory concentrations in the Bactec vials ($\geq$8MICs). For the prediction of bactericidal activity, the model needs to accommodate the inclusion of subinhibitory concentrations which can be justified on scientific grounds, and the exclusion of the short exposure periods (6h).

Delays before effective regrowth commences are conventionally incorporated when times taken for drug-exposed cultures to regrow

to a defined GI reading are determined. Lag periods and effective growth rates and their relationship with CFU/ml in a mathematical logistic model were also examined separately. The model was valid for INH and RMP but good correlations were not found for EMB, SM, OFL and AMK. The validity of the model as well as the relative roles of the lag periods and regrowth rates in predicting bactericidal activity should be viewed with caution because of the variability inherent in the techniques used. Better organism dispersal and other measures to improve technical aspects of future studies may also result in a more reliable evaluation of the method.

The present study suggests that the use of the radiometric regrowth model for screening of candidate drugs for anti-TB activity may be rewarding. However, further studies using control antibiotics with little or modest activity against *M. tuberculosis* such as the macrolides or coamoxiclav are required before this screening proposal can be offered with sufficient confidence. Further modification of the model to include determination of PAEs using the 2h exposure period and achievable peak serum levels would be relatively easy to accomplish. The speed that this technique offers, its simplicity and safety add to its attractiveness as a screening procedure for candidate anti-TB drugs.

The use of the radiometric model for the study of drug combinations yielded findings that were difficult to interpret in relation to published data. Using CFU counts, all but one of the two-drug combinations studied resulted in indifferent interactions using the two criteria utilized to interpret synergy. The other drug combination showed indifferent and antagonistic trends depending on the criteria used to study synergy, and the isolate studied. This reinforces the need for the use of internationally standardized techniques which

would give statistically reliable data. The *in vivo* implications of these killing and regrowth *in vitro* data are unknown but warrant further investigation.

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APPENDIX 1

McFarland Turbidity Standard

The McFarland standard is a standard with which the turbidity of the bacterial suspension is compared to ensure a constant inoculum size.

0.1 ml of 1 % Barium chloride is added to 9.9 ml of 1 % Sulphuric acid to obtain a McFarland no. 1 turbidity standard.

APPENDIX 2

Table A.1 Bactec 7H12 and Middlebrook 7H10 breakpoint concentrations (ug/ml) for standard anti-tuberculosis drugs in the modified proportion method for *M. tuberculosis* susceptibility testing.

Drug	Bactec 7H12 broth	7H10 agar
INH	0.1	0.2
	0.4	1.0
RMP	2.0	1.0
EMB	2.5	5.0
	7.5	10
SM	2.0	2.0
	6.0	10

Adapted from Siddiqi (1988). Bactec TB system. Product and procedure manual.

Table A.2 Tentative interpretation of MICs determined radiometrically for *M. tuberculosis*.

Drug	MIC (ug/ml)			
	Susceptible	Moderately susceptible	Resistant	very resistant
Isoniazid	≤ 0.1	0.5	2.5	>2.5
Rifampicin	≤ 0.5	2.0	8.0	>8.0
Ethambutol	≤ 2.0	4.0	8.0	> 8.0
Streptomycin	≤ 2.0	4.0	8.0	>8.0
Ofloxacin	≤ 1.0	2.0	4.0	> 4.0
Amikacin	≤ 2.0	4.0	8.0	> 8.0

Adapted from Heifets (1991a).

APPENDIX 3

Table A.3 Means and pooled standard errors of the means (SEMs) of $(T-C)_{400}$ regrowth readings and decreases in $\log_{10}CFU/ml$ counts.

Drug[a]	MIC[b]	Exposure time (h)	$(T-C)_{400}$ $\pm SEM^c$ (Range)	Decrease in $\log_{10} CFU/ml^d$ $\pm SEM$ (Range)
INH	1	24	5.3 ± 1.2 (4.1-6.5)	0.8 ± 0.4 (0.4-1.2)
		48	6.5 ± 1.2 (5.3-7.7)	1.8 ± 0.4 (1.4-2.2)
		72	9.2 ± 1.2 (8.0-10.4)	2.3 ± 0.4 (1.9-2.7)
	3	24	$\geq 17.7 \pm 1.6$ (16.1-$\geq$19.3)	0.9 ± 0.4 (0.5-1.3)
		48	11.6 ± 1.6 (10-13.2)	1.7 ± 0.4 (1.3-2.1)
		72	$\geq 17.8 \pm 1.6$ (16.2-19.4)	2.5 ± 0.4 (2.1-2.9)
RMP	1	24	5.3 ± 0.9 (4.5-6.2	1.3 ± 0.3 (1.0-1.6)
		48	8.5 ± 0.9 (7.6-9.4	1.3 ± 0.3 (1.0-1.6)
		72	8.8 ± 0.9 (7.9-9.7)	1.8 ± 0.3 (1.5-2.1)
	3	24	$\geq 17.7 \pm 0.9$ (16.8-18.6)	2.0 ± 0.3 (1.7-2.3)
		48	$\geq 17.8 \pm 0.9$ (16.9-19.7)	1.7 ± 0.3 (1.4-2.0)
		72	$\geq 18.0 \pm 0.9$ (17.1-18.9)	2.4 ± 0.3 (2.1-2.7)
EMB	1	24	4.3 ± 0.8 (3.5-5.1)	0.5 ± 0.3 (0.2-0.8)

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Drug[a]	MIC[b]	Exposure time (h)	$(T-C)_{400}$ $\pm SEM[c]$ (Range)	Decrease in $\log_{10} CFU/ml[d]$ $\pm SEM$ (Range)
		48	4.8±0.8 (4.0-5.6)	0.6±0.3 (0.3-0.9)
		72	4.8±0.8 (4.0-5.6)	0.9±0.3 (0.6-1.2)
	2	24	4.4±0.9 (3.5-5.1)	0.6±0.3 (0.3-0.9)
		48	6.6±0.9 (5.7-7.5)	1.0±0.3 (0.7-1.3)
		72	6.1±0.9 (5.2-7.0)	1.1±0.3 (0.8-1.4)
SM	1	24	1.1±1.0 (0.1-2.1)	0±0.2 (0-0.2)
		48	0.2±1.0 (0-1.2)	0±0.2 (0-0.2)
		72	0.7±1.0 (0-1.7)	0±0.2 (0-0.2)
	3	24	1.3±1.0 (0.3-2.3)	-0.1±0.2 (0-0.1)
		48	1.6±1.0 (0.6-2.6)	-0.1±0.2 (0-0.1)
		72	1.0±1.0 (0-2.0)	-0.2±0.2 (0-0)
OFL	1	24	1.6±0.8 (0.8-2.4)	0.5±0.4 (0.1-0.9)
		48	2.1±0.8 (1.3-2.9)	0.5±0.3 (0.2-0.8)
		72	2.0±0.8 (1.2-2.8)	0.6±0.3 (0.3-0.9)
	2	24	2.3±0.8 (1.5-3.0)	0.7±0.4 (0.3-1.1)
		48	4.1±0.8 (3.3-4.9)	0.8±0.3 (0.5-1.1)

Drug[a]	MIC[b]	Exposure time (h)	$(T-C)_{400}$ $\pm SEM^c$ (Range)	Decrease in $\log_{10} CFU/ml^d$ $\pm SEM$ (Range)
		72	4.5 ± 5.3 (3.8-9.8)	1.0 ± 0.4 (0.6-1.4)
AMK	1	24	1.6 ± 0.9 (0.7-2.5)	0.5 ± 0.3 (0.2-0.8)
		48	0.6 ± 0.9 (0-1.5)	0.1 ± 0.3 (0-0.4)
		72	2.4 ± 0.9 (1.5-3.3)	0.3 ± 0.3 (0-0.6)
	3	24	2.8 ± 0.9 (1.9-3.7)	0.5 ± 0.3 (0.2-0.8)
		48	1.6 ± 0.9 (0.7-2.5)	0.5 ± 0.3 (0.2-0.8
		72	3.2 ± 0.9 (2.3-4.1)	0.5 ± 0.3 (0.2-0.8)

[a]INH = isoniazid, RMP = rifampicin, EMB = ethambutol, SM = streptomycin, OFL = ofloxacin, AMK = amikacin. [b]MIC = minimal inhibitory concentration. [c]SEM = standard error of the mean [d]CFU = colony forming units
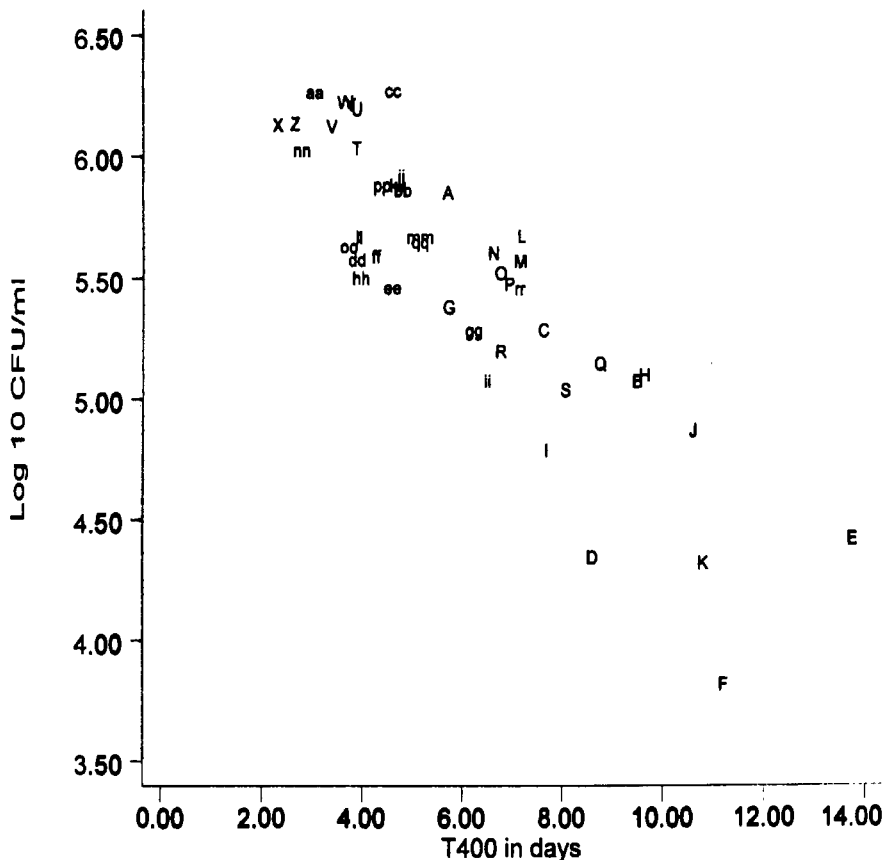
APPENDIX 4

Figure A.1: Relationship between bacterial survivors in $\log_{10}$CFU/ml versus T_{400} regrowth times in days following exposure to isoniazid (INH), rifampicin (RMP), ethambutol (EMB), streptomycin (SM), ofloxacin (OFL) and amikacin (AMK). A-F shows exposure to INH at 1MIC, 6h; 3MIC, 6h; 3 MIC, 24h; 1 MIC, 48h; 3 MIC, 48h; 1 MIC, 72h; respectively. G-K denote exposure to RMP at 1 MIC, 6h; 3 MIC, 6h; 1 MIC, 24h; 1 MIC, 48h; 1 MIC, 72h, respectively. L-S show exposure to EMB at 1 MIC, 6h, 2MIC, 6h; 1 MIC, 24h; 2 MIC, 24h; 1 MIC, 48h; 2 MIC, 48h; 1 MIC, 72h; 2 MIC, 72h, respectively. T-Z and aa show exposure to SM at 1 MIC, 6H; 3 MIC, 6h; 1 MIC, 24h; 3 MIC, 24h; 1 MIC, 48h; 3 MIC, 48h;1 MIC, 72h, 3 MIC, 72h, respectively. Letters bb-ii show exposure to OFL at 1MIC, 6h; 2 MIC, 6h; 1 MIC, 24h; 2 MIC, 24h; 1 MIC, 48h; 2 MIC, 48h; 1 MIC, 72h, 2 MIC, 72h, respectively. Amikacin exposures are shown by jj-rr at 1 MIC, 6h; 3 MIC, 6h; 1 MIC, 24h; 3 MIC, 24h; 1 MIC, 48h; 3 MIC, 48h; 1 MIC, 72h, 3 MIC, 72h; 8 MIC, 72h, respectively.
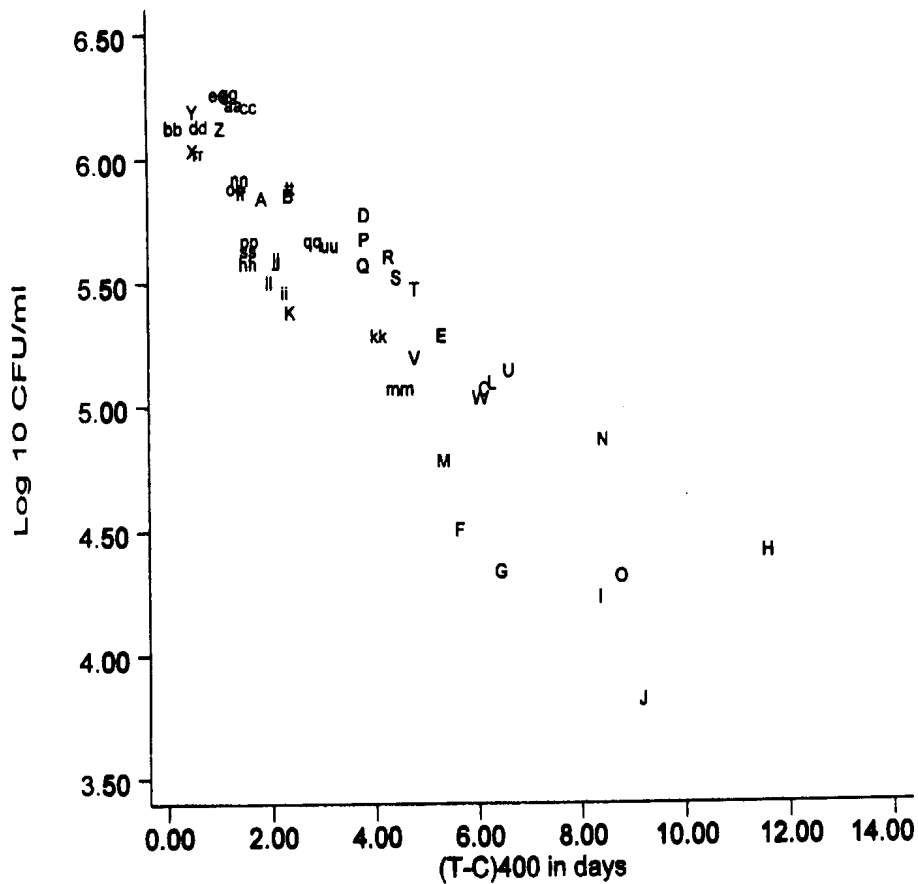
Figure A.2: Relationship between bacterial survivors in $\log_{10}$CFU/ml versus $(T\text{-}C)_{400}$ indices in days following exposure to isoniazid (INH), rifampicin (RMP), ethambutol (EMB), streptomycin (SM), ofloxacin (OFL) and amikacin (AMK). A-J shows exposure to INH at ½ MIC, 6h; 1MIC, 6h; 3 MIC, 6h; ½ MIC, 24h; 1 MIC, 24h; ½ MIC, 48h;1MIC, 48h; 3MIC, 48h; ½MIC, 72h; 1MIC, 72h, respectively. K-O denote exposure to RMP at 1 MIC, 6h; 3 MIC, 6h; 1 MIC, 24h; 1 MIC, 48h; 1 MIC, 72h. P-W show exposure to EMB at 1 MIC, 6h, 2MIC, 6h; 1 MIC, 24h; 2 MIC, 24h; 1 MIC, 48h; 2 MIC, 48h; 1 MIC, 72h; 2 MIC, 72h, respectively. X-Z and aa-ee show exposure to streptomycin at 1 MIC, 6h; 3 MIC, 6h; 1 MIC, 24h; 3 MIC, 24h; 1 MIC, 48h; 3 MIC, 48h;1 MIC, 72h, 3 MIC, 72h, respectively. Letters ff-mm show exposure to OFL at 1MIC, 6h; 2 MIC, 6h; 1 MIC, 24h; 2 MIC, 24h; 1 MIC, 48h; 2 MIC, 48h; 1 MIC, 72h, 2 MIC, 72h, respectively. Amikacin exposures are shown by nn-uu at 1 MIC, 6h; 3 MIC, 6h; 1 MIC, 24h; 3 MIC, 24h; 1 MIC, 48h; 3 MIC, 48h; 1 MIC, 72h, 3 MIC, 72h, 8 MIC, 72h, respectively.