Issues in Infectious Diseases
Editors: H. Zeichhardt, B. Mahy
Vol. 2

Mycobacteria and TB

Editors
S.H.E. Kaufmann
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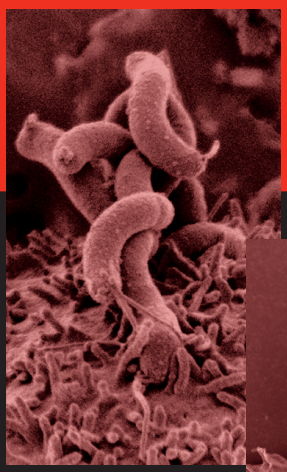
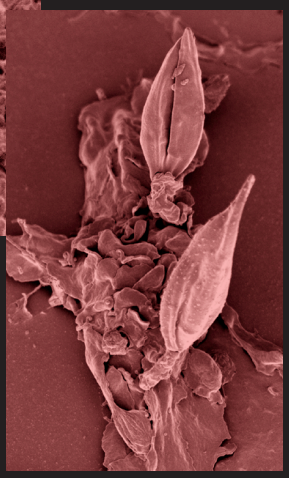
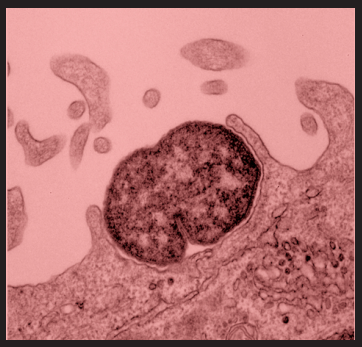
Mycobacteria and TB







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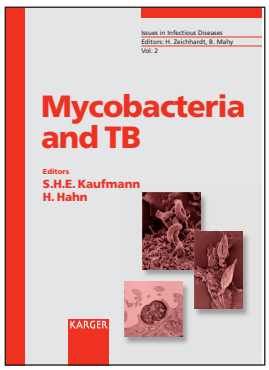

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Tuberculosis remains one of the main fatal infections in humans. With annual morbidity and mortality rates worldwide of 8 and 2 million cases respectively, the disease is far from being eradicated. In fact, the dangerous liaison between TB and HIV, and the increasing incidences of multi-drug resistant strains of *Mycobacterium tuberculosis* are aggravating the problem. The latest epidemiological data indicate that new drugs and a novel vaccine are urgently needed to control TB adequately.

This volume summarizes the state of the art in the prevention, diagnosis and therapy of TB. In addition, the molecular biology of *M. tuberculosis* and the immunology of the host response are presented. Researchers are beginning to understand how the immune response controls the pathogen quite efficiently, yet fails to eradicate it completely in the 2 billion people worldwide who are infected but do not develop the disease. Finally, recent strategies towards the development of new vaccines are reviewed.

Scientists investigating the epidemiology, immunology and molecular biology of TB or engaged in vaccine and drug development as well as physicians and social workers treating TB patients will benefit from this timely overview.

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Mycobacteria and TB

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This volume appears at the time when the International Course

The Immune System in the Protection

and

Susceptibility to Tuberculosis

is being held at the Scuola Superiore d'Immunologia Ruggero Ceppellini in Napoli/Italy, marking its 10th year of activity.

Since most of the authors of this volume, including the editors, are involved in this course, we are proud to recognize the activities of the School and its never tiring director, Prof. Serafino Zappacosta.

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Foreword

'Issues in Infectious Diseases', the new title of a series that started as 'Infectiology', aims to cover the most recent developments in infectious diseases from modern molecular biology, immunology, clinical sciences, vaccination, drug development, epidemiology to diagnostics. The two new series editors of 'Issues in Infectious Diseases' have set themselves the aim for the 'Issues' to cover the whole spectrum of infectious agents in state-of-the-art overviews of current interest.

The present issue focuses on mycobacteria and tuberculosis, and provides an impressive overview of the multiple facets of an infectious disease that remains one of the most serious and challenging infectious disease problems of the present day.

Heinz Zeichhardt, Free University of Berlin
Brian W.J. Mahy, Centers for Disease Control, Atlanta

Preface

One hundred and twenty years ago, on March 24, 1882, Robert Koch described his discovery of *Mycobacterium tuberculosis* that killed over 40% of the working class in Germany. Similar figures were recorded for all major cities in Europe and the USA. Of course, this is different nowadays, and many Western European countries consider tuberculosis a minor problem. However, the annual morbidity and mortality rates worldwide are in the order of 8 and 2 million cases, respectively, demonstrating that this disease is far from eradicated. In fact, the dangerous liaison between tuberculosis and HIV, the causative agent of AIDS, and the increasing incidences of multi-drug resistant (MDR) strains of *M. tuberculosis* in several parts of the world are worsening the problem. To date, 50 million people are already co-infected with HIV and *M. tuberculosis*, the risk of developing active disease in these individuals being dramatically higher. A similar number of people are infected with MDR strains of tuberculosis, and in certain countries over 10% of all tuberculosis cases are caused by MDR strains and therefore cannot be treated adequately.

Clearly, we do have drugs at hand to treat tuberculosis. However, the prolonged treatment time of up to 6 months with a combination of three different drugs renders chemotherapy unfeasible in developing countries and frequently results in the failure of patient compliance worldwide, which in turn fosters the development of MDR strains. Aside from the cost in lives, this causes the cost of treatment to increase by 100-fold, rendering the treatment of MDR strains in developing countries infeasible. As far as BCG vaccination is concerned, it protects against tuberculosis in newborns and young children only, but not

against the most prevalent form of the disease, pulmonary tuberculosis in adults.

It is generally accepted that novel chemotherapeutic regimens as well as a novel vaccine are urgently required in order to control tuberculosis adequately, unless we accept a death toll of over 20 million in the first decade of this century.

Novel therapeutic and preventive measures will be best developed on the basis of our increasing knowledge about the molecular biology of the pathogen and the immunology of the host response. We now have a better understanding of the survival strategies of the pathogen as well as its unique Achilles' heel. Similarly, we are now beginning to understand how the immune response controls the pathogen quite efficiently but fails to eradicate it completely in the 2 billion people worldwide who are infected but do not develop the disease. It is the aim of this book to provide the reader with the latest scientific information about the two opponents in the trench warfare that takes place during *M. tuberculosis* infection.

We would like to cordially thank our colleagues who, by contributing to this volume, have generously shared their knowledge. We are also grateful to Lucia Lom-Terborg for her excellent help in editing this book.

Stefan H.E. Kaufmann
Helmut Hahn
Berlin

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Tuberculosis as a Global Public Health Problem

Donald A. Enarson

International Union Against Tuberculosis and Lung Disease, Paris, France

Although great advances have been made in the control of tuberculosis (TB), deaths from this disease are still the equivalent to the crash of a Boeing 747 aircraft every hour of every day [1]. One third of all humans are thought to be infected with the causative organism, *Mycobacterium tuberculosis*. TB kills more women than any cause of maternal mortality. The total cost of implementing the global control strategy is equivalent to building one large hospital each year in an industrialized country. TB control is the most cost-effective development assistance for health, and yet, only 0.2% of investment in health in poor countries has been spent on TB services in recent years. The neglect of TB is a scandal that has gone on for decades and is only now beginning to be addressed.

Transmission of TB from one person to another takes place from an infectious case (the smear-positive pulmonary TB case is the most potent source) to a susceptible, uninfected person. Cases of TB arise either from this large pool of infected persons or from persons with previous disease which had become inactive prior to becoming reactivated. Once involved with this cycle of infection and disease, the only escape leading to no further disease is by death, by modern specific chemotherapy of active disease or by preventive therapy in the absence of current disease. Even then, an individual may become reinfected after having been cured of previous infection or disease. Although cure of infection or of disease reduces the probability of disease to a very low level, it never eliminates the possibility entirely.

Infection with Tuberculosis

To become infected, a susceptible individual must come into contact (usually prolonged and close contact) with the causative organism expelled from an

infectious case. The likelihood of an uninfected individual having such contact varies considerably depending upon the social and cultural characteristics of the society in which the individual lives. Studies of contacts over the past several decades in Canada have shown that the number of contacts per case of infectious TB appears, in general, to be declining. In the mid-1970s the number of contacts per case in the general population was 7; at present there are, on average, only 3 contacts per case.

The likelihood of infection with TB, given contact with an infectious case, is largely a function of the duration and intensity of the contact. Thus, individuals living in the same household with an infectious case have the greatest risk of becoming infected. The likelihood of infection from casual contact is substantially less. Given contact with an infectious case, approximately 1 out of 6 persons will become infected [2]. This means that 5 out of 6 will not; it is much more likely that a contact will remain uninfected than that such a person will become infected after contact with a single infectious case. Repeated contact undoubtedly increases this risk. On the other hand, contact with a single bacillus is sufficient to cause infection, however small that probability is.

Development of Clinical Tuberculosis

Once infected, the likelihood of developing disease is not different whether one has been infected through casual or close contact, or been infected by a smear-positive or smear-negative source. Some studies have suggested that individuals infected by smear-positive source cases are more likely to develop disease. This conclusion is probably incorrect, resulting from the determination of the proportion of infected contacts arising from remote infection based upon population surveys of tuberculin sensitivity which likely underestimates the prevalence of remote infection among the subset of the population having contact with a case of TB (and particularly that group whose contact is with smear-negative, culture-positive cases). The likelihood of developing disease is greatest immediately following infection and declines exponentially from that point [3]. The incidence of clinically significant disease within the first year after infection is approximately 1.5%. Within the first 5 years after infection (during the steep slope of the decline) the cumulative risk of disease is between 5 and 10%; the remainder of the lifetime risk (under stable social and immunologic conditions) is probably around 5%. Thus the total lifetime cumulative risk of developing disease after becoming infected by contact with an infectious case is perhaps 15% (about 1 in 6 persons). The corollary is that 5 out of 6 persons who have become infected do not develop disease at any time in their lives and that the likelihood of not developing disease is much higher than the

likelihood of developing disease at any time during the infected individual's life. These estimates are valid where the likelihood of subsequent contact with another infectious case is low. Where repeated contacts with infectious cases are likely, the probability of developing disease may be substantially higher. The overall probability of developing disease, given contact, then is a multiple of 1 in 6 × 1 in 6, which is 1 in 36.

The Social Impact of Tuberculosis

TB is a disease of young people. This is due to the fact that the greatest predictor of disease due to TB is the amount of time that has passed since infection with TB, this risk being greatest within 5 years of first becoming infected with TB. Even in countries where TB is uncommon and most disease arises from remote infection, most individuals who will be infected become infected by the age of 20 years [4]. The association of TB with youth makes this disease an important factor in the economy of many countries, which simultaneously experience a low gross national product and a high incidence of TB, because the disease affects the most economically productive sector of the economy. As a result, an organized TB control programme, unlike many other health-related activities, is a net contributor to economic development [5].

Measuring Tuberculosis in the Community

Mortality Rate

Where TB is common and specific treatment is not available, the mortality rate is a good indicator of the size of the TB problem in a community. This is the measure previously used from the time that detailed information on TB began to be available in Great Britain in the 1840s until the early 1940s when specific chemotherapy for TB was introduced. From these data, the profile of TB as a disease of young people was noted [6–8]. In Canada the rate of decline prior to the introduction of chemotherapy was steady at approximately 3% per annum. After 1945, the rate plummeted and then began to stabilize again around 1960. The rapid fall in mortality rate occurred at exactly the same time in the countries of Western Europe and North America and coincided with the introduction of streptomycin in 1946. The major portion of the decline in mortality rate was over by the latter year, even though adequate treatment for TB, preventing subsequent relapse, was only just introduced in that year and not yet fully utilized.

Prevalence of Infection with M. tuberculosis

A second measure of TB in a community was introduced in the early part of the 20th century with the development of the tuberculin skin test. Initially introduced as a form of therapy, this test provides the possibility of studying infection with TB independent of clinical disease. Much of the earliest epidemiologic use of this test was undertaken in Scandinavia in studies of student nurses who, in the course of their work, were exposed to infectious cases of TB. Heimbeck [9], in Norway, found that many young women entering training were uninfected but became infected during the course of their training. The likelihood of developing clinical TB was highest among those young women who were uninfected at the time they entered their training. The risk of becoming infected and subsequently experiencing the very high rate of disease immediately following infection explained why they were so much more likely to get the disease than their colleagues who had previously been infected.

Incidence (Risk) of Infection with M. tuberculosis

Since these early studies, the tuberculin test has been used to determine the prevalence of infection in samples of the general population of many countries. The skin testing of new recruits to the military service of the Netherlands (a country which has never had a national policy of BCG vaccination) provided the data from which the epidemiologic concept of annual risk of TB infection was developed [10], although the concept was first described in the United States 30 years previously. This estimate is derived from calculating the proability of becoming infected within a given year, either estimated from the age-specific prevalence of tuberculin skin sensitivity or directly measured by repeating the test in the same population at several points in time and calculating the rate of development (incidence) of tuberculin skin sensitivity (indicating infection with TB). The mathematical principle of the calculation of the annual risk of infection (ARI) is similar to the calculation of compound interest on a bank deposit and is based on an algebraic formula $ARI = 1 - (1 - p)^{1/a}$, where 'p' is the prevalence of infection and 'a' is the average age of the group tested. This has been a very useful measure to determine and monitor the epidemiologic situation with regard to TB infection and provides a reliable estimate of the incidence of disease within the community.

The average ARI estimated from a single prevalence survey is a relatively insensitive measure of short-term changes in the trend of TB. This is due primarily to the fact that it is an *average* estimate over the whole lifespan of the individual tested. Thus, in children tested at the age of 10 years, the estimate is the average of the lifetime experience centred on a point 5 years previously. This is why, for example, no effect was observed on the trend in the average ARI in Europe during the period of the world wars even though the mortality

rate sharply increased. An average experience over 18 years (the age of the tested army recruits) could not detect a change over 5 years (the period of the wars).

Prevalence of Active TB in a Community

Surveys of representative samples of the general population of some countries have been conducted to determine the number of cases of active TB within the community at a given point in time. In some countries, the prevalence surveys have been repeated periodically. Under stable conditions and in the absence of human intervention, the prevalence rate is a reasonable indicator of the size of the TB problem in a community. It, however, implies a stable pattern of transition from infection to disease and of disease to either death or spontaneous cure. It gives a very good indication of the 'vector of transmission' in that it identifies all infectious sources (the smear-positive cases) at a point in time. Under stable conditions, the ratio of prevalence to incidence in a community is 2:1 (that is, the average duration of a case is 2 years).

The introduction of specific chemotherapy of TB has resulted in a disruption of the ratio of prevalence to incidence, by shortening the duration of cases. A well-treated patient will remain a case for an average of 3–4 months (2 months prior to diagnosis and 1–2 months after the commencement of treatment). Alternatively, if poor treatment is given, and the patient survives but does not become cured, the duration of a case may actually be increased. Since the introduction of chemotherapy for TB, the prevalence rate has become an indicator of the quality of the TB control programme within a community.

Annual Notification Rate of Active TB

In countries where notification of TB cases is quite complete, the incidence of active TB can be approximated, based on the notification, and has been used to monitor the epidemiologic situation. This measure requires the supervision and review of all notifications by TB experts to ensure the validity and completeness of the registration of cases. Where this expertise is not part of the procedure, the information is much less useful for epidemiological monitoring and must be used only with caution. The incidence of all active cases of TB is approximately twice that of smear-positive pulmonary cases. An ARI of 1% is approximately equal to an annual incidence of active cases of 100/100,000 [11].

Benchmarks in TB

For purposes of discussion, various 'benchmarks' of TB may be identified as follows (in rates per 100,000 per year) as shown in table 1 [12].

Table 1. Benchmarks in the epidemiology of TB (in thousands)

1,000	Above this rate, TB can be said to be 'epidemic'
100	Above this rate, groups can be defined as at 'high risk' for TB
10	Below this rate, groups can be defined as at 'low risk' for TB
1	Below this rate, TB programmes are entering the elimination phase
0.1	At this level, TB can be said to be eliminated

Estimates of the Current Size and Future Trend of the Problem

TB continues to be a very large problem throughout the world. Estimates of the size of the problem have been made and indicate that, in the early 1990s, there were thought to be as many as 16 million cases of TB in the world with 8 million new cases per year (one half of which are infectious cases) and 3 million deaths each year due to the disease [13]. It is further estimated that as many as 1 billion (1,000 million) persons may be infected with TB.

Composite Estimates

The STOP TB initiative has prepared best estimates of the number of cases of TB for the year 1997 [14] for each of the countries of the world and has identified 22 countries that, together, contain an estimated 6.4 million patients, more than 80% of all estimated TB patients in the world [15]. These countries include: India 1.8 million cases, China 1.4 million cases, Indonesia 583,000 cases, Bangladesh, 300,000, Pakistan, 261,000, Nigeria 253,000, Philippines, 219,000, South Africa, 170,000, Russia, 157,000, Ethiopia, 156,000, Vietnam 145,000, Democratic Republic (DR) Congo, 129,000, Brazil, 122,000, United Republic of Tanzania 97,000, Kenya 84,000, Thailand 84,000, Myanmar 80,000, Afghanistan 74,000, Uganda 66,000, Peru 65,000, Zimbabwe 63,000 and Cambodia 57,000 (fig. 1).

In relation to regions (according to WHO designation), the largest number of cases occurs in the South-East Asia region with 2.8 million cases (45%), Western Pacific region with 2.9 million (29%), Africa region 1 million (16%), Eastern Mediterranean 0.3 million (5%), American region 0.19 million (2.9%) and Europe region 0.16 million (2.6%). This distribution can be expected to change over the coming years (fig. 1) due to varying demographic patterns, economic development patterns and widely varying impact of the human immunodeficiency virus (HIV) epidemic. Even with control measures applied, it is quite possible that the number of cases may actually increase as much as 2–3 times over the next several decades. This effect will be greatest in the Africa region due to the immense impact of the HIV epidemic. Even with emerging

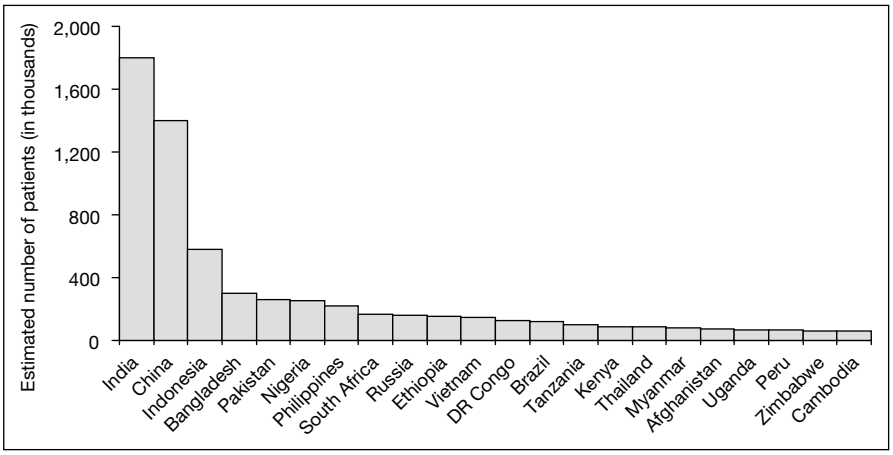

Fig. 1. Estimated number of TB patients in 1997 in the 22 countries with the largest number of patients [adapted from 15].

possibilities of stemming this epidemic, it is likely that the impact of the massive increases in cases already seen will continued to be in evidence for years to come. Natural population growth associated with slowing of economic development and some impact of HIV will be associated with a continuing rise in number of cases in the South-East Asia region. To a lesser degree, lateness in implementing control measures associated with socioeconomic dislocation will be associated with a rise in cases in the Eastern Mediterranean region (primarily in the subcontinent).

The Course of Tuberculosis in Human Populations

A conceptual model of the course of TB in a human population has been proposed [16]. This model indicates that, after introduction of the disease to a group previously without experience of the disease, the incidence of active TB in the population rapidly rises to reach a peak of incidence in the population. This reflects the high rate of clinical disease after primary infection of the large proportion of the population that is uninfected. As an increasing proportion of the community becomes infected and their risk of developing disease declines with time, the incidence of disease declines from the high peak and may stabilize or continue a steady decline, as has been observed in numerous countries [17].

The rise in rates immediately following the introduction of TB into the community, hypothesized by this model, has rarely been observed in human

populations. Among Aboriginal groups of western Canada [18], immediately following the institution of the reservation system, mortality rose from 1% per annum in 1881 to 9% per annum in 1886 and only slowly declined again to 1% per annum by 1900. Studies of Sudanese recruits to the Egyptian army in the early 1900s [19] illustrated a similar phenomenon. Young men, who had come from a low prevalence area and who had previously been uninfected, upon moving into a high prevalence area, were exposed to infectious cases, rapidly developed fulminant disease and died.

Variation of Epidemiological Characteristics at Different Levels of TB

The clinical and epidemiologic features of TB vary at different stages of the course of TB in the population. When TB is very common, most cases result from recent infection and the disease is most common in young people (especially women). Rates are very high (>100/100,000 per annum) and high-risk groups are absent, the whole population being at a high risk of disease. When TB is uncommon in the community (such as at the present time in Canada), most cases are the result of remote infection and the disease affects predominantly old men. TB cases arise from high-risk groups whose risk is determined mainly by the likelihood of having been infected in the past.

TB at High Levels of Prevalence

The Inuit of North America have been historically identified as a group with very high rates of TB [20, 21], prior to the introduction of the epidemic of HIV infection. In the Northwest Territories of Canada, where the largest group of Inuit reside, information regarding TB status was retained on more than 80% of the entire population. This system was in place from 1962 until 1985. In the early 1960s the incidence of active TB in the entire community was between 1 and 2% per annum. The incidence was highest among young people (particularly women) as is usual in such situations, reflecting a high rate of transmission of TB with most disease due to recent infection. This picture had changed dramatically by the 1980s when highest rates were seen in old men. During this period, in addition to active case finding and case holding until cure, there was an aggressive programme of preventive therapy that was most active between 1968 and 1972. Preventive treatment, which often consisted of 12–18 months of fully supervised, two-drug therapy, was given to eligible individuals. By 1984, 43% of those with previous TB (fibrotic lesions) and 34% of those without evidence of previous disease, but who had been infected with TB (never having received BCG) had been treated.

In 1970–1972, the greatest number of cases and highest incidence rate of active TB was in the group with previous TB. Lower rates were seen in those who were previously uninfected and in those who had been vaccinated. By 1980–1982,

incidence rates in all groups had fallen but the decline was most dramatic in the group with previous disease. Within this group, only a small number of cases was observed and such cases as were seen occurred only among those who had not been given preventive chemotherapy. The decline in incidence of active TB in the Inuit of the Northwest Territories is the most rapid decline in incidence of TB ever documented in a human population (the highest rate of decline occurred between 1968 and 1972 when it exceeded 20% per annum). The transition of the Inuit of the Northwest Territories from a high-risk to a low-risk group has been accompanied by a change in the epidemiologic profile of TB in the group.

TB in Low-Risk Populations

Even when TB is very uncommon, the probability of becoming infected among those who will do so is highest in those under age 20. In such locations, if the incidence of active TB is determined only in the subgroup of the population, which has been infected with TB, and displayed by age and sex, the familiar peak in young people, higher in women is again observed [22]. When TB incidence declines, high-risk groups emerge in the community. In Canada in the 1970s and 1980s, these groups were identified and studied in detail [23]. The segments of the general population that experienced rates of active TB at least 10 times higher than the national average were considered 'high-risk' groups for purposes of this investigation (the rates exceeded 100/100,000 per year in each of the groups). The relative risk, as compared with the general population, was 13 in the aboriginal groups in the mid-1970s. Other high-risk groups were: contacts of active cases, relative risk 62; previous cases (fibrotic lesions), 38; silicosis cases, 39; residents of urban slums, 20; Asian-born Canadians, 15. Since 1982, a new high-risk group has emerged with the highest relative risk for TB ever recorded, namely, patients with the acquired immunodeficiency syndrome (AIDS) with a relative risk estimated in Vancouver, British Columbia, to be >500.

All high-risk groups together, in the 1970s and 1980s in Canada, accounted for 80% of all the cases diagnosed at that time: 33% occurred in Canadians born in Asia; 17% in individuals who had previously had TB; 12% in aboriginal Canadians; 8% in contacts of active cases; 7% in residents of urban slums and 3% in patients with AIDS.

The Urban Poor

In a study of notifications of active TB in the province of British Columbia, from 1970 to 1985 [24], we found a much higher rate in the urban area. Over the period of study, the discrepancy in rates widened. The annual rate of notification of active TB between 1980 and 1982 varied across the sections of the city from the lowest socioeconomic level (with a notification rate of 242) to the

highest level (with a notification rate of 2). This association between notification rate and income was observed in other cities as well. Among the socioeconomic characteristics, we found that unemployment was the single most important predictor of level of notification rate in the city. Moreover, in comparing the notification rate of TB in the low-income census tracts with similar reports in poor areas of Buffalo, New York, reported before the introduction of adequate chemotherapy for TB [25], we found that the rates were quite similar for the two cities. These findings suggested to us that the socioeconomic conditions of the area led to an increase in current transmission of TB, a finding subsequently confirmed in US cities [26].

Immigrants

Immigrants now account for a high proportion of all cases in countries of Western Europe and, in many countries of Northern Europe, the majority [27]. In the early 1970s [28] we showed that the notification rate of active TB among foreign-born persons corresponded very closely to the rate among their countrymen who had not emigrated, for every country for which we had information. Indeed, we showed that the rates were very similar among comparable birth cohorts according to place of birth [29], in spite of decades of residence in Canada. Similar observations have been made in Australia where the probability of tuberculous infection in children was related to location of birth of the child and not of the parents [30]. In other locations, where there is regular travel between the country of immigration and the country of origin [31], disease rates were shown to be associated with visits to the country of origin. When notification rates among foreign-born persons in Canada were calculated by individual country of birth and compared between 1971 and 1981 (the years in which reliable population estimates were determined), there was a lower rate in 1981 in every instance as compared with 1971 [32].

We studied immigrants to British Columbia from 1982 to 1985 from five countries of Asia (Japan, Korea, the Philippines, China, and India) [33] and found that the prevalence of previous TB among the Asian immigrants was about 6 times that in residents of British Columbia (6% compared with 1%). In the years following immigration, a larger number of cases occurred in the group of immigrants who had evidence of previous TB than in those who did not (33 cases compared with 30). In those with evidence of previous TB, the greatest number of cases were discovered on the initial examination after entry into Canada. Most of these patients were asymptomatic at the time of examination and the radiographic appearance was unchanged. The notification rate of active TB was determined in the two subgroups of immigrants and was over 5 times higher in those with evidence of previous TB (even when the initial 'prevalence' cases were not included). Moreover, all the cases in those with

previous TB among the immigrants occurred in those who had either inadequate or no previous chemotherapy for TB. The notification rate in those who had no previous chemotherapy was very high (1% per annum for bacillary cases) and this group accounted for 11 of the 12 cases in the group.

Progress in Control Measures

The historic decline in TB in countries that are currently industrialized remains unexplained. A review of existing information from urban centres in Europe documented that this decline had already been underway since at least the latter half of the 18th century in some locations [34]. Some authors [35] have suggested that TB had been infrequent in Europe prior to the Middle Ages and that it had appeared, risen and peaked by mid-18th century. The explanation for this trend is not obvious. Some have suggested that it is related to an improvement in 'socioeconomic' factors, but this has no real basis in evidence or logic. It is quite possible that the combination of increased space and ventilation in houses accompanied by decreasing family size might have contributed to this trend, but this is not proven. Whatever the case, there is a natural underlying trend in the disease that must be taken into account when assessing any interventions.

Prior to 1990, it is quite possible that there was also a steady decline in TB in many low-income countries. Once again, the explanation is not self-evident. Since 1990, however, there are clear indications of increases in TB in areas in which general health services have declined (such as the countries of the former Soviet Union) or in regions heavily affected by the HIV epidemic.

In areas where health services have declined, the mismanagement of TB cases has converted many of them into chronic, drug-resistant cases that promote the transmission of tuberculous infection, a possibility that had been predicted previously [36]. The additional factor that drove the epidemic in the former Soviet Union as the overcrowding in institutions, particularly the penitentiaries, which further fuelled the epidemic. This transformed the new epidemic into an almost solely male experience with rates of disease substantially higher in men as compared with women. While mismanagement had likely slowed the natural decline in TB in other locations such as China, Taiwan and Korea, the additional nosocomial factor substantially increased the negative impact and drove case rates upward at an alarming pace.

With the adoption of the DOTS strategy and its expansion, substantial progress has been made in the global fight against TB [37]. Figure 2 demonstrates progress toward targets by 1998 among the 23 countries with the highest

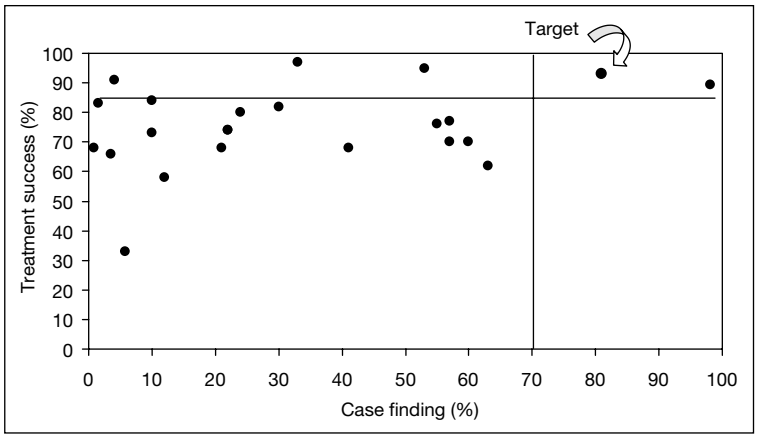

Fig. 2. Progress toward targets in TB control, as determined from routine reports to the WHO [adapted from 15].

burden of TB, containing over 80% of all TB patients in the world. Two countries, Peru and Vietnam, have already achieved the WHO targets of 85% success in treatment and 70% case detection. Cambodia is on the verge of achieving these targets. A group of countries, Afghanistan, Indonesia, Uganda, Pakistan, Russia, Thailand and Mozambique, are lagging far behind with treatment success rates under 70%. Among these countries, however, some (Uganda, Pakistan, Thailand and Mozambique) have demonstrated substantial progress in treatment success since 1998 and have or will likely have treatment success rates in excess of 70% by 2001. Major challenges remain in Afghanistan, in Indonesia and in Russia.

One group of countries (Zimbabwe, Kenya, Tanzania and DR Congo) had treatment success rates in the 70–79% range and case detection rates just under 60%. This group of countries is distinguished by the fact that they are all heavily affected by the HIV epidemic. Consequently, the high case fatality rate associated with HIV reduces treatment success substantially. In each of these countries, when this factor is taken into account, the efficiency of treatment is substantially better. Another important group of countries, including some of the countries with the largest populations, while a good level of treatment success has been achieved, the expansion of the DOTS strategy has not yet been sufficient to increase case finding. This group includes India, China, Brazil, Nigeria, Bangladesh, Philippines, Ethiopia, Myanmar and South Africa. In many of these countries, case finding has expanded substantially since 1998.

Challenges for Elimination

The observation that TB is a disease that has declined 'naturally' (independent of specific medical intervention [38]) in many locations suggests that it should be possible to control such a disease. Targeting elimination requires that the goal of our efforts must be to eliminate TB [39]. Current strategy for TB control aims to achieve an infection-free generation through cure of the most infectious cases. That the DOTS strategy [40] can protect a generation from infection with *M. tuberculosis* in a poor country has now been demonstrated [41].

There are substantial challenges to achieving control of TB. As the strategy is based on clinical care of individual cases, there is a question of the feasibility of service delivery to the whole population. It has been demonstrated to be feasible in countries where it would have seemed virtually impossible [42]. Nevertheless, the extent to which it can be expanded to cover all the cases remains an open question. Progress in the fight against TB has been wiped out in some areas by the impact of HIV [43]. This has been most notable in sub-Saharan Africa where TB case rates in some countries are now reaching historical heights. It is impossible to speak about control of TB in such communities, without the control of transmission of HIV. Anyone serious about the fight of TB in Africa today must be fully committed to the fight against HIV. The emergence of drug resistance [44], and particularly of multi-drug resistance, poses a threat to TB control, especially in countries of the former Soviet Union. Patients from low-income communities, whose disease is caused by such organisms, are very difficult, if not impossible, to cure [45]. The cause of this tragedy is medical malpractice. It can be avoided, even in low-income countries, through scrupulous observance of the DOTS strategy [44].

While these challenges will be regionally important and may prevent progress in the control of TB in some areas, several other factors may prevent progress on a global scale. The first of these challenges is the loss of focus on clear objectives in the strategy and the adoption of dangerous policies and priorities. The capacity of chemotherapy to worsen the epidemiological situation if improperly applied was demonstrated when multi-drug chemotherapy was first introduced in Edinburgh nearly half a century ago [46, 47]. Since that time, there has been a litany of errors in judgement that has compromised the global fight against TB, particularly in low-income countries [48]. The DOTS strategy is highly dependent on maintaining political commitment and declining political commitment results in regress in the fight against TB [49]. Can such political commitment be sustained for a full generation at the global level [50]?

Eliminating TB will require new knowledge, tools and strategies. First of all, we need a better understanding of immunopathogenesis of TB. Advances in

molecular genetics of TB should make this possible. This improved understanding is essential for the development of an effective vaccine and for shortening the duration of chemotherapy. The development of effective vaccines which can prevent infection with *M. tuberculosis* and which can interrupt the transition from infection to disease must have the highest priority. Elimination of disease, to date, has been feasible primarily when there is a vaccine-based strategy.

As TB declines, it increasingly affects subsets of the population [51] that become increasingly hard to reach. Molecular epidemiological tools have enhanced our ability to track transmission [52] and to identify high-risk groups [53]. These tools, now demonstrated, must be applied to aid us in targeting our interventions. Without this ability to target intervention, even new tools for treatment and prevention of TB cannot be efficiently applied. The characterization of the nature of transmission and the identification of target groups in the population are essential to the elimination phase of TB. Along with this enhanced surveillance, there is a need to improve the tools for detection of both disease and of infection. Finally, our tools for preventive therapy must be improved.

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New Perspectives in the Molecular Epidemiology of Tuberculosis

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The development of DNA fingerprinting techniques for typing *Mycobacterium tuberculosis* isolates during the last decade has led to an increasing number of studies of the molecular epidemiology of tuberculosis. Molecular typing techniques allow investigators to determine whether the strain causing disease in one case is identical to that causing disease in another case, and hence whether transmission could have occurred. In addition, the level of genetic relatedness of *M. tuberculosis* isolates can be determined.

The advanced way transmission of *M. tuberculosis* is investigated has for example resulted in improved detection of nosocomial and institutional outbreaks [1, 2] and a better understanding of the role of laboratory cross-contaminations in the laboratory diagnosis of tuberculosis [1]. These advanced typing methods have also led to discovery that recent transmission, including reinfection, is more important in the epidemiology of tuberculosis than was assumed previously [3–7]. This finding has important implications for control of tuberculosis.

Strain typing in different geographic areas has revealed the population structure of *M. tuberculosis* differs significantly between settings with a low and a high incidence of tuberculosis. This has led to extensive research on selective advantages of predominant genotypes, such as the 'Beijing' genotype of *M. tuberculosis*, which is highly prevalent in Asia. Selection of predominant *M. tuberculosis* genotypes may have implication for research on the development of new tuberculosis vaccines.

DNA fingerprinting techniques have also led to improvements in the identification and recognition of subspecies of the *M. tuberculosis* complex, such as *Mycobacterium bovis* BCG and *Mycobacterium microti* [8, 9], which were previously difficult to identify using biochemical procedures. Two new species have been identified within the complex, namely *Mycobacterium canettii* [10, 11] which is the most divergent subspecies within the complex, exhibiting a smooth colony morphology and a rapid growth in vitro, and *M. tuberculosis* subsp. *caprae* subsp. *nov*. which has been isolated mainly from goats [12].

The recent introduction of whole genome analysis has provided possibilities to associate the presence or absence of particular genes present in bacteria with pathogenicity and transmissibility [13]. This genetic marker, with a low turnover, will also provide new insight in the evolutionary development of the *M. tuberculosis* complex and offers possibilities for a renewed identification of taxons within the complex.

In this chapter, we review the currently most important methods for typing *M. tuberculosis* complex isolates. The major conclusions for the epidemiology of tuberculosis at the population-based and the global level are summarized. Furthermore, future prospects of typing methods at the genomic level are discussed.

Methods for Typing *M. tuberculosis* Complex Isolates

Standard DNA Typing Method for M. tuberculosis *Complex Isolates*

The most widely applied and standardized molecular typing method for *M. tuberculosis* complex isolates is IS*6110* restriction fragment length polymorphism (RFLP) typing which is based on the detection of the repetitive sequence IS*6110* which can be found in most isolates of the *M. tuberculosis* complex [1, 2, 6, 7, 14–33]. In low-incidence regions like The Netherlands, Denmark, and Norway, this typing methodology is applied to all *M. tuberculosis* complex isolates. In the USA, genotyping is performed on a large scale in more than 25 states. In other countries, e.g. Germany, Italy, Spain and France, routine typing is mostly reserved for resistant isolates.

An update of the detailed protocol on the use of IS*6110* RFLP typing has been published recently [33]. To generate IS*6110* RFLP patterns, a well-grown *M. tuberculosis* complex isolate is required. DNA is extracted, purified and digested with the restriction enzyme *Pvu*II. This restriction enzyme cleaves the IS*6110* element at a single site. The *Pvu*II restriction fragments are separated overnight on an agarose gel and subsequently transferred to a DNA membrane. IS*6110*-containing restriction fragments are visualized by adding a peroxidase labeled probe with a DNA sequence complementary to the right-hand part

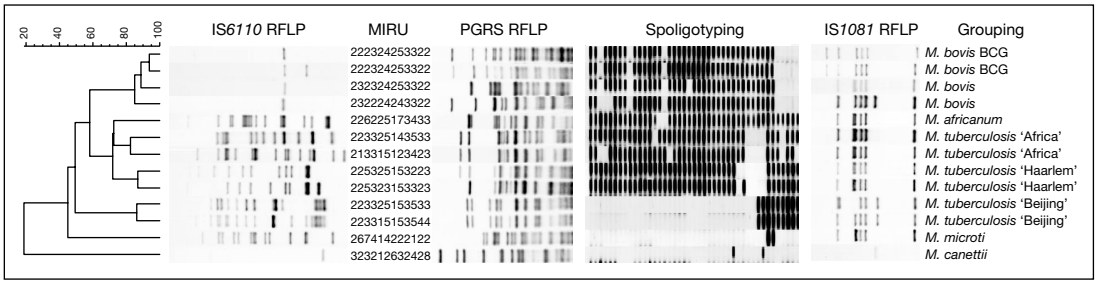

Fig. 1. DNA fingerprint patterns of representative strains of six subspecies of the *M. tuberculosis* complex. These DNA fingerprint patterns were prepared by IS*6110*, PGRS and IS*1081* RFLP typing and spoligotyping. From left to right, bands in the respective RFLP patterns are displayed from high to low molecular weight and the spoligo patterns from spacer 1 to spacer 43. The MIRU typing patterns are represented by numbers, each digit represents the number of repetitive units at a different locus. The dendrogram was prepared using the Dice-UPGMA method and reflects the combined equal-weight similarity of the strains by the five methods displayed. The methods are depicted from most (left) to least discriminative (right).

of the IS*6110* sequence in a hybridization buffer onto the DNA membrane. The hybridizing restriction fragments are detected by a chemiluminescence reaction initiated by two substrates and the RFLP patterns are detected on a light-sensitive film. Figure 1 shows typical IS*6110* RFLP patterns of *M. tuberculosis* complex strains.

To facilitate the inter-laboratory comparability of IS*6110* RFLP patterns, the most critical aspects of this procedure, the restriction enzyme, the probe and the markers have been standardized [32]. Standardization of the internal and external molecular weights has facilitated accurate computer-assisted comparison of RFLP patterns. The computer-assisted analysis of DNA fingerprints has been described extensively elsewhere [34].

M. tuberculosis complex strains differ in the number of IS*6110* copies present in their genome, ranging from 0 to 25, and also in the genomic positions where these elements are inserted [16, 20, 35–41]. By mapping IS*6110* insertions sequences, Warren et al. [42] recently demonstrated three mutational mechanisms underlying IS*6110*-associated RFLP: IS*6110* insertion, chromosomal mutation and deletion.

For *M. tuberculosis* complex isolates with a small number or no IS*6110* copies, IS*6110* RFLP typing is insufficiently discriminative [15, 35, 43]. The DNA of strains with less than five copies of IS*6110* is therefore digested with restriction enzyme *Alu*I and, after electrophoreses and Southern blotting, subjected to a hybridization to a polymorphic GC-rich sequence (PGRS) probe [15].

In figure 1 the discriminatory power of PGRS RFLP is illustrated for the *M. bovis* and the *M. bovis* BCG strain with identical single-copy IS*6110* RFLP patterns. There are several typing methods (described elsewhere in this chapter) for strains with fewer than five IS*6110* copies, such as spoligotyping [44], variable numbers of tandem repeats (VNTR) typing [45], and fluorescent-amplified fragment length polymorphism (FAFLP) typing [46].

Soon after the introduction of IS*6110* RFLP in the early 1990s it was noticed that random transpositions of IS*6110* result in band shifts in the IS*6110* RFLP patterns of isolates from epidemiologically linked cases [16, 47]. This led to concern about the use of IS*6110* RFLP for identifying the spread of strains, given that the marker needs to evolve fast enough to create distinct patterns among non-epidemiologically related isolates, and yet slowly enough to yield identical RFLP patterns for strains of epidemiologically related cases. Three studies have since estimated the stability of this genetic marker using serial isolates from tuberculosis patients [48–51]. In one study, 29% of the paired isolates from 49 patients, taken at least 90 days apart, showed only minor alterations in the IS*6110* RFLP patterns [49]. In contrast, 544 serial isolates from patients in the Dutch study were found to be more stable and the half-life was estimated to be 3.2 years [48]. This means that on average, half of the strains exhibit a band shift in their IS*6110* RFLP patterns in a period of 3–4 years [48–51]. This interval is sufficient for distinguishing epidemiologically related and unrelated isolates and therefore supports the use of IS*6110* typing in epidemiological studies of recent transmission of tuberculosis.

Presently it is unclear what factors influence the stability of IS*6110* RFLP. In one study of drug-resistant isolates, instability in IS*6110* RFLP was significantly correlated with increased time intervals between isolates though changes in IS*6110* RFLP were not related to changes in the resistance profiles of isolates [51]. In another study, instability of IS*6110* RFLP was found in some multidrug-resistant strains, but not in others [52], suggesting that the pace of the molecular clock of IS*6110* RFLP may be strain-dependent and indirectly linked to resistance.

Within the framework of an European Union Concerted Action Project on Molecular Epidemiology of Tuberculosis, a standard set of long-term sequential isolates is currently being compiled to facilitate an international comparison between the stability of current and future markers for typing of *M. tuberculosis* complex isolates.

Low-intensity bands also hamper the interpretation of strain typing results. A single low-intensity band was found in about 7% of 1,277 IS*6110* RFLP patterns of *M. tuberculosis* complex strains examined in The Netherlands [53]. To study this phenomenon, single-colony cultures of strains with low-intensity bands in their IS*6110* RFLP patterns were grown. None of the RFLP patterns of

single-colony cultures from eight isolates showed the low-intensity bands of the respective parental strains, but did show either no band at all, or a band with a normal intensity at the position of the low-intensity band in the parental strain. This suggests that low-intensity bands in IS*6110* RFLP usually reflect mixed bacterial populations with slightly different RFLP patterns [53]. Given the growing application of DNA fingerprinting techniques for typing *M. tuberculosis* isolates and the problems associated with band shifts and low-intensity bands, some standardization of the interpretation of these phenomena is clearly needed [53, 54].

Spoligotyping

Spacer oligonucleotide typing or 'spoligotyping' is based on the detection of DNA polymorphism in the direct repeat (DR) region in the genome of *M. tuberculosis* complex [44]. This *M. tuberculosis* complex-specific genomic region contains 36 bp DRs, interspersed by unique DNA spacers of 35–41 bp in length. The numbers of DRs per strain and thereby the presence of particular spacer sequences differs significantly between strains. To visualize the DNA polymorphism in the DR region of *M. tuberculosis* complex, the spacer sequences are initially amplified by PCR, using primers based on the DR sequence. The reverse primer is biotin-labelled and, hence, also the PCR product. In the first generation spoligotyping, 43 oligonucleotides are used, based on the DNA sequence of spacers in *M. tuberculosis* strain H37Rv and BCG vaccine strain P3. These oligonucleotides are covalently linked to a Biodyne C membrane in parallel lines. The denatured PCR products are allowed to hybridize perpendicular to the oligo lines in the reversed line blot hybridization [55]. The hybridization is detected by adding streptavidine-peroxidase conjugate and a substrate, which results in a chemiluminescence reaction that is detected on film.

Spoligotyping is relatively easy to perform, cheap, rapid and reproducible [44, 56]. However, although strains with less than five IS*6110* copies can often be further subdivided by this technique, it is far less discriminating for other strains than is IS*6110* RFLP typing. Particular predominant spoligo patterns are shared by strains with totally different IS*6110* RFLP patterns [36]. In any case, spoligotyping can be used as a screening method to differentiate between strains since differences in spoligo patterns are almost always indicative of differences on the basis of other genetic markers.

In order to optimize the level of discrimination, novel spacer sequences, mainly derived from *M. canettii*, were recently evaluated in spoligotyping. This gave only a slight improvement in the differentiation [57].

Spoligotyping can be directly applied to clinical material thus allowing epidemiological typing of bacteria from stored clinical specimens [58] and even paraffin-embedded samples [59]. Spoligotyping can simultaneously detect and type the bacteria: *M. bovis* [44], *M. microti* [9, 60], *M. canettii* [10] and *M. caprae*

[12] can be readily recognised by distinct characteristic patterns. In figure 1, characteristic spoligo patterns of *M. tuberculosis* complex subspecies are shown.

Spoligotyping can also be applied to *M. tuberculosis* DNA from naturally mummified tissue, as shown recently by Donoghue et al. [61], who obtained samples from 168 18th century mummies and were able to generate spoligo patterns for a part of them. Interestingly, almost all spacers in the panel of 43, but spacers 33–36, gave a positive signal in the spoligotyping of the mummy samples, resembling the present *M. tuberculosis* patterns.

Similarly, spoligotyping has been successfully applied to a 17,000-year-old skeletal specimen of a bison, originating in North America. The spoligotype pattern revealed that the respective bacteria were probably not related to the modern *M. bovis* or *M. microti*, as indicated by the presence of spacers 39, 40, 41 and 43. The bison spoligo pattern fitted best to *Mycobacterium africanum* and *M. tuberculosis* patterns [62].

The existence of an *M. tuberculosis* complex-specific DR locus offers the possibility to study the evolution and the world-wide spread of these bacteria. Sola et al. [63] recently described a nomenclature and a phylogenetic reconstruction of the 259 shared types (clustered patterns) found among the spoligo patterns of 3,319 isolates from 47 countries. Among the shared types, which represented 84% of the total number of patterns analysed, seven major genetic groups represented 37% of all clustered strains. Of the 259 shared types, 59 were exclusively found in the USA, whereas 50 were unique for Europe. The differences in distribution between the continents of 14 other shared types were also significant. The Beijing genotype, with a characteristic nine-spacer pattern, represented 18% of the isolates and constituted a separate branch in the phylogenetic tree constructed by pairwise comparison of patterns [63].

Standardization of the description of spoligo patterns has been recently recommended, which should facilitate comparison of results from different laboratories [64]. It is recommended that all patterns receive an arbitrary number after submission to an existing database, and either an octal or hexadecimal code.

New Molecular Typing Techniques

Several typing methods have been introduced in recent years that are less technically demanding than IS*6110*-based RFLP typing. Those specifically developed to type *M. tuberculosis* complex are spoligotyping [44], the mycobacterial interspersed repetitive unit (MIRU) typing [65], Fast Ligation-mediated PCR (FLiP) [66], VNTR typing [45], mixed-linker PCR [67], double repetitive element PCR (DRE-PCR) [68], heminested inversed PCR [69], IS*6110* ampliprinting [70], IS*6110* inverse PCR [71] and ligation-mediated PCR (LM-PCR) [72]. More broadly applicable methods that were used to

type *M. tuberculosis* complex isolates are arbitrarily primed PCR (APPCR) [73], pulsed field gel electrophoresis (PFGE) [74], enterobacterial repetitive intergenic consensus sequences PCR (ERIC-PCR) [75] and FAFLP typing [46].

In the whole-genome fingerprinting technique FAFLP typing [46], two restriction enzymes – *Eco*RI and *Mse*I – are used to digest DNA of *M. tuberculosis* isolates. *Eco*RI and *Mse*I adapters are ligated to the double-digested DNA. The fragments are amplified in PCR by using a non-selective forward primer for the *Mse*I adapter and an A-, G-, C-, or T-selective reverse primer for the *Eco*RI adaptor site. Latter selective primers are labelled with different fluorescent dyes. The amplification products are separated on a 5% denaturing polyacrylamide gel, e.g. on an automated DNA sequencer, and the sizes of the fragments are determined directly with reference to internal lane standards.

FLiP is an optimized variant of the mixed-linker PCR using fluorescent detection of PCR products and automated detection of the fingerprint patterns [66, 76]. In this method, mycobacterial DNA is digested with *HhaI*, and subsequently a double-stranded oligonucleotide, designated mixed-linker, is ligated to the outer ends of the fragments. The mixed-linker contains a GC overhang at the 3′ end compatible with the restriction fragments. One strand of the linker contains uracil instead of thymidine and is subsequently removed by uracil *N-glycosylase* treatment. Restriction fragments containing the IS*6110* sequence are amplified by PCR with a linker- and an IS primer, followed by a nested PCR with fluorescently labelled primers. The detection of PCR products is similar to that of FAFLP typing, allowing three different samples to be run in one lane if these samples are labelled with different dyes [66].

Another new, automated high-throughput genotyping technique is the MIRU typing [65]. This typing method is actually based on the variability in the numbers of tandem repeats and another group has earlier described the similar VNTR typing [45]. MIRUs are 40- to 100-bp elements often organized as tandem repeats and dispersed in intergenic regions of *M. tuberculosis* complex strains. Twelve out of 41 MIRU loci in *M. tuberculosis* strain H37Rv were found to be variable [77]. In MIRU typing, these loci are multiplied in a multiplex PCR using sets of primers of which one of each is labelled with a different fluorescent dye. The PCR products are analysed on an automated sequencer, on basis of internal molecular weight standards, with software programs for automated genotyping, allele calling and database construction. An advantage of the MIRU and VNTR typing methods is that the results can be displayed by a number, which facilitates inter-laboratory comparison of the patterns (see fig. 1). Each digit of the number represents the number of repetitive units at a particular locus.

Differentiation and Reproducibility of Genetic Markers

In 1999, the discriminatory power of most typing methods available at that time were evaluated using a set of 90 DNAs of *M. tuberculosis* complex strains and, in addition, the intra-laboratory reproducibility was evaluated using 31 blinded duplicate DNA samples [56]. The RFLP typing methods using IS*6110* [32], IS*1081* [8], DR [15], PGRS [15] and GTG_5 [78] and the mixed-linker PCR [67] proved to be highly (100%) reproducible. VNTR typing [45] (97% reproducible) and spoligotyping [44] (94%) were also reproducible, whereas in contrast, DRE-PCR [68], IS*6110* inverse PCR [71], IS*6110* ampliprinting [70] and APPCR typing [73] were found poorly reproducible. The discriminative power was the highest for IS*6110* RFLP typing with 84 types, subsequently followed by mixed-linker PCR, AP-PCR, PGRS RFLP, DRE-PCR, spoligotyping and VNTR typing [56].

Additional typing methods were later evaluated with the same set of DNAs used by Kremer et al. [56]. MIRU and FLiP typing were demonstrated to be reproducible and only slightly less discriminative than IS*6110* RFLP resulting in 78 and 81 types, respectively [65, 76]. On the contrary, LM-PCR [72] was found to be poorly reproducible (81%) and yielded 71 types [unpubl. data].

The reproducibility and the discriminatory power of the few methods which were not included in the initial inter-laboratory study have since been estimated elsewhere. The heminested inverse PCR has been documented to be reproducible [69, 79]; the discriminatory power of LM-PCR was found to be more discriminatory than spoligotyping and less discriminatory than IS*6110* RFLP [80], and the discriminatory power of ERIC-PCR was found to be higher than that of IS*6110* RFLP typing [75].

Epidemiological Applications of RFLP Typing

Quantifying the Extent of Recent Transmission in a Population

Since the early 1990s, many studies have used DNA fingerprinting techniques to calculate the proportion of all cases in a population who share identical isolates (are 'clustered'). High (30–40%) levels of clustering have been found even in some low-incidence settings [6, 7, 30], suggesting that the proportion of disease attributable to recent transmission is far higher than previously thought. This finding has important implications, especially for control of tuberculosis: it is easier to control tuberculosis by interrupting transmission if much of the morbidity is attributable to recent transmission than if it is mainly attributable to reactivation of an infection acquired many years ago. However, conclusions about the extent of recent transmission are complicated, since several factors influence the amount of clustering in a population [81]. For example, the amount

of clustering in any population increases both with the fraction of the cases included [82] and study duration [6, 83]. Long studies are more likely to identify transmission-linked cases than are those spanning only a few months or including a fraction of cases in the population. Clustering is also generally less common among old cases than young cases [6, 36, 83], given that old cases are more likely to have been infected many years ago than are the young. Overall clustering estimates in a given population will therefore depend on its age distribution. They will also depend on the molecular clock of the genetic marker used to define DNA fingerprint patterns. In one rural area for example, clustering was found even among epidemiologically-unlinked cases, which has generated speculation that the rate of change of DNA fingerprint patterns may be slower during latent infection than during ongoing transmission [84]. Anecdotal evidence illustrating that DNA fingerprint patterns can remain unchanged even 33 years after an individual has been first infected has been published recently [85].

There are several methods for estimating the amount of recent transmission from crude clustering statistics. The 'n-1' method is the most widely applied and assumes that each cluster includes one case attributable to reactivation, which leads in/directly to the remaining cases in the cluster [7]. Though easy to apply, this method is not ideal, since cases can be misattributed to reactivation if they were infected just before the start of the study. An alternative ('transmission index') method addresses this problem and relies on incidence data to calculate the probability that a given case is a source of infection of other cases in a cluster [29]. This method has been successfully applied to calculate the extent of transmission within and between different nationalities in The Netherlands and to illustrate that younger individuals are more likely to lead to secondary cases than are old cases [29, 86]. Analyses of 'genetic distances' between isolates may also provide reliable insight into the extent of recent transmission in a population, though this approach has not yet been used extensively [87]. Studies are currently underway, using modelling techniques to identify other methods for identifying the extent of recent transmission from clustering statistics, as part of the European Concerted Action Programme on the Molecular Epidemiology of Tuberculosis.

Despite problems with accurately quantifying the extent of recent transmission, population-based DNA fingerprinting has nevertheless been helpful in identifying risk factors for clustering and hence for recent transmission. Risk factors for clustering often include young age [36], homelessness [6], incarceration [84] and previous hospitalization [7]. HIV has been found to be a risk factor for clustering in some studies [7, 27] but not in others [6, 88, 89]. To facilitate comparison between different studies on the molecular epidemiology of tuberculosis, and to improve the understanding of the factors influencing clustering, the European Concerted Action Programme on the Molecular

Epidemiology of Tuberculosis has developed a set of guidelines for the minimum amount of data which should be provided when designing or writing up molecular epidemiological studies [81]. These include details of case ascertainment, case definition, geographical area from which the isolates were collected, study period, definition of clustering, and the cluster size distribution; at the very least, data need to be broken down by age, sex and immigration status.

Estimates of the Relative Importance of Reactivation and Reinfection

Though it is recognised that reinfection with *M. tuberculosis* can occur in HIV-positive individuals, its contribution to morbidity among HIV-negative individuals is often disputed. To date, several studies have applied DNA fingerprinting techniques to elucidate this question [3, 90–92] by calculating the proportion of cases whose isolates from second or subsequent disease episodes differed from the isolate from their initial (cured) episode. A very high (75%) proportion of disease was attributed to reinfection in a 6-year study in a high-incidence area in Cape Town, where the isolates from the recurrent and previous disease episode differed for 12 out of 16 HIV-negative cases [3]. A similarly high (46%) proportion of disease was attributed to reinfection among HIV-negative cases identified between 1991 and 1996 in the Canary Islands [92], which is considered to have a moderate disease incidence. In contrast, only one 1 of 18 recurrent episodes (6%) during the period 1995–1998 among HIV-negative South African goldminers, where the prevalence of tuberculosis (1,536/100,000) is perhaps higher than in any other setting today, were attributed to reinfection [91].

There are probably several reasons for the different findings in these settings, including chance and differences in study duration. For example, a 6-year study such as that in Cape Town is more likely to identify cases experiencing reinfection than for example a study lasting 3 years. Laboratory contamination does not appear to have contributed to the findings in any of the studies [3, 90–92].

Du Plessis et al. [93] examined intra-patient strain diversity in autopsied, HIV-negative individuals resident in a high-incidence community. They performed RFLP typing using IS*6110*, DR and MTB484 as probes on 6–15 samples of 12 autopsy cases and 1 pneumonectomy case. In 2 cases, pulmonary infection by two distinct strains suggested dual infection. In another case, identical strains were isolated from primary and secondary lesions, suggesting reinfection of the primary infection. In the remaining cases, all isolates were identical, suggesting reactivation, but in these cases the primary lesions could not be identified with certainty.

Two recent studies [94, and unpubl. data] have estimated the contribution of reinfection by calculating the amount of clustering among cases who experienced disease many years ago (e.g. before 1980 and before DNA fingerprinting techniques were available). Overall, 16% of Dutch cases who had onset

between 1994 and 1997 and who had had onset before 1980, were clustered with other cases during the 1990s [unpubl. data]. These findings are consistent with those from Norway, where about 13% of cases who experienced recurrent disease in 1995 were in a cluster [94].

All of the above studies (excepting that in the goldmines [91]) suggest that the proportion of disease attributable to reinfection, at least among recurrent cases, is higher than previously thought. However, it is presently unclear as to whether the contribution of reinfection is similarly high among cases who have experienced previous infection without disease. This question could ultimately be resolved by linking long-term molecular data to information on previous exposure, typically collected during contact tracing.

While several studies have applied DNA fingerprinting techniques to examine the contribution of reinfection to tuberculosis morbidity, very few have analysed the relative importance of multiple or simultaneous infections. The most detailed study found that none of the 1,277 IS*6110* patterns identified between June 1997 and May 1998 had multiple low-intensity bands and concluded that multiple infections are rare, at least in The Netherlands [53]. Other studies [95–97] have been based on very small (<10) numbers of cases. One recent study highlighted that simultaneous infection with more than one strain may account for conflicting drug-sensitivity findings, identifying both drug-susceptible and -resistant colonies within an isolate having different RFLP patterns [96].

Other Epidemiological Applications of DNA Fingerprinting Techniques

The availability of molecular data on isolates from all cases since the early 1990s in different settings, e.g. The Netherlands, Denmark, San Francisco and Cape Town, means that it is now possible to study several important aspects in the epidemiology of tuberculosis. In The Netherlands, for example, these data have been used to study the serial interval (time interval between successive cases in a chain of transmission) and the incubation period for tuberculosis [98]. Considering clusters in which both the primary and secondary cases in a chain of transmission during the period 1993–1996 had been confirmed through contact-tracing, the geometric mean serial interval and incubation period was found to be about 30 and 21 weeks respectively; neither sex, age, site of TB nor nationality were found to be risk factors for short serial intervals or incubation periods. Given a longer study period, it is likely that the incubation periods and serial intervals would have been even more consistent with those from early observational studies [99], which found that 80% of individuals who develop disease within 7 years after initial infection do so within 2 years after infection.

The same Dutch data set has also been used to demonstrate, for the first time, that cases transmit preferentially to individuals similar in age [100]. Considering Dutch cases who were clustered only with one other Dutch case

during the period 1993–1996, for example, the mean age difference of cases in the same cluster was found to be about 13.9 years, as compared with the 25.5 years which might be expected in a random sample of all possible pairs in the data set.

The large data set of isolates from patients collected since 1991 in San Francisco has been used to investigate the relative infectiousness of smear-negative and smear-positive cases [101]. Approximately 17% of cases with onset between 1991 and 1996 were attributed to smear-negative sources of infection, which confirmed findings from previous studies that smear-negative cases are less infectious than those who are smear-positive.

Transmission of Drug-Resistant Strains

DNA fingerprinting techniques have also been helpful in identifying and tracing outbreaks of multidrug-resistant tuberculosis [27, 102–105].

The rapid detection of such outbreaks, especially if they involve HIV-positive individuals, is important, given the potentially short time interval between infection, disease (and, sometimes, death) among HIV-positive individuals. The largest outbreaks involved the W strain in New York, where it accounted for 22% of multidrug-resistant tuberculosis cases in 1992, 82% of whom were HIV-positive [106]. Large multidrug-resistant tuberculosis outbreaks involving HIV-positive cases have been reported in several European countries, the largest of which appears to have been in Milan between 1991 and 1995, in which 82/92 of the strains available for typing were in the same cluster [105].

Despite these large outbreaks, it is still unclear as to whether drug-resistant strains are as transmissible as those which are drug-sensitive. According to a large population-based study in The Netherlands, patients with isoniazid resistance were significantly less likely to be in a cluster as compared with cases diseased with isoniazid-sensitive strains, though the finding was not significant when the analyses were restricted just to Dutch cases [6].

Another factor associated with the transmissibility/pathogenicity of resistant variants is the type of mutation underlying especially isoniazid (INH) resistance. From guinea pig experiments in the 1950s it is known strains that have become INH-resistant, and thereby also catalase-negative, are less virulent than susceptible isotypes. Using a PCR-based method, it was investigated to what extent a mutation at amino acid position 315 (AA315) in the *katG* gene of *M. tuberculosis* is associated with the level of INH resistance, the resistance profile, and tuberculosis transmission [107]. Of 4,288 *M. tuberculosis* isolates, 295 (7%) exhibited INH resistance, 278 of whom (94%) were studied for the AA315 mutation. Of 148 AA315 mutants, 89% had minimum inhibitory concentrations (MIC) of 5–10 g/ml, while 75% of the other 130 INH-resistant strains had MICs of 0.5–1 g/ml. Of the AA315 mutants, 33% had mono-resistance

compared with 69% of the other INH-resistant strains ($p < 0.0001$). Multidrug resistance was found among 14% of the AA315 mutants and 7% of the other strains ($p > 0.05$). The probability of being in a RFLP cluster was similar in AA315 mutants and INH-susceptible strains, but – as stated above – was reduced in the other INH-resistant strains. This means the AA315 mutation is associated with poly-resistance and appears to lead to secondary cases as often as INH-susceptible strains.

A recent study in a high-incidence community of Cape Town used DNA fingerprinting techniques to investigate the validity of clinical criteria for defining primary and acquired drug-resistant tuberculosis [108]. Overall, 29% (18/63) of cases identified between 1992 and 1997 were defined, by clinical criteria, as having primary drug-resistant tuberculosis, which implies recent transmission. In contrast, a larger proportion, 52% (33/63) of cases were attributed to recent re/infection by 'n-1' rule and overall 25/45 cases appeared to have been incorrectly classified as having acquired drug-resistant tuberculosis.

Identification of Laboratory Cross-Contamination

Laboratory cross-contamination can result in incorrect diagnosis of patients and unnecessary long-term treatment [109]. The introduction of DNA fingerprinting techniques has led to the recognition that sampling and laboratory errors occur more frequently than previously suspected [110, 111]. Laboratory cross-contaminations partly occur because of the high viability of mycobacteria despite less optimal environmental conditions [112], the complexity of the sampling procedures, batch processing [113], and the use of liquid culture medium [111, 114].

It has been suggested to suspect a false-positive *M. tuberculosis* culture if the DNA fingerprint of a culture, isolated from a patient without clear tuberculosis symptoms, is identical to the isolate of another patient, processed within a 1-week period in the same laboratory [111]. Using this criterion, in The Netherlands, 2.4% of the 8,889 positive cultures were confirmed as false-positive cultures in the period 1993–2000 [unpubl. data].

Contribution of Molecular Techniques to our Understanding of the Population Structure of *M. tuberculosis* Complex Strains

Improved Identification of M. tuberculosis *Complex Isolates*

Several of the methods for typing *M. tuberculosis*, such as RFLP typing [35, 115], VNTR typing [45], spoligotyping [43, 44, 116] and PFGE [117] can also be applied to study transmission of *M. bovis*, the causative agent of bovine tuberculosis. This disease is uncommon in humans in industrialized countries,

largely as a result of the introduction of the pasteurization of milk in the early 1950s. According to IS*6110*/PGRS RFLP typing studies, human-human transmission of *M. bovis* is rare [unpublished observation from the Dutch RFLP database since 1993], though outbreaks of *M. bovis* among HIV-positive individuals can occur [118]. The size of the problem of bovine tuberculosis in developing countries is presently unknown due to the lack of well-funded and representative studies. It is also widely recognised that the presence of *M. bovis* in wildlife reservoirs like the possum (*Trichosurus vulpecula*) in New Zealand, and the badger (*Meles meles*) in the UK makes bovine tuberculosis difficult to control in cattle populations.

The application of molecular typing methods has led to the recognition that some *M. tuberculosis* complex isolates do not fit the previous classifications based on biochemical tests and growth characteristics. For example, strains, which are intermediate between *M. bovis* and *M. tuberculosis*, have been isolated from wild seals stranded off the coast of Argentina. These strains contain the so far *M. tuberculosis*-specific *mtp40* sequence [119], but cannot grow on glycerol-egg containing medium, which is characteristic of *M. tuberculosis* [120]. In a study in Guinea-Bissau, Källenius et al. [121] found that 140 out of 229 *M. tuberculosis* complex strains could be allocated into one of three biovars, representing a spectrum between the classical bovine- (biovar 1) and the human (biovar 5) tubercle bacilli, using biochemical criteria. The different groupings found in that study could be subdivided further according to the number of IS*6110* copies present and the absence of particular spacers in the spoligo patterns.

Some of the strains observed in the study of Källenius et al. [121] were *M. africanum*, which is a separate subspecies within the complex, but which is often hard to identify due to its extensive phenotypic heterogeneity. The prevalence of *M. africanum* appears to be the highest in Africa, reaching e.g. 60% in Yaoundé, though the bacterium has also been isolated from European patients without any links to Africa [122]. Investigators have since used genotyping using IS*6110* RFLP, VNTR typing and spoligotyping to discriminate further groups within isolates, mainly from African countries, that were identified as *M. africanum* using phenotypic methods [122, 123]. The majority of the isolates were characterized by a specific spoligotype pattern, that was intermediate between *M. bovis* and *M. tuberculosis*, lacking hybridization to spacers 8, 9 and 39 [122]. In a collection of 105 phenotypically identified *M. africanum* isolates, 24 proved to belong to other species of the complex when genotyping was applied; 19 were *M. tuberculosis*, 3 were *M. bovis*, and 2 were of the recently described species *M. canettii* [122].

Through the application of molecular typing methods, *M. canettii* was described for the first time as a separate species within the complex in 1997 [10].

So far, it has only been detected in patients exposed in Africa [124]. This species exhibits abnormal smooth colony morphology and a shorter multiplication time than other members of the complex. It also has the highest degree of evolutionary divergence within the *M. tuberculosis* complex [10], with isolates typically containing only one IS*1081* element. This is exceptional: all of the 2,000 *M. tuberculosis* complex isolates tested so far have contained 5–7 bands [11]. It is likely that with the development of new unidirectional evolutionary markers, based on genomic deletions, the grouping in the *M. tuberculosis* complex will be better understood.

Application of IS*6110* RFLP typing has led to the recognition that *M. microti*, first identified in 1937 [125], and since found to be common in voles, wood mice and shrews, can cause disease in humans. In total, 9 human cases of tuberculosis caused by *M. microti* have been identified: 5 in The Netherlands [9, and unpublished observation], 2 in Germany [126] and 1 in Switzerland [11] and the UK [60]. The two most frequently found types of *M. microti* can be distinguished using spoligotyping: one type shows hybridization to only spacers 37 and 38 in the panel of spacers currently used in spoligotyping (see fig. 1) and the other is a type most frequently found in cats in the UK, reacting with the spacers 4–7, 23, 24, 37 and 38 [60]. It is possible that *M. microti* infections may be more common in humans than is implied by these studies, given that primary culture of *M. microti* from clinical material is difficult and takes about 8–12 weeks and that no studies among humans have been carried out outside of Europe.

Population Structure of M. tuberculosis

The population structure of *M. tuberculosis* differs significantly by geographic area, as illustrated by figure 2. IS*6110* RFLP patterns typically show a high degree of polymorphism in areas with a low incidence of tuberculosis [6, 7]. This may be partly attributable to the fact that immigration from countries with a high incidence of tuberculosis has increased in the past decades, and thus new strains from many diverse settings are being introduced. It is also influenced by the fact that much of the disease incidence in the native population in low-incidence areas occurs among old individuals who are experiencing reactivation of an infection acquired many years ago. The diversity of strains observed in the population thus depends on the diversity of strains which existed many years ago, and, of course, the rate of change of DNA fingerprint patterns during latent infection, which is presently unknown.

The *M. tuberculosis* isolates found in most countries with a high incidence of tuberculosis are genetically much more homogeneous, as compared with those in low-incidence settings, though exceptions have been reported [127]. The IS*6110* RFLP patterns of isolates from the Central African

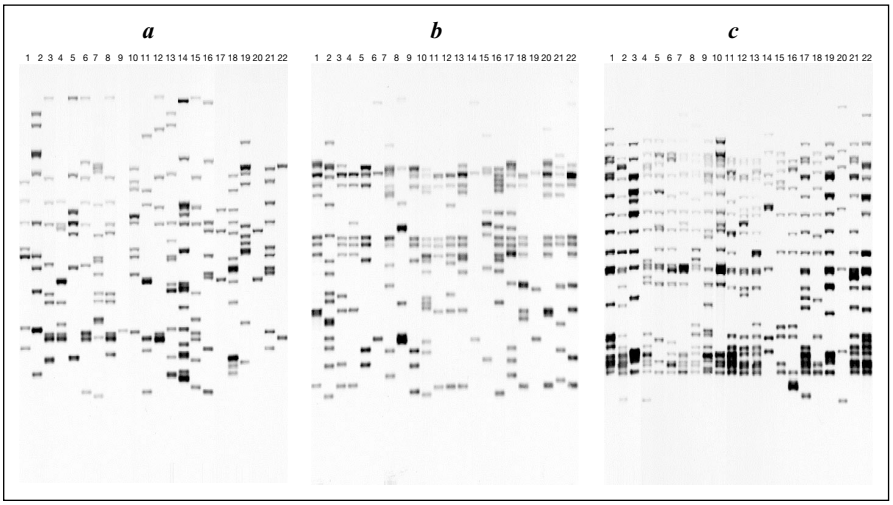

Fig. 2. IS*6110* RFLP patterns of 22 representative *M. tuberculosis* strains isolated in The Netherlands (***a***), Zambia (***b***) and China (***c***). Note the differences in similarity between the patterns of the strains from these areas; the strain diversity is the highest amongst strains from The Netherlands and the lowest among the strains from China.

Republic, Rwanda, and Burundi showed a high degree of similarity [16]. Studies have also demonstrated that the majority of the IS*6110* RFLP patterns in Tunisia and Ethiopia belong to three and four genotype families sharing at least 65% similarity within a genotype, respectively [128]. The most pronounced example of genetic conservation among *M. tuberculosis* isolates was found in the Beijing region, where more than 80% of the IS*6110* RFLP patterns showed a similarity of more than 80% [129]. The strains were therefore designated the 'Beijing genotype' of *M. tuberculosis*. All of the Beijing genotype strains exhibited identical spoligo patterns consisting of a positive reaction on the last nine spacers in the set of 43 used in the first generation spoligotyping (see fig. 1, 2a).

Beijing genotype strains have also been found in other parts of Asia [129–132], Estonia, Russia [133–135], and elsewhere [36]. In some studies [136–138], they have been significantly more frequently resistant to tuberculostatics [36, 130, 133, 135, 136], as compared with other strains. The 'W' strain, a multidrug-resistant strain that caused a large outbreak in North America in the early 1990s, constitutes an evolutionary branch of the Beijing genotype [139]. In Vietnam the occurrence of the Beijing genotype was significantly correlated with young age, suggesting that it has been introduced there only recently [130].

Evolutionary Development of the M. tuberculosis *Complex*

The unusually high degree of sequence conservation in their housekeeping genes [140] suggests that members of the *M. tuberculosis* complex underwent an evolutionary bottleneck 15,000–20,000 years ago. In contrast, high degrees of polymorphism have been detected using numerous repetitive sequences, such as IS*6110*, DR, PGRS, and MIRUs. Comparative genomics have revealed that there are several variable genomic regions in the *M. tuberculosis* complex. In 1996, subtractive genomic hybridization was employed for comparing *M. tuberculosis*, *M. bovis* and BCG [141] and identified that three distinct regions (designated RD1–RD3), representing 23 kb in total, had been deleted from *M. bovis* BCG. Seven additional deleted regions were identified when whole-genome comparisons were undertaken using bacterial artificial libraries of *M. tuberculosis* H37Rv and *M. bovis* BCG, together with the complete genome sequence of H37Rv [142]. In the same study, two *M. tuberculosis* H37Rv loci, RvD1 and RvD2, were found to have been deleted from the genome of *M. tuberculosis* relative to *M. bovis* and *M. bovis* BCG. Comparison between the genomes of H37Rv and the attenuated H37Ra by PFGE techniques revealed three additional deleted regions in H37Rv [143]. A sixth deletion in *M. tuberculosis* was demonstrated in *in silico* comparisons of the near complete *M. bovis* genome sequence and the *M. tuberculosis* H37Rv sequence [unpubl. data].

Behr et al. [144] performed comparative hybridization experiments on a DNA microarray, containing 4,896 spotted PCR products, representing 99.4% of the open reading frames (ORFs). They demonstrated that eleven regions of H37Rv were absent from one or more virulent *M. bovis* strains and five additional regions were present in *M. bovis*, but absent from BCG. Furthermore, they demonstrated variability among BCG daughter strains in the presence of four deleted regions.

In another microarray experiment, a *M. tuberculosis* high-density oligonucelotide array was used to detect small-scale genomic deletions among 19 clinically and epidemiologically well-characterized isolates of *M. tuberculosis*. This yielded identical deletion patterns in epidemiologically related isolates and different patterns for unrelated isolates, suggesting that deletion analysis can be useful in epidemiologic studies [13]. Among 16 unrelated isolates, 25 different deleted sequences were detected, comprising in total 1.7% of the *M. tuberculosis* H37Rv genome. The spatial distribution of these deletions was not random in the genome; in the region from 1.3 to 2.7 Mb, deletions were more often present than expected if the deletions were distributed randomly [13]. Because IS*6110* is thought to be an evolutionary driving force, this is concordant with the absence of preferential insertion sites of IS*6110* in the genome upstream of the origin of replication found by

Sampson et al. [145], and suggests that the genes in this region are relatively important. The microarray experiment of Kato-Maeda et al. [13] demonstrated convincingly that as the amount of deletions increased, the likelihood that bacteria will cause pulmonary cavitation decreased. No correlation was found between the percentage of deletions and either the transmission or pathogenicity indexes.

Genetic Variability of M. tuberculosis *Complex Bacteria and Pathogenicity*

The latest findings on major genomic deletions, already encompassing hundreds of ORFs, indicate that the genome of the *M. tuberculosis* complex bacteria may be less conserved than previously assumed [144]. The evolutionary implications of this are presently unclear. Some of these deletions may be explained by the fact that the genome of these bacteria still contain a high number of redundant genes. On the other hand, the study of Kato-Maeda's group [13] in San Francisco has found that loss of genes is associated with a lower degree of pathogenicity. This variability in pathogenicity has been confirmed recently by testing the pathology and immune response of a panel of different genotypes of *M. tuberculosis* in a mouse model. The survival of BALB/c mice after infection with a *M. tuberculosis* strain of the Beijing genotype was significantly shorter than that of the reference strain Erdman and that of *M. canettii* [unpubl. data]. It was also demonstrated that the Beijing genotype strain induced the most severe pathology in the lung of these mice together with a significantly reduced immune response.

Another indication that genotypes of *M. tuberculosis* differ in their ability to induce immune responses in humans was found in Indonesia, where patients infected with Beijing genotype strains were two times more likely to exhibit a febrile response as compared with patients infected with strains of other genotypes [132]. It is conceivable this implies that strains from different genotypes interact differently with the host defence system, and thus immunological research is currently being conducted to explore this in further detail.

BCG vaccination has been found to have an efficacy of 75% in countries such as the UK, and appears to provide little protection in other settings, such as Malawi and South India [146]. This variable efficacy has been attributed to many factors, however, to date, the contribution of genetic variability in *M. tuberculosis* strains has not yet been explored. Some investigators have proposed that BCG-induced protection may differ according to genotype and the introduction of BCG could have resulted in shifts in the population structure of *M. tuberculosis* in Asia [129] and Central Africa [128]. It will be impossible to find conclusive evidence to support this hypothesis using only molecular epidemiology.

A New Evolutionary Scenario for the M. tuberculosis *Complex*

Brosche et al. [unpubl. data] have proposed a new evolutionary scenario for the *M. tuberculosis* complex, based on the distribution of 20 variable regions from insertion-deletion events in 100 strains comprising the various species of the complex. It was shown that most of the polymorphisms did not occur independently, but rather result from ancient, irreversible genetic events in common progenitor strains. *M. tuberculosis* strains could be subdivided into two groups – the 'old' and the 'modern' tuberculosis, on the basis of absence or presence of a particular deletion, designated TbD1. The Beijing, Haarlem and African family described previously [56] represent modern branches of the evolutionary tree of *M. tuberculosis*. Successive loss of RD9 and other subsequent deletions was identified for an evolutionary lineage consisting of *M. africanum*, *M. microti* and *M. bovis*. This evolutionary lineage must have diverged from the common progenitor before TbD1 occurred. *M. canettii* and the 'old' tuberculosis show no deleted regions and therefore appear to be direct descendants of the progenitor strain. The common ancestor could therefore have been a human pathogen. The proposed evolutionary scenario is supported by spoligotyping of samples of a 17,000-year-old bison, which revealed a *M. tuberculosis*/*M. africanum*-like spoligo pattern rather than a *M. bovis* characteristic pattern [62]. The new evolutionary scenario contradicts the conventional hypothesis that *M. tuberculosis* has evolved from *M. bovis*, by the adaptation of *M. bovis* to the human host after the domestication of bovines [147].

Future Approaches for the Identification and Typing of M. tuberculosis *Complex Isolates*

Simultaneous species identification and resistance testing have already been successfully combined in different high-density DNA probe microarrays [148, 149]. Ideally, these methods should be combined with molecular strain typing at various evolutionary levels, using markers with different molecular clock speeds. The 'slow' evolutionary markers could be used to distinguish broad taxons within the complex, whereas fast genetic markers could be used to identify the representative genotype family to which the isolate belongs. In principle, the fastest evolutionary marker could also be used to identify successive cases in a chain of transmission, provided that the time interval between these cases is short. Given the long-term interval required to culture the necessary quantity of bacilli for each multiple genetic marker assay, these techniques at present may be unpractical for answering urgent questions regarding e.g. nosocomial transmission.

Ideally, the whole genome of each isolate should be visualized to facilitate a broad characterization, but it may take some time before this will be possible. However, a combination of genetic markers useful to examine the epidemiology

of tuberculosis at different levels at once is in reach. Genomic deletions as unidirectional evolutionary markers appear able to broadly distinguish different taxons in the complex. Depending on the techniques used for such a multiple molecular marker assay, MIRUs [77] and spacers from the DR region [44] could serve as the next tools used in the molecular characteriszation of *M. tuberculosis* complex isolates. The polymorphism associated with one of the most discriminative markers – IS*6110* – should also be included, especially in combination with the above-described genetic markers. The use of IS*6110* as an evolutionary marker is restricted by the fact that some IS*6110* elements have been found at preferential insertion sites [145]. For example, Fang et al. [150] have found independent occurrence of IS*6110* insertions at the same sites in the genome of epidemiologically unrelated *M. tuberculosis* isolates and this has been supported by the demonstration of convergent evolution on the basis of mutational events of IS*6110* [42, 151]. Given the rapidly improving possibilities for developing an efficient multiple molecular marker assay for *M. tuberculosis* complex isolates, this does not seem to be a problem.

Conclusions

In the last decade, an unprecedented level of genetic polymorphism has been identified among *M. tuberculosis* complex bacteria. Our ideas on the structure of the *M. tuberculosis* genome have evolved greatly: whereas we previously believed that the *M. tuberculosis* complex was genetically highly conserved, as demonstrated by the pioneers of the restriction enzyme analysis technique [152], we have since recognised tens of thousands of different *M. tuberculosis* strains on the basis of DNA polymorphism. This has contributed significantly to an improved description of subspecies in the complex and has also provided the basis of the development of molecular epidemiological typing methods, which have altered the way we study transmission of *M. tuberculosis.* In the long run this should lead to both an improved understanding of the natural history of tuberculosis and control of this 'global emergency'.

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Vaccination – The Current Status of BCG

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History of BCG Vaccine

BCG vaccine is one of the oldest vaccines as well as one of the most controversial (table 1). Even the name 'BCG' opens the door to one of the world's oldest and most fascinating of vaccines. Originally it was called 'Bilieux de Calmette et Guérin'. The letter 'b' signified bile because Calmette and Guérin used a culture medium with beef bile to diminish the bovine bacillus virulence that was obtained after 231 passages or subcultures between 1908 and 1919 at the Pasteur Institut, Lille, France. The original virulent bovine bacillus strain was lost during the German occupation on northern France during the World War I [1]. The original *Mycobacterium bovis* strain was isolated in 1901 by Nocard from the milk of a cow with tuberculous mastitis and donated to the Pasteur Institute in Lille in 1901. This strain was kept by Calmette in the laboratory and the attenuation process was initiated in 1908 [2]. The organism used in the vaccine was finally obtained in 1919 through a long process of attenuation subcultures and was first given to a human as an oral vaccine in 1921. Despite its long history, the mechanism of protection, its composition and its protective effect against adult disease remain incompletely understood. Notwithstanding these gaps in understanding, BCG is the mostly widely administered vaccine in the world today, with over 100 million doses given each year, mostly to newborn infants.

Part of Tuberculosis Prevention and Control

Tuberculosis (TB) emerged as a major concern in the aftermath of World War II, and the use of BCG was subsequently encouraged in many countries,

Table 1. Strengths and weakness of BCG vaccine

Strong points	Effective against disseminated forms of disease in infants
	Long track record of safety
	Low cost
	Heat stable in freeze-dried form
	Potent adjuvant
	Scar indicates a dose received
	Positive effect on control of other diseases
	No alternative vaccine available
Weak points	Difficult route of administration, especially in infants. Needs special route (intradermal), special syringe and needle and special dose (0.05 ml for the newborn and neonates, for older infants, children and adults the dose is 0.1 ml)
	Uncertain protection against adult disease
	Significance of no scar remains uncertain
	Frequent mild side effects
	Concern in HIV-infected areas
	Problems related to reconstitution and potential for unsafe injection practices
	Fragile vaccine supply
	Difficulties with quality control

stimulated in particular by UNICEF and by Scandinavian Red Cross Societies, and then by the World Health Organization (WHO). Major trials were set up by the British Medical Research Council (BMRC) and by the United States Public Health Service (USPHS) in the early 1950s. It was soon evident that the procedure employed by the BMRC (Copenhagen strain, given to tuberculin-negative 13-year-olds) provided high efficacy against TB. In contrast, that used by the USPHS (Park or Tice strains given to tuberculin-negatives of various ages) provided very little protection. On the basis of these results, the respective public health agencies recommended BCG as a routine for tuberculin-negative adolescents in the UK, whereas BCG was not recommended for routine use in the USA, but restricted to certain high-risk populations.

The majority of the world followed the lead of Europe and the WHO, and introduced BCG, first in campaigns targeted at all children, and then as a routine vaccination according to various schedules (e.g., at birth, school entry or school leaving). However, the Netherlands and the USA decided against routine BCG vaccination. Instead, they based their TB prevention strategy entirely upon reduction of sources of infection, by case finding and treatment, and including contact tracing and the use of tuberculin to identify infected individuals eligible for preventive therapy. BCG was incorporated into

the infant immunization schedule of WHO's Expanded Programme on Immunization (EPI) in 1974 [3].

BCG Vaccines

The original BCG seed was a live *M. bovis* strain attenuated by passage on culture medium that contained glycerol, potato slices and beef bile. It was distributed to several laboratories from which each produced its own BCG and maintained it by serial passage. A stabilizer was introduced in the process of freeze-drying in the late 1950s. The strains suffered spontaneous mutational changes during the periodic passages (every 3 or 4 weeks) through culture media in such a way that after several years there were clear differences even in the macroscopic properties among the several strains kept in different laboratories. In the late 1950s, during the freeze-drying tests, the properties of the strains were purposely modified to make them more resistant to the freeze-drying process, since freeze-drying kills many bacilli. No adjuvant or preservative is used [4]. The diluent is either saline solution or distilled water [5]. Four main strains account for more than 90% of the vaccines currently in use worldwide.

The modern vaccine is a freeze-dried powder that must be reconstituted before use and is generally contained in a multidose vial. Once reconstituted, the vaccine must be protected from ultraviolet light (sunlight), kept cool and discarded within 6 h, whether or not all the doses have been used, thus minimizing chances of contamination. In addition, once reconstituted, the BCG bacilli start dying in such a way that the viability is gradually reduced.

Protection against Disease

It has been problematic to establish the precise protection afforded by BCG. The Indian trial at Chingleput was intended to test the efficacy of BCG in a large population, 360,000 persons of all ages. However, the efficacy was measured through the incidence of culture and smear-positive pulmonary TB. Since very few cases were positive in children under 5 years, they were excluded from the analysis and thus provided no data on efficacy in infancy [6, 7]. Its results, therefore, did not influence EPI policy, and BCG use continued to increase.

All the evidence showed that BCG was effective in infancy. The variation in efficacy was observed in older children and adolescents. The hypotheses that explained the lack of efficacy of BCG in some areas did not apply to infants

and young children – infants and young children are vaccinated before they can become infected with environmental mycobacteria.

There are no reliable immunological markers of protection against TB, making the definition of end-points in studies difficult. For this and other reasons, estimates of efficacy have varied widely. Clinical efficacy in preventing pulmonary TB has ranged from zero protection in the southern United States and in Chingleput, South India, to approximately 80% in the UK [6, 8]. This variation does not apply to infants [9].

There is no consensus on the reasons for this variation. Efficacy is not readily predictable based on strain or manufacturer. Some studies suggest that efficacy is reduced if there has been prior sensitization by environmental mycobacteria, but the evidence is not consistent. The degree of protection has not correlated with the degree of tuberculin test sensitivity induced by immunization, nor with BCG scar size. Studies of TB in adults who were vaccinated with BCG in infancy have not shown a clear protective benefit [10, 11]. As a result, the mainstay of TB control programmes has focused on case finding and treatment.

While questions have been raised about the overall efficacy of BCG in protecting against adult forms of the disease, there is convincing evidence that it is highly successful in protecting against disseminated disease in the first year of life. Protection conferred against tuberculous meningitis and miliary TB is estimated to be 75–80% [12, 13].

In Zambia, the percentage of children with BCG scars (83%) was the same for HIV-infected children with and without TB, indicating no evidence of protection from BCG vaccination [14], but this study did not have sufficient power to evaluate protection against tuberculous meningitis or miliary disease.

Vaccine Safety

Reactions to BCG are predominantly related to infection by the live attenuated bacterium, and to errors in achieving correct intradermal inoculation – a difficult field technique. While many vaccines have come under criticism from anti-vaccine lobbyists in the last decade [15], BCG has largely escaped. This is probably because the most active anti-vaccine groups operate in countries where the vaccine is not administered routinely to infants.

Side Effects

It has always been acknowledged that BCG causes local reactions at the site of injection. In 90–95% of vaccine recipients, BCG causes a specific lesion that starts as a papule 2 or more weeks after vaccination. This then becomes

ulcerated and heals after several months, leaving a scar. Particularly in African and Asian individuals a keloid scar may develop. In some children, the nodule has its full evolution without causing the skin ulceration nor leaving a scar. Thus the lack of scar does not imply lack of protection – the bacilli have nonetheless multiplied within the body and induced the immunity. The duration of suppuration may alter the willingness of mothers to allow their children to receive other antigens [16]. More serious local reactions have also been described [17]: limited lupoid reaction, lasting a few months, keloids, and tuberculous lupus (1/200,000 inoculations) has been reported [18, 19].

Mild Adverse Events

Mild reactions are mostly local with or without regional manifestations. Local reactogenicity differs between vaccines, varying with both strain and number of viable bacilli. Thus the Pasteur and Copenhagen strains have generally been found to be more reactogenic than the Tokyo, Glaxo or Brazilian (Moreau) strains [5]. There were several reports in the late 1980s of 'outbreaks' of BCG reactions, manifested as large ulcers and local lymphadenopathy or suppurative lymphadenitis. At this time, changes in vaccine availability led many programmes to switch from the less reactogenic Glaxo1077 to the more reactogenic Pasteur 1173P2 strain without staff being notified of the necessary change in dosage it implied (in Austria [20], India [21], Jamaica [22], Mozambique [23] and Zimbabwe [24]).

Axillary or cervical lymphadenitis usually heals spontaneously and it is best not to treat the lesion if it remains non-adherent to the skin. An adherent or fistulated lymph gland, however, may be drained and an anti-TB drug may be instilled locally. Some authors recommend systemic treatment of severe persistent lesions with erythromycin [25], while others have tried systemic treatment with isoniazid [26] and local streptomycin with aspiration [27]. However, lesions have persisted for 1 month after therapy with either drug [26].

Local and regional *suppurative lymphadenitis* is now becoming rare, especially when BCG inoculations are performed by well-trained staff, with a standardized freeze-dried vaccine and a clearly stated individual dose depending on the age of the vaccinated subjects.

Severe Adverse Events

Osteitis: Osteitis/osteomyelitis is a rare and severe consequence of BCG vaccination that occurs some years after administration of the vaccine and is typically associated with changes in BCG vaccine strain. An increase in osteitis to 35 per million occurred in Czechoslovakia after a shift from the Prague to Russian strain BCG [28]. Both Finland and Sweden reported increases in

osteitis after 1971, when they shifted to a Gothenburg strain produced in Denmark. Sweden reported rates as high as 1 in 3,000 vaccine recipients, which declined rapidly when the national programme shifted to a Danish (Copenhagen 1331) vaccine strain [28]. Osteitis in Scandinavian countries seems to be mostly linked to the Göteborg strain [29]. The incidence rate of such complications ranged from 15 to 73 per 100,000 vaccinated between 1971 and 1978. Dittmann [30] quotes a frequency between < 0.1 and 30 per 100,000 vaccine recipients.

Tuberculous meningitis: This has been described [31], but is exceptional.

Generalized infection: This is rare and is generally limited to children with severe immune deficiencies. A multicentre study has identified the syndrome in children with severe combined immunodeficiency (SCID), chronic granulomatous disease, Di George syndrome and homozygous complete or partial interferon-γ receptor deficiency [32–34]. Its frequency is reported as less than 5 per million vaccine recipients, reflecting the rarity of the underlying conditions [28]. If not properly managed, these cases may be fatal. The first case was reported in 1953 [35], 30 years after BCG had first been given to man. Between 1954 and 1980, 34 cases were published in the global literature, one study [36] estimated the incidence as 2.19 per 1 million vaccine recipients. Severe and generalized BCG infection that may occur in immunocompromised individuals may be treated with anti-tuberculous drugs including isoniazid and rifampicin [37].

BCG in HIV-infected infants: More than 28 cases of disseminated BCG have been reported in HIV-infected children and adults [38, 39]. This complication has undoubtedly occurred in more HIV-infected individuals than reported in the published literature since the diagnosis cannot be made on clinical criteria alone and requires laboratory facilities to culture the organism and differentiate it from other mycobacteria [40, 41]. Disseminated disease usually occurs several months to years after vaccination, but was reported in one 30-year-old person with HIV infection who was vaccinated at birth [41]. Disseminated BCG infection is more likely to occur when the vaccine is administered to individuals with clinical AIDS or advanced immunosuppression. With progressive immune suppression, reactivation of latent BCG organisms can occur causing regional or disseminated disease [41, 42]. In one study, however, no cases of disseminated BCG were found among 155 adult patients with AIDS who received BCG in infancy and whose blood was cultured for mycobacteria [11].

Around 1.5 billion subjects had already been vaccinated before EPI was launched in 1974. Around 100 million neonates have been vaccinated each year with BCG since then. Remarkably few adverse events have been reported, considering these figures.

Tuberculin Testing

A number of diagnostic skin tests (e.g., Heaf, Tine, Mantoux) have been devised using antigenic components of the organism called tuberculins [43]. After injecting a small amount of a tuberculin into the skin (usually on the forearm), the resulting indurated area is measured. From this, some estimate can be made of the body's past exposure to tuberculous infection, environmental mycobacteria or BCG vaccine – environmental mycobacteria infections interfere with the interpretation of the tuberculin sensitivity as a result of the cross-reactions they induce.

Tuberculin testing has value in diagnosing active infection, and in deciding whether or not to administer an adolescent or adult dose. However, all neonates are tuberculin-negative; therefore, there is no point to recommend tuberculin test prior to vaccination.

WHO does not recommend any screening prior to administration of BCG in the first year of life with the exception of avoiding the vaccine in those suspected of being symptomatic for HIV infection. Administration of BCG to tuberculin-positive children does neither good nor harm. But not giving a tuberculin tests prior to BCG avoids the cost of the tuberculin test as well as avoiding missing the vaccination in those children who may not come back for the reading of the tuberculin test.

The tuberculin skin test is the only practical tool for determining the response to BCG vaccination (there are other possible immunological markers), but the diameter of the skin test following immunization is not a good predictor of protection against *Mycobacterium tuberculosis* disease [44]. In Rwanda, only 37% of HIV-infected infants developed a skin test response >6 mm diameter after BCG vaccination, as compared with 57% for HIV-uninfected infants born to HIV-infected women and 70% for infants born to HIV-uninfected women [45].

Public Health Strategies

Improved living standards (where children no longer slept in close proximity to parents with open pulmonary disease), coupled with anti-tuberculous medication resulted in reasonable control of the disease up to the 1980s in many countries. However, TB was declared a global emergency by the WHO in 1993, *M. tuberculosis* then being considered to be responsible for more adult deaths than any other pathogen (it has since been displaced to second place by HIV infection). Control of TB now relies upon chemoprophylaxis and the ascertainment and treatment of cases, in particular employing the 'directly observed therapy – short course' (DOTS) approach [46].

It is clear from the huge number of doses of BCG administered, and the continuing toll from TB, that BCG cannot be relied upon as the main public health tool for combating TB. Nonetheless, its demonstrable ability to protect against the deadly disseminated spread of the disease in infants and children has assured BCG a place in national immunization programmes. WHO recommends that all countries with a high incidence of TB infection should offer a single dose of BCG at or soon after birth. Multiple repeat doses of BCG are not recommended [47].

Schedule

BCG vaccination policies differ greatly between countries, as shown in table 2. The various policies may be broken down into five groups: (1) *BCG only at birth* (or first contact with health services): this is the current recommendation of the EPI, and is the policy in most of the world today, in particular in developing countries; (2) BCG once in childhood or adolescence; (3) repeated/booster BCG; (4) no routine BCG use, and (5) high-risk individuals.

These five categories reflect national schedules, but countries also differ in the level of legal compulsion attached to their vaccination programmes, and as to whether formal written 'informed consent' (by a parent or guardian) is required before vaccination. In those European countries recommending universal BCG vaccination, the vaccination is considered compulsory in 29 countries, and voluntary in 7 others. All these policy differences are based upon regional differences in patterns and perspectives of TB, regional variations in health systems (economics, relative emphasis on preventive and curative services, manpower), and local history (personalities and 'schools' of opinion). Justifications for the various policies are embedded in the medical and public health teaching and traditions of the countries involved. Whether it is optimal to vaccinate in the first few days of life, or preferable to wait 1 or 2 months, has often been discussed but remains unresolved. Immunological studies are currently underway to compare the immune response to BCG when given at birth or at 2 or 4 months of age. This subject will need to be reviewed in the context of continuing research on cellular immune responses in early infancy and childhood.

Repeat Vaccination

Though many countries still recommend repeat vaccination, this is not endorsed by WHO. There is surprisingly little information on which to base this

Table 2. Summary of number of countries reporting on particular BCG immunization policies, by WHO Region (compiled from a survey in 1995 by a WHO Regional Office, a special survey by EPI Geneva in 1998, and data routinely reported to EPI Geneva)

WHO Region (total number of countries)	Number of countries reporting by special survey[a]	First BCG given at birth	First BCG given after infancy	Recognized contra-indications (number of countries)	Special risk groups (number of countries)	Booster given	Indications for booster	Vaccine coverage in millions (%)[b]
AFR (46)	Nil	46	0	No info	No info	0	–	16.9 (66)
AMR (44)	Nil	30	2	No info	No info	0	–	14.9 (97)
EMR (24)	21	18	3	Mothers with AIDS (7) HIV (9) Household contacts of AIDS (2) Tuberculin-positive (10) BCG scar (12) Other (4)	Medical (1) TB contacts (1) Students (1) Industrial workers (1)	7	No scar (6) Tuberculin-negative (1)	14.7 (89)
EUR (49)	47	34	8	No info	No info	30	Risk group (2) Military (1)	9.6 (89)
SEAR (10)	8	10	0	Mother with AIDS (1) HIV (2) Household contact of AIDS (1) Tuberculin-positive (2) BCG scar (5) Other (1)	None reported	3	No scar (3) Tuberculin-negative (1)	35.3 (97)
WPR (25)	Nil	18	1	No info	No info	8	No info	26.6 (94)

[a] Countries not reporting officially to WHO on BCG: AMRO (12); EMR (3); EUR (5); WPR (6).

[b] Vaccine coverage as reported to WHO for 1997 as a percentage of newborns, and an estimate of the number (millions) of infants immunized and not taking into account infant death. The number of doses consumed will be much higher due to high vaccine wastage rates in some countries. Additionally, the coverage is calculated only on those countries that report – large countries not reporting can skew the average.

decision, one way or another. One of the few formal evaluations of repeat BCG was carried out in Malawi, where the study found the second dose had no effect on (pulmonary) TB, but provided increased protection against leprosy [48]. Another more recent study in Hong Kong showed no benefit, but may have been less sound in its design [49].

Scar and Protection

The implications of the presence or absence of a post-vaccination scar for protection against disease have yet to be determined, though one study found no evidence of a relationship between scar size and protection against either TB or leprosy [50]. In a review of ten randomized controlled trials, it was concluded that BCG efficacy probably decreases by 5–14% a year and therefore it does not give any further protection 10 years after vaccination [51]. Such information is essential both for estimating the impact of BCG vaccination programmes and for rational decisions on the utility of repeat vaccination.

Vaccination of High-Risk Individuals

Countries identifying populations at high risk of TB have frequently chosen to offer BCG only to these high-risk groups or populations. Alternatively, countries have offered BCG to adolescents when they are considered to be entering a higher risk period of their lives. Other high-risk groups are as follows:

Health Care Workers

Health care workers have long been recognized as being at high risk from TB. They are also particularly likely to be exposed to drug-resistant *M. tuberculosis*. A review of the effectiveness of BCG in tuberculin-negative nurses and physicians noted that the cumulative data were consistent in showing appreciable protection, though the studies were not always methodologically rigorous [52]. A cost-benefit model explored the utility of BCG in a health care setting in the USA, and concluded that even a vaccine with only 13% efficacy would be worthwhile [53]. Such arguments have been voiced to encourage a return to a policy of selective use of BCG among health care professionals in the USA (a policy abandoned in 1988). The special case of health care workers will need continued attention as additional countries give up routine BCG, particularly if the incidence of drug-resistant infection increases.

Infants of Immigrants from High Incidence Countries

Industrialized countries accepting immigrants from countries where the incidence of TB is high may offer BCG to newborn infants of such immigrants, and skin testing and immunization as appropriate to older children of immigrants.

HIV-Infected Individuals

Symptoms of immunodeficiency rarely appear before several months of age in neonates infected at birth. The study conducted by O'Brien et al. [38] confirmed the absence of severe adverse events in asymptomatic children infected with HIV and immunized with BCG at birth. Evidence to date supports the current policy of withholding BCG from those with clinical evidence of immunosuppression, and not on the grounds of HIV sero-positivity alone [54, 55]. A requirement to screen infants for HIV infection before vaccination is not feasible. In addition, the current policy has the advantage of providing protection to HIV-positive and -negative children who are at high risk of exposure to TB because their mothers may have HIV. The extreme rarity of reports of systemic BCG-osis in adult AIDS patients [56] may reflect that viable BCG does not remain long in most vaccinated individuals, or that the diagnosis is frequently missed.

To prevent any risk of generalized infection with BCG in these patients, WHO recommends giving BCG to neonates as soon as possible after birth in countries where TB is an important public health problem, *except* in individuals with clinical symptoms of AIDS [54, 55].

Babies Born to Infected Mothers

Babies born to mothers who have developed TB disease shortly before or shortly after delivery will not be protected by BCG given at birth quickly enough to avoid the risk of becoming infected. Such newborns should be given prophylactic chemotherapy using daily isoniazid (5 mg/kg) for 6 months. BCG can then be given after chemotherapy ends. BCG is not contraindicated in these infants, but if given, it will be inactivated by the prophylactic chemotherapy and the infant will need re-immunizing later [57].

Criteria for Discontinuation

Despite WHO's encouragement to continue with the use of BCG, a number of industrialized countries have shifted from routine to selective BCG vaccination. The International Union Against Tuberculosis and Lung Disease (IUATLD) [58] has suggested criteria under which it may be reasonable for a

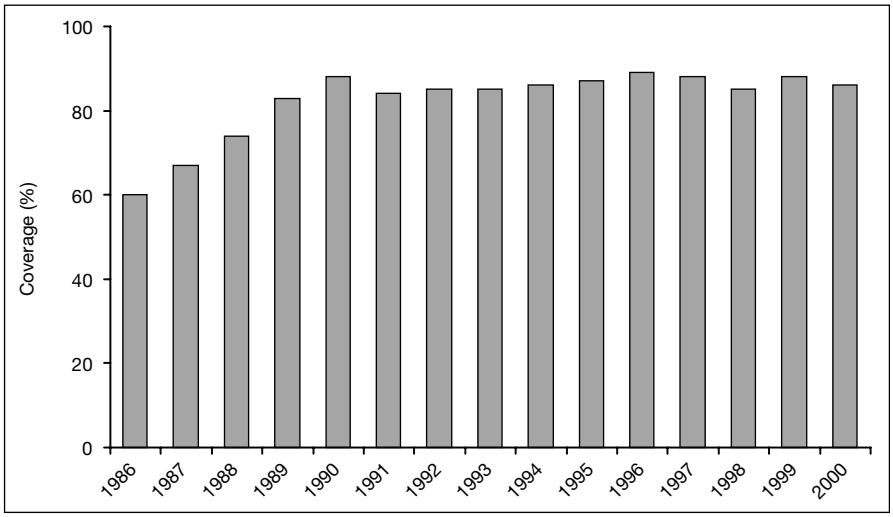

Fig. 1. Annual global coverage (%) of infants with BCG vaccine by 12 months of age as reported to WHO, 1980–2000 (source: WHO Vaccine-Preventable Disease: Monitoring System. 2001 Global Summary. WHO/V&B/01.34. Geneva, WHO, 2001).

country to shift away from routine BCG vaccination only if: an efficient notification system is in place, *and either* the average annual notification rate of smear positive pulmonary TB is less than 5 per 100,000, *or* the average annual notification rate of tuberculous meningitis in children under 5 years of age is less than 1 per 10 million population over the previous 5 years, or the average annual risk of tuberculous infection is less than 0.1%.

Further work is needed on the benefit-cost ratio of BCG as opposed to other approaches to TB control. One argument favouring discontinuation of BCG is based on the advantages inherent in the absence of non-specific BCG-induced tuberculin sensitivity. This would facilitate the use of tuberculin testing for contact tracing, source of infection identification and selection of individuals for preventive therapy. Although this is a valid argument, many years would have to pass after discontinuation of routine BCG vaccination before a population was completely replaced by unvaccinated individuals.

Global Uptake of BCG

Approximately 100 million children annually now receive one or another BCG vaccine (fig. 1).

Administration

Historically, BCG vaccination was first administered orally. This route was favoured by Calmette and Weill-Hallé in France in the 1920s [59], but workers particularly in Sweden soon began to experiment with intradermal administration [61]. Intradermal or percutaneous administration was ultimately favoured for four reasons: (1) oral vaccination required much larger doses of BCG (e.g., from 10 to 300 mg, compared with 0.1 mg for intradermal injection) and hence was more expensive; (2) it proved difficult to control the effective dose with oral administration, as some viable bacilli were inactivated in the stomach; (3) intradermal administration proved much more efficient at inducing tuberculin conversion, and (4) there were reports of cervical lymphadenopathy attributed to oral administration.

The last country to use oral administration of BCG was Brazil, where this practice was discontinued only in 1973.

Most current BCG vaccines are given by the intradermal route, generally by injection with a 25- or 26-gauge needle, in the deltoid insertion region of the upper arm. Some countries (e.g., Japan, South Africa) have employed percutaneous administration with special multipuncture devices. Other techniques have been tried, but found inferior either because of inconsistent dose delivery or adverse reactions (e.g., with jet injectors [61] or low tuberculin conversion rates (bifurcated needle) [62]).

Most manufacturers (including all those providing vaccine for UNICEF) recommend a 0.05-ml dose for infants. Children and adults generally receive twice this amount, i.e. 0.1 ml.

Intradermal administration is problematic because it is technically harder to give the full dose into the correct tissue layer of a newborn infant who may have a small arm circumference and little or no subcutaneous fat. If given correctly, a bleb or 'peau d'orange', a pitted area of skin is raised at the injection site. A special low-volume syringe is used capable of delivering accurately 0.05 ml to infants and 0.1 ml to children and adults.

Over the years, the type of syringe has altered. Originally it was a glass re-usable syringe and re-usable needle. It was then replaced in the 1980s with a disposable needle and syringe, and currently an autodisable syringe has already been approved for supply by UNICEF and is expected to become the syringe of choice in the first decade of this century.

In pursuit of an easy and safe way of administering the vaccine, a number of alternative methods of giving the vaccine have been tried in the past (e.g., aerosol, liquid vaccine and a multiple puncture gun, scarification). In theory, transcutaneous immunization sounds practical, but has not yet been tested.

Contraindications

Because it is a live organism and could theoretically infect the placenta and damage the fetus, BCG is generally contraindicated in pregnancy. Industrialized countries tend to have stricter guidelines on other contraindications than do developing countries, generally reflecting the different abilities of their health services to ascertain relevant information and to provide alternative preventive services to individuals in particular categories. For example, BCG is contraindicated in the UK for individuals with impaired immunity, with malignant conditions, and also to anyone who is HIV-positive, pregnant, tuberculin-positive, febrile, or with a generalized septic skin condition. This contrasts with the current WHO guidelines for BCG use in infant immunization schedules that mention only 'symptomatic HIV infection (i.e. AIDS)' as a contraindication for BCG. Importantly, being HIV-positive in the absence of clinical signs of impaired immunity is not considered a contraindication by WHO.

Protection against Other Diseases

BCG has long been recognized as an immune modulator, and has been tried in the treatment of various diseases. It has been used in the treatment of neoplasms [63]. At least two diseases related to TB have been reported to be controlled to some extent by administration of BCG.

Leprosy: The rapid decline of leprosy observed in many countries of Africa has coincided with the introduction of wide-scale use of BCG there. A number of controlled trials and approximately ten observational studies have all shown some protection against leprosy, ranging from 20 to 80%. Comparisons between protection by BCG against TB and leprosy in the same populations suggests protection is greater against leprosy [64].

Buruli ulcer: There is also evidence that BCG provides some protection against Buruli ulcer (*M. ulcerans* infection), and against glandular disease attributable to various other 'environmental' mycobacteria, in particular *M. avium intracellulare* [65].

Non-specific effect: A study in Guinea-Bissau suggested that BCG may have a non-specific protective effect on the survival of infants, irrespective of any protection afforded against TB, its complications or other TB-like infection [66]. This observation remains to be confirmed by other studies.

As a Vaccine Delivery Vehicle

There is much interest in the use of BCG as a live vector to deliver a variety of recombinant antigens. Such potential offers another reason for maintaining BCG in the infant immunization schedule, thereby readying it for use, should a strategic vaccine be developed that requires BCG as a delivery vehicle.

Vaccine Manufacture

Strains

Four main strains account for more than 90% of the vaccines currently in use worldwide: the French Pasteur strain 1173P2, used in 14 countries for their own production, the Danish (Copenhagen) strain 1331, the Glaxo strain 1077 derived from the Danish one and the Tokyo strain 172. Despite WHO's attempts to standardize production and vaccine characteristics, by stabilization and lyophilization, there is a wide range of live particles per dose. According to immunogenicity in animal models, some vaccines (Pasteur 1173P2 and Danish 1331) are called 'strong' strains, whereas Glaxo strain 1077 and Tokyo 172 are called 'weak' [67]. But it is difficult to demonstrate that one strain is clearly superior to another in the protection of human beings. Adverse events differ somewhat between 'strong' and 'weak' strains.

BCG vaccines are currently produced by 40 or more manufacturers around the world. The major commercial producers in terms of export volume are Pasteur-Mérieux-Connaught, Evans-Medeva, and the Japan BCG Laboratory, which together accounted for 85% of over 200 million (infant, 0.05-ml) doses provided through UNICEF annually.

BCG has never been cloned, and there are now several different BCG seed strains ('sub-strains') in use in BCG manufactures, and several different methods of BCG culture. Given the continued controversies over BCG vaccines, and the possibility that differences among vaccines may be responsible for some of the observed differences in efficacy, it is important to appreciate the variation inherent in today's BCG vaccines.

Aside from small quantities of liquid BCG produced for local use, all of today's BCG vaccines are provided in freeze-dried form. The freeze-drying process, in addition to the particular culture methods employed by different manufacturers, leads to considerable differences in the numbers and proportions of viable and dead organisms per dose of vaccine. It is recognized that this has implications both for reactogenicity (measured in terms of the size of the local lesion) and for the induction of delayed type hypersensitivity [68].

Table 3. BCG vaccine quality by economic status of producing country in 2000

Place of manufacture	Percentage of total producers (n = 18)	Quality of BCG vaccine	Percentage of total production
Industrialized	22	All pre-qualified for sale to UN agencies	42.6
Developing country or economy in transition	78	Pre-qualified for sale to UN agencies	12.8
		Assured quality[a]	8.9
		Unknown quality	35.7
Total	100		100

[a]Assured quality means the vaccine is manufactured in a country with a fully functional national regulatory authority to ensure the quality of the vaccine before release.

Source: Information supplied to WHO, 2000. The information was drawn from a study undertaken for WHO by Ms Susan McKinney in 2000.

Vaccine Quality, Supply and Distribution

The current situation regarding a reliable global supply of high-quality vaccine is extremely fragile. BCG vaccines are currently produced by approximately 18 manufacturers around the world (table 3). Three quarters of the number of doses are produced in developing countries or countries with an economy in transition, mostly for local consumption. In 2000, one quarter of the world's BCG production was purchased on a competitive basis by UNICEF, although the amount the Fund purchased was slightly less than half of that offered by pre-qualified manufacturers. Much of the remaining vaccine is produced within the countries themselves, for local use. Over the last few years, the number of manufacturers producing BCG vaccine globally has dropped by half, increasing dependency on the remainder. Currently, only five manufacturers make BCG vaccine that is WHO pre-qualified for sale to UN agencies (including UNICEF). This is potentially a serious situation – if only one or two key manufacturers were to withdraw from the UNICEF purchase process, global supply of high-quality BCG would be seriously compromised. The element of commercial competition is diminished, with the potential for pushing up prices.

Table 4 does not immediately cause concern with regard to the maximum global production capacity. However, the maximum capacity indicated by manufacturers represents an extreme case and for it to be achieved, would require a shift to the production of BCG away from the production of other vaccines.

The quality of BCG production leaves much to be desired in many facilities. Very few manufacturers are able to implement even the most basic aspects

Table 4. Sub-strains of BCG currently used in manufacture, by doses produced per year, as of 2000[a]

Sub-strain	Number of manufacturers reporting	Doses produced per year (millions)	Doses produced that were pre-qualified for sale by UN agencies, %	Maximum production capacity per year (millions)
Russian	2	34	0	150
Glaxo 1077	2	54	100	70+
Pasteur 1173	5	54.1	0	108.8
Sofia (SL 222)	1	60	100	60
Tokyo 172	1	65	100	100
D2PB302	5	80	0	90+
Copenhagen 1331	2	120	66	300
Total	18	467.1	55.4	879+

[a]Based on information supplied to WHO during 2001.

of Good Manufacturing Practice. Instead, BCG is produced as a laboratory product with each lot showing variability, thus consistency of production and of efficacy are impossible to demonstrate.

The current quality control measures emphasize viable counts and the ability to induce tuberculin sensitivity. While intuitively reasonable, there is little or no evidence that these measures are meaningful, let alone optimal, as correlates of protection. Studies are now underway to evaluate other potential correlates, such as the ability to induce cytokine secretion on exposure to mycobacterial antigens. It is hoped that these will lead to more relevant means of testing vaccines. Moreover, in addition to the shortcomings in the quality control tests being used, many BCG vaccines are still subject only to final product testing as a means of assuring quality.

New Vaccine Development

It is estimated that only about 5% of potentially vaccine-preventable, TB-related deaths are actually averted by BCG. This illustrates the need for an improved vaccine. Recent scientific advances, such as the complete sequencing of several mycobacterial species including a laboratory strain and a clinical isolate of *M. tuberculosis*, have significantly increased the likelihood for the construction of a superior TB vaccine. Furthermore, the highest level of political commitment as well as new sources of support from public funding agencies and private foundations have all created an institutional and economic climate

favourable for such development. Major challenges include developing, evaluating and producing a vaccine that: (1) will protect against adult pulmonary disease; (2) can be used in populations where BCG has already been used widely; (3) can be used where there is a high prevalence of non-specific tuberculin sensitivity, and (4) can be used where a high proportion of adults have already met the tubercle bacillus.

TB vaccine development is making rapid progress; dozens of candidate antigens are currently passing through different phases of pre-clinical research, and phase I clinical trials are imminent. Through its Department of Vaccines and Biologicals, WHO is playing an active role in many aspects of this process, notably in identifying immune markers of protection as well as designing phase III trials, and identifying appropriate sites where these studies can be performed. It is not yet clear whether candidate vaccines need to be tested in infants who have already received BCG, thus testing the new vaccine in its ability to boost the priming achieved by the BCG vaccine. Until such time as an alternative, superior vaccine is developed, it is likely that BCG will maintain its place in infant schedules as a somewhat mysterious but effective vaccine – possibly for decades to come.

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Laboratory Diagnosis of Tuberculosis

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In the industrialized world, the time has gone when laboratories needed weeks to months to diagnose tuberculosis from clinical specimens. Currently, the mycobacteriology laboratory is experiencing more changes than ever before. In the past decade, new methods for culture and susceptibility testing as well as molecular tools have been introduced which allow a rapid laboratory diagnosis of tuberculosis. Determining which assays will be most useful is a challenge for clinicians and laboratorians who are both aiming at providing the best care for the patient.

Mycobacterium tuberculosis Complex – A Multifaceted Group of Organisms

In contrast to the many non-tuberculous mycobacteria which are capable of replicating in the inanimate environment, the major ecological niche for *Mycobacterium tuberculosis* complex organisms are tissues of humans and warm-blooded animals. This group of obligate pathogens includes several closely related species which display >95% DNA/DNA homology: *M. tuberculosis, Mycobacterium bovis*, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), *Mycobacterium africanum*, *Mycobacterium microti*, and *Mycobacterium canettii* [1–4].

Like all mycobacteria, members of the *M. tuberculosis* complex are aerobic, non-spore-forming, non-motile, slightly curved or straight rods (0.2–0.6 × 1.0–10 μm). The high content of complex lipids of the cell wall, its most important component being mycolyl-arabinogalactan, presents a hydrophobic permeability barrier that prevents penetration of common aniline dyes. Once stained with special procedures, mycobacteria are not easily decolorized, not even with acid-alcohol, i.e., they are acid-fast. Due to a very long generation time (16–18 h

to undergo one cycle of replication), *M. tuberculosis* yields visible colonies only after growth of several weeks on solid media. Colonies of *M. tuberculosis* complex are non-pigmented to buff; colony morphology is predominantly flat and rough (fig. 1a).

M. tuberculosis is the most prominent member of the complex, and globally, among the leading causes of infectious disease [5]. In addition to the classical organism, there exists an 'Asian variant' of *M. tuberculosis* [6], and a new subspecies, *M. tuberculosis* subsp. *caprae* which has been isolated from goats [7]. *M. bovis* causes disease in warm-blooded organisms including primates and humans, while *M. bovis* BCG is still being used for a vaccine in many parts of the world and for intravesical BCG immunostimulation against bladder cancer. In immunocompromised patients, BCG may cause disease [8]. Different phenotypes of *M. africanum* cause tuberculosis in tropical Africa. Although its colonies resemble those of *M. tuberculosis*, the physiological and biochemical properties position *M. africanum* between *M. tuberculosis* and *M. bovis* [9]. *M. microti*, well known for its difficulties to grow on conventional media for acid-fast bacilli (AFB), is evidently not restricted to rodents and other animals since it has recently also been identified as a causative agent of tuberculosis in both immunocompromised and immunocompetent humans [10]. The youngest member of the *M. tuberculosis* complex, *M. canettii*, has been reported to cause lymphadenitis and generalized tuberculosis as well [3, 4]. Its round and smooth colonies differ, however, from those of all other members of the complex and can be mistaken for those of a non-tuberculous mycobacterium (fig. 1b).

Safety

Because of its low infectious dose (minimum approx. 10 bacteria inhaled) and its capacity to survive in droplet nuclei, *M. tuberculosis* has a high potential to cause laboratory infections. Therefore, special safety precautions are paramount when working with specimens potentially containing mycobacteria [11]. Precautions must exceed those normally used in microbiology laboratories. Ideally, a laboratory analyzing *M. tuberculosis* complex must conform to Biosafety Level 3 which includes special measures such as proper directional air flow (negative inside pressure), biological safety cabinets (preferably class 2), leak-proof containers for centrifugation, and other special safety equipment. Persons handling specimens must have previous experience in working in microbiological laboratories and must continuously undergo safety training. Also, personnel health policies should at least include an initial tuberculin test and clinical evaluation of persons with positive tests.

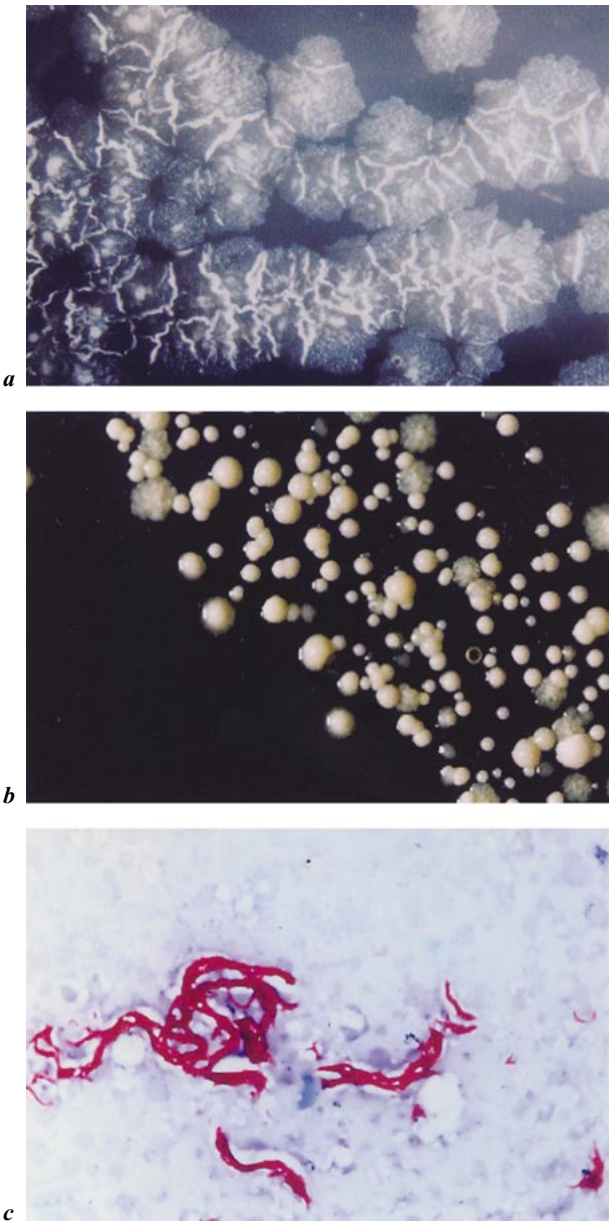

Fig. 1. a *M. tuberculosis* on Middlebrook 7H10 agar. Note the dry and rough colonies with sometimes a nodular or wrinkled surface. ***b*** *M. canettii* on Middlebrook 7H10 agar. Note the predominantly domed and smooth colony morphology (photograph kindly provided by D. van Soolingen, RIVM, Bilthoven/NL). ***c*** Acid-fast staining (Ziehl-Neelsen) of *M. tuberculosis* showing serpentine cording.

Collection and Pretreatment of Specimens for Smear and Culture

Many different types of clinical specimens may be collected for mycobacterial analyses [12–14]. Most often, specimens originate from the respiratory tract, but urine, gastric fluid, tissues, biopsies and normally sterile body fluids are also common. Generally, as much specimen as possible should be submitted to the laboratory. Transport media or preservatives are usually not needed owing to the robust nature of mycobacteria. Also, it is important that no fixatives be added. Swabs are, in general, not optimal for the recovery of AFB since the amount of material to be analyzed is limited and the hydrophobic cell envelope of mycobacteria compromises a transfer of the organisms from swab to media. If processing is delayed for more than 1 h, specimens should be refrigerated at 4°C, except blood and bone marrow.

Normally, sterile specimens such as biopsies or body fluids other than urine and gastric juice usually do not require pretreatment if collected aseptically [12–14]. Tissue and biopsies should be ground in sterile physiological saline before inoculation and body fluids concentrated to maximize the yield of mycobacteria. To check for possible contaminants, any specimen may first be inoculated on a chocolate agar plate.

The majority of specimens submitted for mycobacterial culture, however, consists of a complex organic matrix contaminated with a variety of microorganisms. Such specimens need to be homogenized and decontaminated by adequate procedures [12–14]. Prior to inoculation to the media, pretreated specimens have to be concentrated. Since harsh decontamination can kill 20–90% of the mycobacteria present in a clinical specimen, it is important to apply pretreatment which is efficient in removing unwanted organisms, but at the same time, guarantees maximum survival of mycobacteria. Excellent results are achieved by decontamination with *N*-acetyl-*L*-cysteine/NaOH, sodium dodecyl (lauryl) sulfate (SDS)/NaOH or 4% NaOH alone [12–15]. Once specimens are pretreated, the contamination rate of media should be around 5%. If it is lower, pretreatment was too harsh, if higher, pretreatment was either too weak or digestion incomplete.

Direct Detection by Acid-Fast Microscopy

Smear microscopy is still among the most rapid and inexpensive ways to diagnose tuberculosis and a rapid means to identify the most contagious patients.

While *M. tuberculosis* may be invisible by Gram stain or only faintly staining as ‘ghosts’ (and thus, be missed), special acid-fast staining procedures are necessary to promote the uptake of dyes [12–14]. Phenol and higher temperature

as used in the classical Ziehl-Neelsen staining allow penetration of the carbol-fuchsin stain more easily. When counterstained with methylene blue, the red-to-pink stained AFB are highlighted against a blue background which facilitates their microscopic recognition. Mycobacteria are also capable of forming stable complexes with some arylmethane dyes such as auramine O. The latter is used for fluorescence microscopy which allows a more rapid screening of the slides because they can be read at a lower power. As shown recently, Kinyoun's carbol-fuchsin method is inferior to both the Ziehl-Neelsen and fluorochrome techniques [17].

The reliability of smear microscopy is highly dependent on the experience of laboratory personnel and on the number of organisms present in the specimen. 10^6 AFB/ml usually result in a positive smear. However, only about 60% of the smears are positive if 10^4 AFB/ml are present. The overall sensitivity of the smear ranges from 22 to 80% [18]. In follow-up specimens of patients under treatment, the diagnostic value of the smear is limited since such patients may shed dead bacteria for a long period of time while culture shows no growth.

Specificity of the smear for detection of mycobacteria is very high. Assessing AFB morphology for presumptive identification of mycobacteria at the species level is, however, dangerous. Even though in liquid media *M. tuberculosis* shows serpentine cording (fig. 1c), one has to be aware that cords are also seen with some non-tuberculous mycobacteria (NTM) species [19]. Sometimes, only a minor part of cells of rapidly growing mycobacteria ($<10\%$) may be acid-fast. It is reported that they may not stain with fluorochrome stains [20]. Conversely, there are non-mycobacterial organisms which exhibit various degrees of acid-fastness such as *Rhodococcus*, *Nocardia*, *Legionella micdadei* as well as cysts of *Cryptosporidium*, *Isospora*, *Cyclospora*, and *Microsporidium* spores.

Sputum smears should be done from pretreated, concentrated specimens to increase sensitivity. It is best to confirm positive smears by having them reviewed by another, experienced technician. Good laboratory practice includes the confirmation of a fluorochrome-positive smear by a carbol-fuchsin staining method (e.g., Ziehl-Neelsen) and reporting the result within 24 h upon arrival of the specimen in the laboratory [21].

Direct Detection of *M. tuberculosis* Complex by Nucleic Acid Amplification-Based Methods

Direct detection of *M. tuberculosis* complex in clinical specimens by nucleic acid amplification (NAA)-based assays represents one of the most

significant improvements in mycobacterial diagnostics. Theoretically, tuberculosis can be diagnosed on the day of arrival of a specimen in the laboratory. Home-brew polymerase chain reaction (PCR) protocols amplifying a large variety of chromosomal DNA elements have disclosed new ways to diagnose *M. tuberculosis* complex and, to a lesser extent, NTM [for references, see 22].

This technique, together with other methods, e.g., transcription-mediated amplification (TMA), ligase chain reaction (LCR) and strand displacement amplification (SDA), have been developed and are marketed in user-friendly, kit-based formats, targeting either DNA or RNA to guarantee a high degree of reproducibility and to facilitate its application in a clinical mycobacteriology laboratory. Although NAA-based techniques have the potential to detect mycobacteria other than *M. tuberculosis* complex, current experience is largely based on the diagnosis of tuberculosis with emphasis on respiratory specimens. Only a very few studies have addressed the use of NAA-based assays for extrapulmonary specimens [23]. Most often, these specimens contain small numbers of AFB only. As a consequence, smears are often negative and culture yields *M. tuberculosis* after several weeks only, if at all.

Compared to culture and the clinical picture, sensitivity and specificity for these molecular methods are usually very high in smear-positive specimens (close to 100%). Performance characteristics for smear-negative specimens are, however, lower [for references, see 22], precluding, thus, the general use of NAA-based assays as a screen to safely rule out tuberculosis.

Molecular tests should be requested by the clinician when a rapid confirmation of suspected tuberculosis is important. Because of the high predictive value of a positive smear of respiratory specimens for *M. tuberculosis* ($>$90% [16]), such specimens do not necessarily have to be tested with NAA-based methods. With a positive smear, such tests may, however, be welcome, if (a) chest X-ray is abnormal; (b) the patient is immunosuppressed (HIV-infected, transplant recipient, etc.); (c) there are epidemiological concerns, or (d) there are clinical symptoms but culture remains negative. In the smear-negative situation, NAA-based tests can be very helpful if tuberculosis is part of the differential diagnosis [24].

Results of molecular methods, like other laboratory findings, should always be interpreted in conjunction with the patient's clinical data. At present, NAA-based assays cannot be used as stand-alone techniques, i.e., a back-up by culture is necessary.

When performing NAA-based tests laboratories should make sure that they are proficient in their use. In an international collaborative quality control study that involved 30 laboratories, the reliability of NAA-based methods in detecting *M. tuberculosis* complex was assessed [25]. Results were disappointing

inasmuch as only 16% of all laboratories proved capable to correctly identify presence or absence of *M. bovis* BCG in the samples. Irrespective of the targets and technology used, this study showed that lack of specificity was more a problem than was lack of sensitivity. As a whole, test reliability was not associated with any particular method. Remarkably, many of the participating laboratories did not use adequate quality controls. These facts underline the need for good laboratory practice and reference reagents to monitor the performance of NAA-based assays (including pretreatment of clinical specimens [see also 21]).

Culture – The Backbone of the Diagnosis of Tuberculosis

Despite the advances in direct detection of *M. tuberculosis* complex in clinical specimens by molecular methods, at present culture is indispensable for a number of reasons since it (a) enhances diagnostic certainty, particularly when NAA-based results are available; (b) provides biomass for further identification and antimicrobial susceptibility testing, and (c) detects NTM, in addition to *M. tuberculosis* complex.

For detection of mycobacteria in clinical specimens, the current 'gold standard' consists of a combination of solid and liquid media. Solid and semi-solid media have been known for decades. More attractive are liquid media which offer significantly shorter turnaround times for detection of mycobacteria. In detecting as few as 10^1–10^2 viable organisms/ml of specimen, culture is more sensitive than smear. Also, it is the only reliable means to monitor the effectiveness of therapy in TB patients [26].

Solid Media

Egg-based media such as Löwenstein-Jensen (LJ), Ogawa, or Stonebrink have the capability to bind and neutralize toxic compounds encountered in clinical specimens. Usually, they contain malachite green, a dye which inhibits growth of contaminating organisms. Egg-based media support good growth of *M. tuberculosis* but need, on average, 18–24 days. Since they are non-synthetic, the quality of the ingredients may vary considerably which may affect the reproducibility of results.

Agar-based media, e.g., Middlebrook 7H10 or 7H11, are chemically better defined and show growth of *M. tuberculosis* complex colonies within 10–14 days. Visual examination of the colonies is much easier. Negative aspects of agar-based media are limited shelf-life, higher costs of preparation, and requirement for a CO_2-enriched atmosphere.

Liquid Media

Apart from the biphasic Septi-Chek System (Becton Dickinson Microbiology Systems, Sparks, Md., USA) the radiometric, semi-automated BACTEC 460TB System (Becton Dickinson Microbiology Systems) represented the most efficient and rapid technique to culture mycobacteria for more than two decades and is still used by many laboratories to date. By this method, ^{14}C-labeled palmitic acid as a carbon source in the medium is metabolized by microorganisms to $^{14}CO_2$ which is monitored by the instrument. The amount of $^{14}CO_2$ and the rate at which the gas is produced are directly proportional to the growth rate of the organisms in the medium. For *M. tuberculosis*, an average detection time of 8 days was found for smear-positive specimens, compared to 19 days in non-radioactive, conventional solid media [27]. For smear-negative specimens, an average recovery time of *M. tuberculosis* between 14 days in the BACTEC 460TB and 26 days in conventional media has been reported [28]. Problems associated with the use of radioisotopes and the potential of needle punctures have implied, however, the search for non-radiolabeled and safer alternatives.

Although utilizing different detection principles, the new culture concepts have in common that they are based on non-radiometric liquid media. New developments range from manual systems utilizing simple tubes (MB Redox, Heipha Diagnostica Biotest, Heidelberg, Germany; Mycobacteria Growth Indicator Tube (MGIT), Becton Dickinson Microbiology Systems) to fully automated systems (BACTEC MGIT 960, Becton Dickinson Microbiology Systems; MB/BacT Alert 3D, Bio-Mérieux, Marcy-l'Etoile, France; ESP Culture System II, Trek Diagnostic Systems, Westlake, Ohio, USA).

The non-automated MB Redox technique is based on a modified Kirchner medium which contains a colorless tetrazolium salt as a redox indicator which is reduced to colored formazan by actively growing mycobacteria. AFB can then be detected visually as pink-to-purple pinhead-size particles. Another manual culture method is represented by the MGIT (Becton Dickinson Microbiology Systems) which contains a modified 7H9 broth in conjunction with a fluorescence quenching-based oxygen sensor. Growth of mycobacteria or other microorganisms in the broth depletes the oxygen, and the indicator fluoresces brightly when tubes are illuminated with UV at 365 nm.

Currently, there are several fully automated systems which allow continuous monitoring of mycobacterial cultures: (a) the BACTEC MGIT 960 based on the MGIT technology; (b) the MB/BacT Alert 3D utilizing a colorimetric carbon dioxide sensor in each bottle to detect growth of mycobacteria, and (c), the ESP Culture System II which is based on the detection of pressure changes in the headspace above the broth medium resulting from gas production or consumption due to growth of microorganisms.

All these new, non-radiolabeled systems have similar performance characteristics. In clinical evaluations, recovery rates and time to detection were similar to those of the BACTEC 460TB System and superior to those of conventional solid media (MB Redox [29]; manual MGIT [29]; BACTEC MGIT 960 [30]; MB/BacT Alert 3D [30, 31]; ESP Culture System II [32]). Fully automated systems are certainly less labor-intensive than conventional culture and address safety more appropriately. However, as a consequence of continuous monitoring, these systems are both instrument- and space-intensive. Also, some of the new systems lack the versatility of the BACTEC 460TB System (e.g., no medium available for the inoculation of blood; no other incubation temperature available than 37°C).

Identification of *M. tuberculosis* Complex

The use of non-radioactively labeled nucleic-acid probe kits was a big step forward towards a more rapid identification of mycobacteria 10 years ago. For *M. tuberculosis* complex, probe technology has a sensitivity of nearly 100%, provided that approximately 10^5 AFB/ml are present. Results for culture confirmation are available within 2 h. Specificity of the *M. tuberculosis* complex probe is high as well. It is, however, imperative that tests are precisely done as recommended by the manufacturer. Otherwise, cross-reaction with mycobacteria other than *M. tuberculosis* complex may occur, as documented in rare instances [33]. Nevertheless, gene probes are unable to differentiate within *M. tuberculosis* complex. Identification to the species level is, however, justified for epidemiologic, public health, and therapeutic reasons.

Most strains of *M. tuberculosis* complex encountered in a clinical mycobacteriology laboratory can easily be identified by physiological and biochemical criteria (table 1). For strains with phenotypes which do not match fully with the type strain or do not grow, additional tests and procedures, mainly molecular ones, have to be considered. *M. canettii* and most strains of *M. microti*, for instance, can be confirmed by spacer oligonucleotide typing (‘spoligotyping’ [34]). Other markers are the *oxyR* and *pncA* genes which show intraspecific sequence polymorphisms and are useful for the distinction between *M. tuberculosis* and *M. bovis* [35]. PCR tests for the RD sequences may contribute to a differentiation of *M. africanum*, *M. tuberculosis* and *M. microti* [36]. Recently, a combination of selected conventional methods and comparative genomics based on PCR deletion analysis have been suggested for rapid identification of the members of *M. tuberculosis* complex [37].

Table 1. Tests commonly used for species identification within *M. tuberculosis* complex[a]

Species	NO_3 reduction	Niacin production	Lebek test	T_2H inhibition[b]	Susceptibility to pyrazinamide	Distinct features	Ref.
M. tuberculosis	+	+	–	R	S	~ 5–20 IS*6110* copies	50
						pncA, C at position 164	35
						oxyR, G at position 250	
M. tuberculosis Asian variant	+/(+)	+/(+)	–	S	S	A few to none IS*6110* elements	50
M. tuberculosis subsp. *caprae*	–	–		S	S	*pncA* sequence characteristic to *M. tuberculosis*	7
						oxyR, *katG*, *gyrA* characteristic to *M. bovis*	
M. bovis	–	–	+	S	R/(S)	Lacking spacers 39–43 upon spoligotyping; PZA-susceptible strains, lack in addition, spacers 3 and 16	34, 51
						pncA, G at position 169	35
						oxyR, A at position 285	
M. bovis BCG	−/(+)	−/(+)	–	S	R	Lacking spacers 39–43 upon spoligotyping	34
M. africanum (Sierra Leonean type)	+/(−)	+/−	+	S/(R)	S/(R)		9
M. africanum (Ugandan type)	+	+/−	+/(−)	R/(S)	S		9
M. microti[c]	?[d]	?	?	?	?	'Croissant-like' cell morphology in stained smears; distinct spoligotypes	10
M. canettii[e]	+	−	−	S	S/R	2–3 IS*6110* elements	3, 4
						1 IS*1081* element	
						Distinct spoligotype	

[a]All catalase-negative.
[b]5 μg/ml (T_2H, thiophene carboxylic acid hydrazide).
[c]Grows badly, if at all.
[d]Unknown.
[e]Only two strains characterized for their susceptibility to PZA.

Susceptibility Testing of *M. tuberculosis* Complex

Drug susceptibility testing is mandatory on initial isolates of *M. tuberculosis* and related species from all patients. If culture remains positive over a longer period of time, susceptibility testing should be repeated to monitor a possible development of drug resistance. The new guidelines by the National Committee of Clinical Laboratory Standards (NCCLS) currently recommend to repeat susceptibility testing at least every 3 months [38].

Most experience is available on testing primary antibiotics, i.e., isoniazid (INH), rifampin (RMP), ethambutol (EMB), and pyrazinamide (PZA) by culture-based methods. Although there are three accepted methods for drug susceptibility testing of *M. tuberculosis* (i.e., the absolute concentration method, the resistance ratio method, and the proportion method [39]), the latter is widely used in the Western hemisphere. By this method, for most of the antituberculous drugs, resistance of *M. tuberculosis* is defined as presence of resistant organisms in >1% of a given population of *M. tuberculosis* cells. Therapy will ultimately be successful if the critical proportion of organisms and the critical concentration of a drug (i.e., the concentration which inhibits the wild-type but not the resistant mutants) are strictly defined and accordingly, the strain is fully susceptible to front-line drugs in vitro. Despite more rapid culture-based techniques for drug susceptibility testing, NCCLS, for instance, still considers the agar proportion method the gold standard against which all other, more rapid, culture-based methods have to be tested [38].

Susceptibility results for the primary antituberculosis drugs can be rapidly generated by the BACTEC 460TB. Once resistance to one or more drugs is being detected it is good laboratory practice to confirm it either by a second method (e.g., the agar proportion method) or by another laboratory, since the impact of resistant strains on patient management is obvious [21]. Although susceptibility testing on solid media (e.g., Middlebrook 7H10 agar or LJ) for confirmation of resistance takes quite some time, it is important since it can indicate false resistance due to a mixed population of *M. tuberculosis* and NTM. If resistance to one or several primary drug(s) is being detected, an extended spectrum of drugs may be tested, either by radiometric procedure [40] or the agar proportion method [38]. Above all, susceptibility results should be reported without delay to the health care provider.

In the last decade, antimicrobial susceptibility testing has become a dynamic field spawning many new technologies that may one day prove successful in a clinical mycobacteriology laboratory. Among the leading novel strategies are, again, several of the new, growth-based, non-radiometric methods. Preliminary studies which include susceptibility testing of *M. tuberculosis* to INH, RMP, EMB and streptomycin (SM) by the BACTEC MGIT 960 [41],

the MB/BacT Alert 3D [42] and the ESP Culture System II [43] show excellent overall agreement with the proportion method on both solid and liquid media as well as promising turnaround times comparable to those by radiometry. Susceptibility testing of *M. tuberculosis* to PZA is more difficult due to the requirement of a more acidic pH value in the medium. A first study based on the BACTEC MGIT 960 technique reports good agreement of the results with those generated by the BACTEC 460TB System [44].

Apart from strictly growth-based strategies, an increasing number of approaches have assessed drug susceptibility by identifying alternative markers of drug-resistant metabolic activities. Among those are colorimetry, flow cytometry, bioluminescence (assay of mycobacterial adenosine triphosphate), and quantitation of mycobacterial antigens [for review, see 45]. Mycobacteriophage-based methods appear to be promising as well [46].

Although molecular approaches [cf. 47] would provide the clinician with susceptibility results within 1–2 days, most of these protocols are not yet ready for the clinical mycobacteriology laboratory. Resistance in *M. tuberculosis* complex organisms arise from mutations which are confined to chromosomal DNA and do not involve mobile genetic elements such as plasmids. Molecular research has shown that often more than a single mutation is responsible for drug resistance making the whole issue very complex, particularly since different genes can be involved. Resistance to INH, for instance, appears to be the result of one single or of multiple mutation(s) in the *katG*, *inhA*, *oxyR-ahpC* and/or *kasA* gene(s). Worse, for many of INH-resistant strains, the respective gene bearing the mutation has not yet been found. Of all primary drug resistances, RMP resistance appears to be best understood. Owing to the fact that >97% of all RMP-resistant strains carry a mutation in a specific core region of the *rpoB* gene [48], resistance to that drug can easily and reliably be detected by molecular methods. This may be achieved by gene sequencing or by a commercially available line-probe assay [49]. The latter test detects nucleotide changes in the relevant part of the *rpoB* gene by oligonucleotide probes.

Conclusions

With the advent of new techniques for the mycobacteriology laboratory, the diagnosis of tuberculosis can be made quite rapidly. However, the laboratory has to carefully make its choice for the most accurate and efficient techniques and to be aware of their advantages and disadvantages (table 2). Both clinicians and laboratorians have to use the best available resources to determine the most appropriate care for their patients.

Table 2. A summary of laboratory methods available for the diagnosis of TB in clinical specimens

Method	Turnaround time	Comments	Advantage	Disadvantage
AFB smear	24 h	Positive smear requires 5×10^3–10^4 AFB/ml	High specificity Rapid, cheap Identifies the most contagious patients High positive predictive value for tuberculosis (> 90%) for respiratory specimens	Sensitivity 22–80% No species diagnosis possible Not suitable for monitoring effectiveness of therapy
NAA-based assays	6–48 h[a]	Requires appropriate laboratory equipment and experienced personnel Analysis of multiple specimens per patient recommended	Rapidity of diagnosis Impact on patient care	Sensitivity of smear positives close to 100%, of smear negatives ~ 60–80%; overall specificity ~ 95–100% Viable *vs.* dead cells (no answer) Not suitable for monitoring effectiveness of therapy
Culture	Days to weeks	Positive culture requires 10^1–10^2 AFB/ml 'Gold standard'	*The* method for monitoring effectiveness of therapy Can be automated Colony morphology may be indicative for *M. bovis* or *M. canettii*	Labor- and time-consuming Results may be late A combination of solid and liquid media required
Identification				
By gene probes	A few hours	Can be applied to culture only and not to clinical specimens	Very fast for culture confirmation Sensitivity and specificity close to 100%	Identifies *M. tuberculosis* complex only Additional tests required for species identification
By biochemistry	Several weeks	NO_3, niacin, catalase as key reactions, additional criteria	Identification at the species level possible	Long turnaround time to results

Table 2. (Continued)

Method	Turnaround time	Comments	Advantage	Disadvantage
		may be needed for *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, *M. canettii* (Lebek test, susceptibility to T_2H, susceptibility to PZA, spoligotyping, IS*6110*- and IS*1081*-based RFLP)		
16S rDNA sequencing	24–48 h	Does not identify members within *M. tuberculosis* complex		
Susceptibility testing				
Growth-based	Several days	BACTEC 460 TB applied most widely but currently being replaced by non-radiometric methods	Well established Correlates with agar proportion method	Time-consuming
Molecular-based	1–2 days	Gene sequencing, line probe assay, other molecular methods [see 47]	RMP resistance: > 97% of the mutations on the *rpoB* gene	Detection of resistance to drugs other than RMP not yet ready for the clinical mycobacteriology laboratory

[a]From processed sediment.

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Mycobacteria and TB – Therapy and Drug Resistance

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Chemotherapy for tuberculosis (TB) began in the late 1940s with the advent of streptomycin. Due to the introduction of isoniazid, rifampin and pyrazinamide, duration of therapy has been shortened from 18 to 9 months, and then to the current 6-months regimen. The goal of TB treatment is to eradicate the bacilli in the various environments within the host, and to prevent the emergence of drug-resistant strains. Therapeutic strategies have changed little during the last years, and *still application of multiple drugs* in *adequat*e *dosages* on a *regular basis* for *sufficient duration* with *expert monitoring* are the principles for successful treatment. The drugs must be given to every patient with confirmed TB. Drugs should be available and free of charge, to every TB patient. The treatment should preferably include an initial intensive phase, since an initial intensive course with three or four drugs has the advantage of being more effective in eliminating tubercle bacilli and in minimizing the influence of drug-resistant strains.

Each of the main antituberculous drugs varies in its capacity to kill bacteria, to sterilize lesions and to prevent the development of drug resistance [1]. Isoniazid (H) is the most potent bactericidal drug, acting on metabolically active bacteria. Rifampin (R) also is an effective bactericidal drug with a potent sterilizing effect. In addition to acting on rapidly dividing bacilli, it kills so-called 'persisters' in intermittent periods of their metabolism after short exposure to the drug, a characteristic that is crucial for its strong sterilizing ability. Both drugs are very effective in preventing the emergence of resistance. Pyrazinamide (Z), although bactericidal, is mainly used for its sterilizing capacity since it kills intracellular bacilli sequestered inside macrophages in an acid environment [2]. Ethambutol (E) and streptomycin (S) are less potent drugs, E probably being bactericidal at high concentration.

Table 1. Current recommendations for therapy of probable drug-sensitive TB[1]

Type of TB disease	Initial phase	Duration, months	Continuation phase	Duration, months
Pulmonary	H, R, Z, E	2	H, R	6
Extrapulmonary	H, R, Z, E	2	H, R	6
Meningitis	H, R, Z, E	2	H, R	10

H = Isoniazid, R = rifampin, Z = pyrazinamide, E = ethambutol.
[1]Adapted from the DZK guidelines [7].

Directly observed therapy (DOT) or directly observed therapy short course (DOTS) has been recommended by the WHO as a means for TB control [3, 4]. There is no doubt that completing short-course therapy is the basis for low relapse rates. The efficacy of 6 months' short-course therapy has been shown in a wide range of clinical trials [5]. With such 6-month regimen and compliance properly monitored, over 90% of smear-positive patients should be converted to culture-negative at 2 months, and cure rates in excess of 95% and relapse rates of under 5% are possible [6].

Therapy Regimens

A treatment regimen of 2 months daily H, R and Z, followed by 4 months of H and R, daily or 2–3 times per week is recommended world-wide [7, 8]. In areas where resistance to H is not documented to be less then 3–5%, E or S should be added to the initial regimen until susceptibility to I and R is demonstrated. Therapy regimens for drug-susceptible TB, dosages and possible side effects are listed in tables 1 and 2, respectively.

In cases where H, R or Z cannot be used (resistance or side effects) it has been recommended that TB treatment should be administered for a minimum of 12 months. Therapy regimens in these cases are listed in table 3.

Treatment regimens with second-line drugs should be considered in settings where MDR-TB is relatively high (i.e., >3% among new cases). However, it is essential that second-line treatment regimens should be implemented only if TB control strategies consistent with international standards are also in place. From a public health point of view, attempts to introduce second-line drugs for treatment of MDR-TB in a place where acceptable cure rates even for drug-susceptible TB cases are not guaranteed will most likely lead to disastrous consequences. Drug resistance to second-line drugs will emerge rapidly, resulting

Table 2. Antituberculous medications: doses and side effects[1]

Antituberculous drug	Dose, mg/kg (max. dose)	Possible side effects
Isoniazid	5 (300 mg)	Rash, hepatic enzyme elevation, hepatitis, peripheral neuropathy
Rifampin	10 (600 mg)	Rash, hepatitis, fever, thrombocytopenia, orange-colored body fluids
Pyrazinamide	25 (2.0 g)	Gastrointestinal upset, hepatitis, rash, arthralgias, hyperuricemia
Ethambutol	20–25 (0.8–2.0 g)	Optic neuritis (decreased red-green color discrimination), decreased visual acuity, rash
Streptomycin	15 (1 g)	Ototoxicity (hearing loss or vestibular dysfunction), nephrotoxicity

[1]Adapted from the DZK guidelines [7] and Friedmann and Selwyn [8].

Table 3. Current recommendations for therapy of drug-resistant TB[1]

Resistance against	Initial phase	Duration, months	Continuation phase	Duration, months
Isoniazid	R, Z, E, S	2	R, E	7–10
Rifampin	H, Z, E, S	2	H, E	10–16
Pyrazinamide	H, R, E, S	2	H, R	7
Ethambutol	H, R, Z, S	2	H, R	4
Streptomycin	H, R, Z, E	2	H, R	4

H = Isoniazid, R = rifampin, Z = pyrazinamide, E = ethambutol, S = streptomycin.
[1]Adapted from the DZK guidelines [7].

in greater harm than benefit. Thus, treatment with second-line drugs should follow strictly international guidelines and drugs should be added to failing treatment regimens only after actual drug susceptibility test results are available.

Human immunodeficiency virus (HIV)-positive patients should be treated for the same duration as HIV-negative patients, but a four-drug initial phase should always be used because of the increased likelihood of drug resistance in such patients.

Monitoring Response to Therapy

Patients should be seen every month to monitor response to treatment. DOT is recommended unless there is evidence that the patient will adhere to therapy. In sputum smear-positive patients, at minimum monthly sputum analysis is mandatory, and weekly or twice a month sputum smears with quantification is encouraged strongly. Otherwise, in sputum smear-negative patients, specimens should be obtained each month until culture conversion is documented. After 2 months of therapy with standard regimen containing both H and R, more than 90% of patients with positive sputum cultures should have converted to negative [9]. If not, the patient should be evaluated carefully and drug susceptibility testing should be repeated.

Additionally, baseline evaluation should include a complete blood count with platelets, liver function tests, blood urea nitrogen, creatinine, and calcium. If Z is used, uric acid should be obtained, if E is used, uric acid, visual acuity, and red/green color perception should be assessed, and if S is used, renal and auditory tests should be performed.

Drug-Resistant Tuberculosis – General Aspects

The emergence of drug-resistant *Mycobacterium tuberculosis* strains has become a world-wide health care problem. Since in the early 1990s the emergence of drug-resistant TB has been reported from the USA, especially New York City [10, 11]. Outbreaks of resistant and even multidrug-resistant (MDR) TB have been published from several other countries around the world [12–19]. Thus, the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) started in 1994 the Global Project on Anti-Tuberculosis Drug Resistance Surveillance which further on demonstrates that drug resistance was ubiquitous in the world [20, 21]. Between 1996 and 1999, patients in 58 geographical regions were surveyed, and 28 sites provided data for at least 2 years [21]. Among new cases, the frequency of resistance at least to one drug was between 1.7 and 36.9% with a median of 10.7%. The level of MDR TB ranged between 0% up to 14.9% for patients newly treated for TB with a median of 1%. High rates of MDR TB were observed, e.g. in Henan Province, China (10.8%), the Russian oblast of Ivanovo (9.0%) and Tomsk (6.5%), or in Estonia (14.1%). These data demonstrate that drug resistance and especially multidrug resistance continues to present a serious problem, especially in several Eastern European countries.

This situation emphasizes the need to further extend the number of countries that apply proper TB control programs, e.g. the DOTS system of the WHO

to avoid a further rise in rates of drug-resistant TB. Moreover, it will be necessary to develop improved treatment and TB control strategies for regions with high rates of drug resistance since in such countries the standard DOTS program alone might not be sufficient to reduce the rates of drug-resistant TB [22, 23]. Further necessities are the improvement of the understanding of development and spread of drug-resistant TB, the establishment of rapid techniques enabling the early detection of drug resistance in clinical isolates as well as to force attempts in developing new or improving old drugs in order to successfully combat the disease in 'hot-spot' areas with high rates of resistant TB. However, it might still be questioned if programs which have reduced resistant TB levels in developed countries, e.g. the New York TB program [11], can successfully be transferred to less developed regions of the world since the costs of the necessary multiple interventions are high.

Development and Spread of Drug Resistance

In the case of *M. tuberculosis*, the development of drug resistance does not occur by acquisition of new resistance genes as described for other bacteria, but is the result of random genetic mutations in particular genes conferring resistance. The drug resistance conferred by genetic mutations of *M. tuberculosis* is spontaneous and not dependent on exposure to drugs [24]. The ratio of resistant bacilli varies for different antituberculous drugs, e.g. the probable incidence for mutants showing resistance to rifampin is approximately 1 in 10^8 bacilli, mutants resistant to isoniazid, streptomycin, or ethambutol have a probability of approximately 1 in 10^6 bacilli [25]. Resistant bacilli may be selected if large numbers of tubercle bacilli are present in a patient treated with only one effective drug. Hence, the development of drug resistance in *M. tuberculosis* can occur during improper antituberculous therapy, e.g. because of improper prescription of treatment regimes, addition of single drugs to failing treatment regimens, inadequate drug supply or patients' non-compliance. Resistant *M. tuberculosis* strains are then selected during phases of treatment regimens with one effective drug only [26, 27]. The development of multiple drug resistance results from multiple periods of 'sequential monotherapies' during which resistance to other antituberculous drugs may be acquired. Resistance to isoniazid and rifampin alone, or in combination with other drugs was defined as multidrug resistance (MDR). The development of resistance during therapy is called 'drug resistance among previously treated patients' (formerly secondary resistance or acquired resistance).

In contrast, drug resistance in patients never receiving TB treatment is defined as 'drug resistance among new cases' (formerly primary resistance).

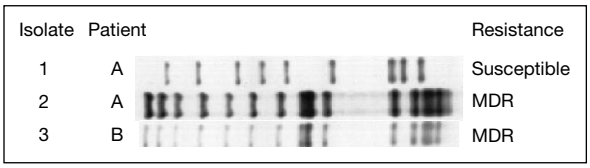

Fig. 1. Exogenous reinfection with MDR TB in a hospital. IS*6110* RFLP patterns of the drug-susceptible and MDR *M. tuberculosis* strain of patient A (isolates 1 and 2) and of the MDR strain of patient B (isolate 3). Patient A was reinfected with the MDR strain of patient B during his stay in a hospital, although receiving regular TB therapy.

These patients presumably have been infected from an index case with drug-resistant TB. It, however, has long been assumed that resistance to antituberculous drugs is associated with a reduced infectivity and pathogenicity of the particular *M. tuberculosis* strains. This is in accordance with several publications describing that outbreaks and transmission of resistant TB are associated with immunosuppression of the patients, e.g. infection with HIV [12, 15]. However, recent studies demonstrate transmission of resistant strains in a large variety of settings, e.g. in hospitals [28] or prisons [29], or even in the public community by applying molecular strain typing techniques such as IS*6110*-RFLP typing [16, 19, 30]. It has moreover been demonstrated that exogenous reinfection with resistant- or MDR *M. tuberculosis* isolates is possible and may even occur in patients receiving regular antituberculous therapy (fig. 1) [19, 30, 31]. These results indicate that in addition to well-known factors contributing to the emergence of resistant TB, transmission of resistant *M. tuberculosis* strains also represents a possible driving force of the emergence and spread of resistant TB. It is therefore necessary to know the relative contribution of primary and secondary resistance to new cases of resistant TB in different settings so that the actual TB control strategies can be evaluated and new strategies for the control of resistant TB can be developed.

Prevention of Drug Resistance

Most of the general aspects preventing the development of drug resistance during antituberculous therapy have been mentioned in the previous sections. There is no way to prevent the spontaneous mutations conferring resistance which occur naturally in the bacilli. The most important points preventing the selection of resistant subpopulations and the development of a resistant TB during therapy are: (1) appropriate therapeutic regimens; (2) drugs of proven

quality; (3) assurance of adherence to therapy, and (4) never add a single drug to failing treatment regimens.

It is well accepted that TB patients must be treated with a combination of at least three drugs, since mutants resistant to drug A will be controlled by drugs B and C as well as mutants resistant to drug B or C will be controlled by the respective other drugs. Even in the case of initial resistance to one drug, a combination of three drugs will be sufficient to control the resulting multiple resistant mutants. It is therefore clear that drugs of unproven quality may not be able to control drug-resistant mutants and may lead to treatment failure and development of resistant TB. The same can result from non-compliance of patients leading to periods during which not all drugs were present in an effective dose and resistant strains may be selected.

In the case of failing treatment regimens it is mandatory to immediately determine the actual drug resistance profile of the isolates to allow the administration of an effective treatment regimen. A single drug, however, should never be added to a failing treatment regimen.

Molecular Mechanisms of Drug Resistance

Recent advances in the understanding of molecular mechanisms of drug resistance resulted in the identification of about 12 genes involved in resistance in *M. tuberculosis* (table 4). The mutations which have been found to be associated with resistance to the particular antituberculous drugs are summarized in the following paragraphs.

Resistance to Inhibitors of Cell Wall Synthesis

Isoniazid is one of the most potent antituberculous drugs, and is used together with rifampin in virtually all modern TB therapeutic regimens [8, 32]. Mutations in *kat*G, *inh*A, *ahp*C, *kas*A, *ndh* have been found to be associated with isoniazid resistance in clinical *M. tuberculosis* strains [33–37]. The prevalence of the particular mutations conferring resistance has been analyzed in several studies. In approximately 50% of resistant isolates, mutations in *kat*G were determined [38–40], between 20 and 30% of resistant isolates contain mutations in *inh*A [35, 38–40] and in 10–15% mutations in *ahp*C have been found [39, 41, 42]. More recently, mutations in *kas*A and *ndh* have been found in isoniazid-resistant *M. tuberculosis* isolates without mutations in *kat*G, *inh*A, and *ahp*C [36, 37], however further studies are necessary to evaluate the role of mutations in these genes in isoniazid resistance. Resistance to ethionamide, which was structurally related to isoniazid, was also found to be associated with mutations in *inh*A [34].

Table 4. Antibiotics against *M. tuberculosis* and drug resistance mechanisms[1]

Site of action	Antituberculous drug	Genes associated with resistance	Approximate frequency of mutations, %	Selected references
Inhibitors of cell-wall synthesis	Isoniazid	*kat*G	40–60	33
		*inh*A	20–34	34
		*ahp*C	10–15	35
		*kas*A	14	36
		ndh	10	37
	Ethionamide	*inh*A	n.d.	34
	Ethambutol	*emb*CAB	50–65	39
	Cycloserine	*alr*A	n.d.	46
Inhibitors of nucleic acid synthesis	Rifampin	*rpo*B	>95	47
	Fluoroquinolones	*gyr*A, *gyr*B	75–95	50
Inhibitors of protein synthesis	Streptomycin	*rps*L,	52–59	53
		rrs (16S RNA)	8–21	54
	Amikacin/kanamycin	*rrs* (16S RNA)	80	55, 56
Unknown	Pyrazinamide	*pnc*A	70–97	58

[1]Adapted from Zhang and Telenti [42] and Riska et al. [52].

Ethambutol also belongs to the first-line drugs used for the treatment of TB. In up to 65% of *M. tuberculosis* isolates resistant to ethambutol mutations in the *emb*CAB gene cluster have been determined, the most common mutations are substitutions in amino acid residue 306 of *emb*B which are associated with high-level resistance [43–45].

Experiments in *M. smegmatis* indicate that resistance to cycloserine in mycobacteria is associated with overexpression of the *D*-alanine racemase (*alr*A) gene which might be induced by mutations in the *alr*A promoter region [46].

Resistance to Inhibitors of Nucleic Acid Synthesis

Rifampin is a critical compound of short-course regimen for TB therapy [8, 32]. In contrast to isoniazid, with several resistance genes involved, resistance to rifampin is mainly based on single-point mutations in a small 81-bp hot-spot region (codons 507–533, *Cluster I*) of the RNA polymerase gene (*rpo*B) which were found in approximately 95% of resistant isolates [47–49]. The most frequent mutations observed were changes of codons 531, 526 and 516 [42, 47–49]. Furthermore, mutation of codon 146 in the beginning of the *rpo*B gene has been found to be associated with high-level resistance in *M. tuberculosis* [49].

Fluoroquinolones such as ciprofloxacin or ofloxacin belong to the second-line antituberculous drugs and were important for the treatment of MDR TB cases [8, 32]. High-level resistance to fluoroquinolones has been found to be related to mutations in the so-called quinolone resistance-determining region (QRDR) in the *gyr*A gene which were found in up to 95% of resistant *M. tuberculosis* isolates [50, 51].

Resistance to Inhibitors of Protein Synthesis

Streptomycin is an aminocyclitol glycoside antibiotic and has been used for treatment of TB since 1944 [52]. Mutations in *rps*L and *rrs* have been identified in 50 and 20% of streptomycin-resistant *M. tuberculosis* isolates, respectively, conferring intermediate or high levels of resistance [38, 53, 54]. The most frequent occurring mutation is an A-to-G transition in codon 43 of the *rps*L gene [38].

Mutations in the *rrs* gene have been found to also be associated with high-level resistance to the second-line drugs amikacin and kanamycin, two other aminoglycosides [55, 56]. Approximately 67% of resistant isolates carried mutation in the *rrs* gene, the most frequent mutation was the base substitution A to G at position 1400 [55, 56]. Cross-resistance may be observed between amikacin and kanamycin as well as between viomycin or capreomycin [55–57].

Resistance to Other Antituberculous Drugs

Pyrazinamide, a derivative of nicotinamide, is recommended in essentially every combination therapy for the treatment of TB and has allowed to shorten treatment regimens to 6 months [8, 32]. The major mechanisms of resistance to pyrazinamide in *M. tuberculosis* are mutations in the *pnc*A gene which have been found in up to 98% of clinical isolates [58–61].

Based on the knowledge on the molecular mechanisms leading to antituberculous drug resistance, molecular assays applying direct sequencing of PCR products or single-strand confirmation polymorphism (SSCP) assays have been established for prediction of drug resistance in clinical *M. tuberculosis* isolates within a few days [62]. Since the resistance to rifampin mainly involves mutations in an easy-to-analyze hot-spot region, the majority of studies were performed evaluating molecular assays for prediction of rifampin resistance. These include the commercial line-probe kit (Inno-Lipa Rif.TB Innogenetics NV, Zwijndrecht, Belgium) that is based on a reverse hybridization assay for detection of resistance mutations and wild-type sequences. PCR-SSCP and the line-probe assay proved to detect more than 90% of strains with rifampin resistance [39, 63], indicating that these techniques have the potential to replace the more time-consuming PCR-DNA sequencing techniques. Resistance to rifampin also

presents a valuable surrogate marker for multidrug resistance which is an tremendous obstacle for TB therapy, since more than 90% of rifampin-resistant *M. tuberculosis* isolates are also resistant to isoniazid [49, 64].

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Molecular Biology of *Mycobacterium tuberculosis*

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Based on 16S rRNA studies, the genus *Mycobacterium* belongs to the actinomycete branch of the Gram-positive bacteria. Mycobacteria have typical characteristics for the group, including a high G + C content in their DNA, aerobic metabolism and a tendency towards mycelial growth, but differ from many members of the group in lacking a spore stage in their life cycle. Most of the actinomycetes, including many non-pathogenic mycobacteria, are soil-dwelling saprophytes indicating that the ancestor of *Mycobacterium tuberculosis* also probably lived in the soil. If so, its genome appears to have been considerably downsized since its free-living days since the related soil-dwelling, spore-forming actinomycete, *Streptomyces coelicolor*, has a genome about twice the size of that of *M. tuberculosis*.

The *M. tuberculosis* Genome

A major landmark in TB research was reached in 1998 when the entire genome of *M. tuberculosis*, H37Rv, was published [1] (fig. 1). The H37Rv genome was sequenced at the Sanger Centre in Cambridge, in collaboration with the Institut Pasteur, Paris. Two recently isolated clinical strains of *M. tuberculosis*, CDC1551 and CSU210 are also subject to DNA sequencing projects at the Institute for Genomic Research (TIGR, Rockville, Md., USA). The genome sequence of the leprosy bacillus has also been published. Genome sequencing projects are ongoing for several other mycobacterial species, including *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium marinum*, *Mycobacterium ulcerans*, *Mycobacterium avium*, *Mycobacterium paratuberculosis* and *Mycobacterium smegmatis*. Comparative genomics of all

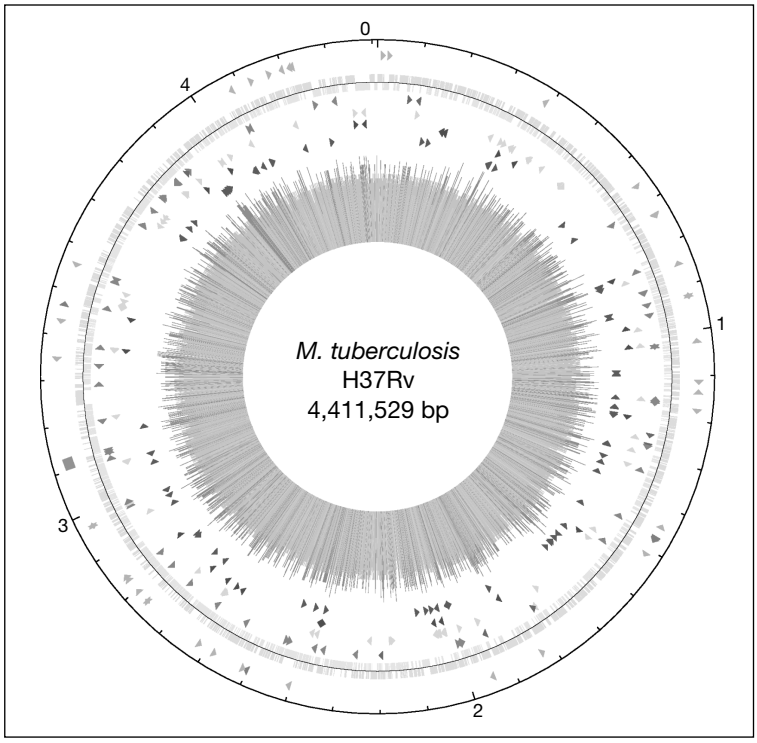

Fig. 1. Circular map of the chromosome of *M. tuberculosis* H37Rv. The outer circle shows the scale in Mb, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes and the DR region (cube); the second ring inwards shows the coding sequence by strand; the third ring depicts repetitive DNA; the fourth ring shows the positions of the PPE family members; the fifth ring shows the PE family members (excluding PGRS), and the sixth ring shows the positions of the PGRS sequences. The histogram (center) represents G + C content. The figure was generated with software from DNASTAR. From Cole et al. [1], with permission. Please refer to original diagram for detailed annotation.

these sequences is likely to yield many new insights into the genome organization and evolution of mycobacteria and could also identify regions involved in the differing characteristics of these pathogens [2]. Behr et al. [3] used DNA microarrays to identify 14 regions (RD1–14) that were absent in *M. bovis* BCG-Pasteur relative to the sequenced H37Rv genome and two regions (RD15 and 16) that were found in only some BCG substrains. Several of the deleted or variable regions encoded known genes including the ESAT-6 secreted antigen, phospholipases C and various transcriptional regulators but many of the genes involved are of unknown function.

M. tuberculosis has a 4,411,529-bp circular genome (fig. 1). The start point for numbering the sequence was arbitrarily chosen to be the initiation codon for the *dnaA* gene involved in genome replication. The H37Rv genome contains 3,924 identified open reading frames, of which 40% were confidently assigned functions on the basis of similarity to known genes and another 44% were assigned a probable function, leaving 16% orphan genes.

Examination of the genome revealed that the pathogen has all the genes necessary for the essential catabolic and anabolic metabolic pathways. A large fraction of the *M. tuberculosis* genome – about 250 genes – was found to be devoted to lipid and polyketide metabolism, strengthening earlier suggestions that the pathogen may adopt a lipolytic lifestyle in vivo. Perhaps surprising considering the aerobic nature of mycobacteria, genes encoding several enzymes involved in anaerobic respiration, such as nitrate and fumerate reductase, were also present. About 10% of the genome is devoted to coding for two large unrelated families of glycine-rich acidic proteins: 99 members of the PE protein family and the 68 members of the PPE protein family (fig. 2). The genes encoding these proteins contain the polymorphic repetitive sequences known as PGRSs, and the major polymorphic tandem repeats (MPTRs) respectively, which are widely used in genetic fingerprinting studies and are described below. Their function is unknown but they have been proposed to be involved in generating antigenic variation and are likely to be major sources of genetic variation in the tubercle bacillus.

A number of secreted proteins were identified that may act as potential virulence factors including a series of phospholipases C, lipases and esterases, as well as several proteases. Proteins involved in storage of oxygen and iron were also identified.

Four copies of the *mce* (mycobacterium cell entry) operon (fig. 3) were identified in the genome. The *mce* gene was first characterized as a macrophage-colonizing factor [4] in *M. tuberculosis*. Each operon encodes a mce-related protein plus additional genes predicted to encode membrane proteins and transcriptional regulators. Inactivation of *mce1A* was found to attenuate the mutant's ability to invade epithelial cells [5], indicating that the genes are likely to be involved in some aspect of virulence. However, the genome of the distantly related, though non-pathogenic, *M. smegmatis* also appears to encode several mce-related genes [6]. To be so widely distributed, mycobacterial must have acquired mce genes very early in their evolution. The genome also contains five copies of a cluster of twelve genes known as the ESAT-6 loci [7]. One of these clusters includes the *esat-6* gene encoding the ESAT-6 secreted protein that is a strong T-cell antigen. This gene is co-transcribed with *lhp* that encodes another small secreted antigen, CFP-10. This particular cluster is contained within the RD1 deletion that is absent in *M. bovis* BCG, accounting for the absence of these

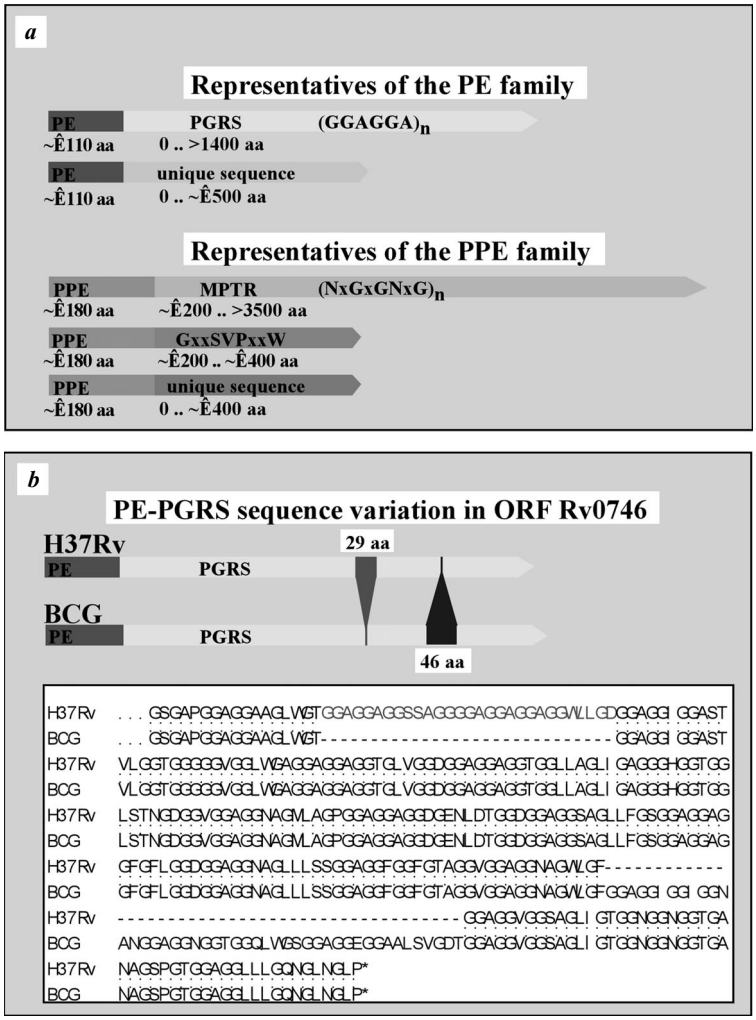

Fig. 2. The PE and PPE protein families. ***a*** Classification of the PE and PPE protein families. ***b*** Sequence variation between *M. tuberculosis* H37Rv and *M. bovis* BCG-Pasteur in the PE-PGRS encoded by open reading frame (ORF) Rv0746. From Cole et al. [1], with permission.

antigens in BCG. Each of the additional ESAT-6 clusters contains (in addition to a copy of a *esat-6* and *lhp*-related gene), genes encoding putative ABC transporters (integral inner-membrane proteins), ATP-binding proteins, subtilisin-like membrane-anchored cell-wall-associated serine proteases and other amino-terminal membrane-associated proteins. The presence of both membrane-

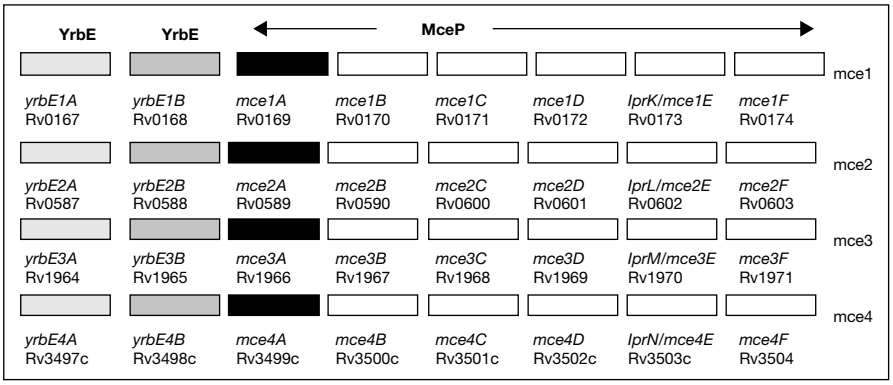

Fig. 3. The *mce* gene clusters. The four *mce* operons are arranged similarly with each operon including approximately 10 kb coding for eight genes. The first two genes code for transmembrane proteins (YrbE A and B) and the remaining eight are of unknown function.

associated and transport proteins in the clusters suggest they are involved in transport, probably secretion, of a substrate through the mycobacterial cell wall. Duplication of genes and gene clusters appears to have been a common event in the evolution of *M. tuberculosis* with as much as 52% of the proteome being derived from gene duplication events [8].

Gene Regulation

The genome contains more than 100 open reading frames proposed to encode regulatory proteins. Thirteen putative sigma factors are present including 2 principal-like sigmas known as SigA and SigB, 10 extracytoplasmic sigmas and 1 stress/sporulation-type sigma known as SigF. Eleven two-component regulator systems comprising a sensor histidine kinase and regulator protein were present, including a *phoP-phoQ* homologue [9], a *devR-devS* system [10], and mtrA-mtrB system [10]. Many of these have been shown to be involved in virulence and the mtrA-mtrB system appears to be essential for viability of *M. tuberculosis*. A large family of serine/threonine protein kinases (STPks) are also present. In eukaryotes, STPKs are involved in signal transduction through phosphorylation and dephosphorylation of regulatory proteins at serine and threonine residues. A number of viral and bacterial virulence factors, such as the *Yersinia* protein kinase A (YpkA, also known as YopO), are known to interact with these systems [11] to subvert host cell function. The *M. tuberculosis* STPKs may similarly be involved in its interaction with host cells [12].

Some of the signals seen by these regulators are known. The sigF gene is upregulated by exogenous stress conditions (e.g., by the administration of antimycobacterial drugs, by entry into stationary phase in vitro or macrophage infection [13]); whereas *SigB*, *sigE* and *sigH* respond to heat shock. The α-crystallin (*acr*) gene is rapidly induced at reduced oxygen tensions. Using a whole genome microarray approach, Sherman et al. [14] identified more than 100 genes whose expression was altered by incubation of cells in defined hypoxic conditions. A microarray study of a *sigE* mutant of *M. tuberculosis* identified genes whose expression was dysregulated in the mutant. The dysregulated genes encode a variety of proteins including transcriptional regulators, enzymes involved in fatty acid degradation and heat-shock proteins.

Transposable Elements and Repetitive Sequences

More than 50 loci in the H37Rv genome were found to contain insertion sequence (IS)-like elements, amounting to about 1.6% of the entire genome [15]. The elements are mostly present as single copies, but the genome does contain 16 copies of the IS3-related element, IS*6110*, and 6 copies of the IS*256*-related IS*1081*. The remainder of IS elements are present as single copies throughout the genome and belong to a variety of IS families including the IS*3*, IS*5*, IS*21*, IS*110*, IS*256*, IS*1535* and IS*L3*. Most of the elements appear to be relatively stable in the genome, although one of the newly discovered elements, IS*1532*, was absent from *M. bovis*, *M. bovis* BCG-Pasteur and from 10 of 29 clinical isolates tested. Although most of the IS elements are found to be scattered, a 600-kb segment of the genome, close to the *oriC*, was found to be entirely lacking in ISs, indicating that insertions in this region are likely to be detrimental to the pathogen's survival [15].

The most variable element in the *M. tuberculosis* genome is undoubtedly IS*6110*, which was first discovered as a repetitive DNA element in *M. tuberculosis* [16–18]. The element consists of 1,358 bp, with 30-bp inverted repeat ends and appears to be a typical member of the widespread IS*3* family of ISs. Like other members of this family, IS*6110* encodes two open reading frames *orfA* and *orfB* and production of a functional transposase is thought to require ribosomal frameshifting between these orf's. As in other IS*3* family members, the putative transposase of IS*6110* includes motifs in common with retroviral integrases. The element is present as a single copy or two copies in *M. bovis* (including the BCG strain in which it was named IS*987*) and in 8–20 copies in most strains of *M. tuberculosis*, with 16 copies in the sequenced strain of H37Rv. However, strains of *M. tuberculosis*, mainly from Asia, have been found with only a single copy or which lack the element altogether [19]. Copies of the

element tend to be scattered throughout the genome of its host; although there are at least two hotspots for integration: one within a region of 36-bp direct repeats (DR) [20] (see below) and the second a 267-bp region termed *ipl* (IS*6100*-preferred locus) [21]. In a laboratory model system, transposition of IS*6110* was shown to be strongly dependent on the nature of the surrounding DNA [22], indicating that the various copies of the element scattered throughout the genome of *M. tuberculosis* genome may have different transposition activity. This may account for the stability of some copies of IS*6110*, such as the one or two copies found in (most strains of) *M. bovis*. The variable copy number and diverse genomic locations found for copies of this element in the chromosome makes IS*6110* an excellent tool for epidemiological analysis of TB (see above, The *M. tuberculosis* Genome). The repetitive nature and high degree of insertion-site variability associated with this element also indicates that it is likely to be (with the PPE and PE protein family) one of the principal sources of genetic variation within the *M. tuberculosis* complex. A comparison of 19 clinical isolates by microarray analysis identified many deletion events resulting in the loss of between 3 and 38 open reading frames [23]. In at least five cases, deletions were associated with IS*6110*. Transposition of IS*6110* in *M. smegmatis* was stimulated by exposure of cells to a microaerobic environment [24], indicating that transposition rates may be modified during passage of the pathogen through different host environments.

Repetitive DNA

In addition to functional or putative transposons, a number of repetitive DNA elements of unknown function have been identified in mycobacteria. The DR region of *M. tuberculosis* consists of a variable number of tandem repeated copies of a 36-bp *M. tuberculosis* complex-specific repetitive sequence [20]. A polymorphic GC-rich repetitive DNA sequence (PGRS) was isolated from *M. tuberculosis* and detected in other mycobacteria [25]. A sequence consisting of repeats of a 10-bp sequence separated by a 5-bp spacer, termed the major polymorphic tandem repeat (MPTR) sequence is also found in *M. tuberculosis* and other mycobacteria [26]. MPTR shows homology to the *E. coli chi* sequence involved in homologous recombination and also the repetitive extragenic palindromic (REP) sequences, found in *E. coli* and other bacteria. These repetitive elements have been widely used for DNA fingerprinting studies (see above, The *M. tuberculosis* Genome). The PGRS and MPTR repetitive elements are now known to be part of the genes encoding the family of glycine- and asparagine-rich proteins, known as the PE and PPE protein families (fig. 2), with the PGRSs contained within some members of the PE family and the MPTRs encoded within

the PPE gene family. The PGRS and MPTR elements are present as tandem repeats within the coding regions of these genes, with variable number of repeats probably generated through a process of recombination or replication slippage.

Inteins

Inteins are a recently discovered class of transposable element that catalyze their own excision, at post-translation level, from the protein encoded by the gene into which they are inserted. It is thought that by this mechanism, inteins avoid insertional inactivation of their target genes. Inteins propagate themselves by 'homing', a gene conversion process initiated by a site-specific homing endonucleases encoded within the intein. An intein was initially found in the *M. tuberculosis recA* gene [27] and another intein was subsequently discovered to be inserted into the *M. leprae recA* gene [28]. The *M. leprae* intein was different in sequence, and located at a different position than the *M. tuberculosis* RecA intein. In the H37Rv genome, inteins are also inserted in two additional genes: the *dnaB* and Rv1461. Inteins are found in both these genes in the *M. leprae* genome and the *dnaB* gene of *Mycobacterium avium-intracellulare* was also found to harbor an intein. The simplest explanation appears to be that inteins were inserted into each of these genes in the common ancestor of mycobacteria and have since been lost, moved and/or diverged in the descendant species.

Prophages

Two closely related prophages, phiRv1 and phiRv2, were found in the H37Rv genome. A third putative prophage was found upstream of the DR region that contains the hotspot, and probably ancestral site, for IS*6110* integration indicating that the element may have found its way into the *M. tuberculosis* genome on the back of a phage. The sequence of pHiRv1 was previously described as the RD3 region and was found to be present in *M. bovis* but absent in *M. bovis* BCG-Pasteur.

Cloning of Genes in Mycobacteria

Most *Escherichia coli* plasmid origins of replication, such as pMB1, do not function in mycobacteria. A number of cloning systems have therefore been developed for mycobacteria. Plasmid vectors based on the origin of replication from the *Mycobacterium fortuitum* plasmid pAL5000 have been widely used

[29–36]. The aminoglycoside phosphotransferase genes from Tn*5* and Tn*903*, which give resistance to kanamycin, function well in *M. tuberculosis* and are most widely used but resistance to sulphonamide, streptomycin, viomycin, hygromycin and fusidic acid, and markers which confer immunity to mycobacteriophage D29 infection have also been utilized. Electroporation is an effective method for introducing DNA into *M. tuberculosis* and transformation efficiencies of up to about 10^5 transformants/μg input DNA are readily achieved. To clone large fragments, an *E. coli* shuttle cosmid vector has been constructed that utilizes the pAL5000 origin and the *cos* site of bacteriophage λ [37]. Temperature-sensitive versions of pAL5000-based vectors have also been developed [33].

Shuttle plasmid vectors have also been constructed based on origins of replication from mycobacteriophages D29, L5 and TM4 [38]. Vectors that utilize the *int* gene and *attP* site of mycobacteriophage L5 to stably integrate genes into a single site (the *attB* site) of the *M. tuberculosis* chromosome [38] have also been constructed. Similar vectors have been constructed that utilize integration systems from mycobacteriophage such as FRAT1 or Ms6. A number of plasmid vectors, which act as phages in mycobacteria and plasmid in *E. coli*, have also been described, mostly based on the mycobacteriophages TM4 and L1 [38]. Conditionally replicating temperature-sensitive derivatives of phage vector systems have also been developed [39].

Although many *M. tuberculosis* genes are known to be regulated (see above, Gene Regulation), inducible expression systems are not well developed for mycobacteria. The most widely used system utilizes the acetamidase gene from *M. smegmatis* which is inducible with acetamide [40].

Transposon Mutagenesis

Transposition is widely used for mutational analysis in bacteria and several transposon mutagenesis systems have been developed in mycobacteria. A suicide vector-based transposon delivery system was used to deliver IS*1096* into the genome of *M. bovis* BCG and generate a transposon mutant library [41]. Both leucine and methionine-requiring auxotrophs were isolated. An artificial composite transposon was constructed consisting of two copies of IS*900* flanking the *aph* gene of Tn*903* and used to stably integrate foreign genes in *M. smegmatis* and *M. bovis* BCG [42, 43]. Both these examples utilized the suicide vector approach in which the transposon is cloned on a vector that cannot replicate in mycobacteria in order that transposition can be selected by its ability to stably integrate the antibiotic marker. The utility of suicide vector systems is however greatly restricted by the relatively low numbers of transformants

obtained. Several conditionally replicating vector systems have therefore been developed for performing efficient transposon mutagenesis in mycobacteria [39, 44]. More recently, a signature-tagged mutagenesis system was developed and used to identify a virulence gene cluster in the *M. tuberculosis* genome [45].

Homologous Recombination in *M. tuberculosis*

The RecA protein was the first recombination enzyme to be identified in *M. tuberculosi*s and its structure was shown to be unusual in containing an intein (see above). Whether the unusual nature of the *recA* gene/RecA protein affects its in vivo function has yet to be definitively answered, but an extra level of regulation for *recA* expression is potentially provided by the requirement for efficient splicing of the protein to gain enzyme activity [46]. Other aspects of regulation of RecA and the SOS response appear to be generally similar to those observed in other bacteria. As in *E. coli*, expression of *M. tuberculosis* RecA is associated with the SOS response that is triggered by DNA damage [47]. In *E. coli*, DNA damage activates RecA which in turn triggers the autocatalytic cleavage of the repressor protein LexA. LexA from *M. tuberculosis* has been cloned and expressed and shown to bind to putative mycobacterial SOS boxes located in the promoter regions of *M. tuberculosis lexA* and *recA* [48]. The mycobacterial SOS boxes are not like those of *E. coli* but are similar to the Cheo box sequence involved in the regulation of DNA damage-inducible genes in *Bacillus subtilis.*

Gene homologues for the RecBCD pathway have also been identified in the *M. tuberculosis* genome although, with the exception of RecA, none of the gene products have been studied to confirm biochemical activity. The presence of chi sites has also not yet been investigated in mycobacteria. From examination of the genome, the most obvious difference in genes involved in homologous recombination from those in *E. coli* is the absence of RecE and RecT homologues in *M. tuberculosis* [49]. This is perhaps not surprising since in *E. coli* these enzymes are encoded by the cryptic *rac* prophage.

Allele exchange in mycobacteria has been most successfully achieved using circular DNA substrates (see below). In *E. coli*, the RecF pathway acts predominantly on closed-circular substrates and is dependent on RecA but is entirely independent of RecBCD. For normal function it requires the gene products from *recF*, *recJ*, *recN*, *recO*, *recQ*, *recR*, *ruvA*, *ruvB*, *ruvC* and *recG* [50]. Most of these genes have been identified in the *M. tuberculosis* genome, with the notable exceptions of RecO and RecR, which in *E. coli* work with RecF, probably during the initial steps of recombination providing ssDNA suitable for RecA function. The exact function of these proteins is not understood

but they are essential to normal activity of the RecF pathway in *E. coli*. In mycobacteria, either alternative enzymes are utilized or RecR and RecO homologues simply have not yet been revealed. A homologue of *recJ* has also yet to be identified in mycobacteria, however, *recN* is present and the gene product is known to have overlapping functions with RecJ. It seems likely that mycobacteria do therefore have a functional RecF pathway, particularly as, in the absence of a RecE-like pathway, there is a need for mechanisms to deal with recombination events on covalently closed DNA molecules, as used in most allele exchange experiments. As in *E. coli*, the *recF* of mycobacteria is likely to be regulated by LexA, as there is an SOS box in the promoter region of the *recF* operon which is identical to that upstream of the *M. tuberculosis lexA* coding sequence.

Allele Exchange

Allelic exchange, or gene knockout (KO), is an essential tool to investigate gene function in bacteria. It is usually achieved through the operation of homologous recombination (HR) systems to replace the target copy of the gene in the chromosome with an inactive copy of the gene located on a vector. The HR event is selected usually through inclusion of a selectable marker within the inactivated copy target gene on a suicide vector. A single crossover will result in integration of the vector into the chromosome and generation of a mero-diploid strain with one functional and one inactive target gene. Complete allele exchange requires two HR events, one on each side of the target DNA, resulting in allele replacement and loss of the vector backbone.

Initial attempts to achieve allele replacement in *M. tuberculosis* were frustrated by the high levels of illegitimate recombination in the pathogen [51]. Instead of inactivating the target gene, the introduced vector integrated randomly in the genome. A number of strategies were devised to overcome this limitation. Linear DNA requires two recombination events to integrate into the genome and Aldovini et al. [52] harnessed this requirement to select for the double crossover events that result in allele replacement of the *uraA* gene in *M. bovis* BCG [52]. The frequency of HR events was, however, very low. A later study that utilized very long linear fragments to transform *M. bovis* BCG obtained relatively high rates of HR [53] and resulted in allele replacement of the *leuD* gene.

Numerous strategies have been devised to obtain efficient allele replacement in *M. tuberculosis*. Most experiments have utilized a plasmid delivery system, although phage systems have also been employed [54]. DNA is usually supplied

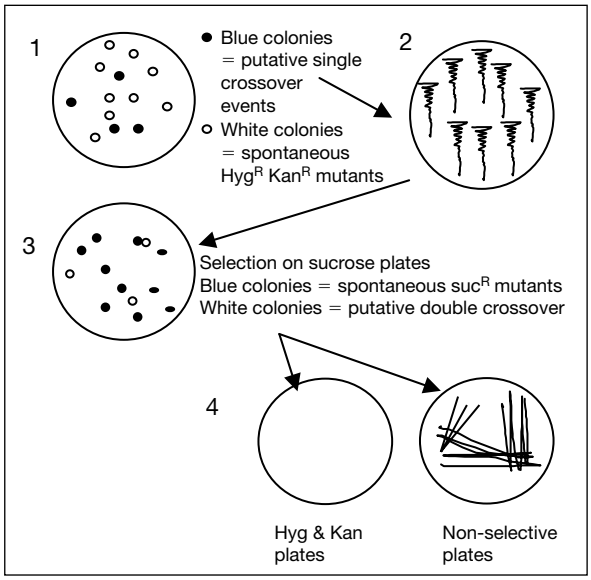

Fig. 4. Allele replacement strategy utilizing the pNIL/pGOAL system developed by Parish and Stoke [59] to generate unmarked mutants. A non-replicative (suicide) plasmid is constructed with the mutated version of the target genes flanked by selectable markers plus the β-galactosidase gene and electroporated into competent cells. 1 = Blue colonies derived from single crossover events are identified on selective plates containing X-gal; 2 = individual colonies are streaked onto non-selective media to allow the second crossover event to occur; 3 = white colonies – potentially deriving from double crossover events – are identified on sucrose/X-gal plates; 4 = the phenotype of putative double crossover knockout mutants are verified by streaking the colonies onto selective and non-selective plates where knockout mutants will be identified as colonies that have lost the selective markers.

in circular form as this has been shown to minimize the occurrence of illegitimate recombination events and is often treated with ultraviolet light to stimulate HR. A counter-selection marker is usually present on the vector backbone to allow selection against both single crossover and illegitimate recombination events that integrate the vector backbone. Although a number of counter-selectable markers have been used, including the *katG* gene [55] and the *rpsL* gene [56], most experiments have utilized the *sacB* gene from *B. subtilis*, which encodes the secreted enzyme levansucrase. Expression of this enzyme is toxic on media containing sucrose [57] so that double crossover events (that have lost the *sacB* gene) can be eliminated by growth on sucrose media. This approach was adopted by Azad et al. [58] to achieve allele replacement of genes involved in cell wall biosynthesis. Several strategies also employ conditional replicating

vectors that can be delivered at high efficiency to *M. tuberculosis* but can thereafter be eliminated. A two-step strategy employing a thermosensitive vector with the *sacB* gene was used to obtain an unmarked mutant of the *purC* gene in *M. tuberculosis* [57]. An alternative strategy to generate unmarked mutants in *M. tuberculosis*, the pNIL/pGOAL system, was developed by Parish and Stoker [59] and was used to generate defined lesions in the *tlyA* and *plcABC* genes. It is illustrated in figure 4. Using these and similar strategies, achieving allele replacement in *M. tuberculosis* is now relatively routine.

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Immunology and Persistence

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As we have begun the new millennium, tuberculosis – together with AIDS and malaria – remain the top three fatal infections of man worldwide. There are numerous reasons why tuberculosis still accounts for extraordinarily high rates of morbidity and mortality. Although effective chemotherapy is currently available, a combination regimen of at least three medications must be continued for 6 months. Lack of compliance to this strict regimen may result in the development of multidrug-resistant (MDR) strains of the bacterium (see Niemann, S. and Rüsch-Gerdes, S., p. 84 and Barry, C.E. III, p. 137). High morbidity and mortality rates result in increasing costs, notably for the developing countries. There, socio-economic factors, the co-infection with the human immune deficiency virus (HIV) and genetic predisposition of the host facilitate the spread of tuberculosis. All of these factors influence susceptibility to the disease. Additionally, several pathogenetic factors of the etiologic agent, *Mycobacterium tuberculosis*, contribute to the difficulties in achieving control of tuberculosis. Of prime importance is the capacity of the pathogen to persist in the host for long periods of time in the presence of an active immune response, and to adapt rapidly to the changing conditions within as well as outside the host.

Course of Infection

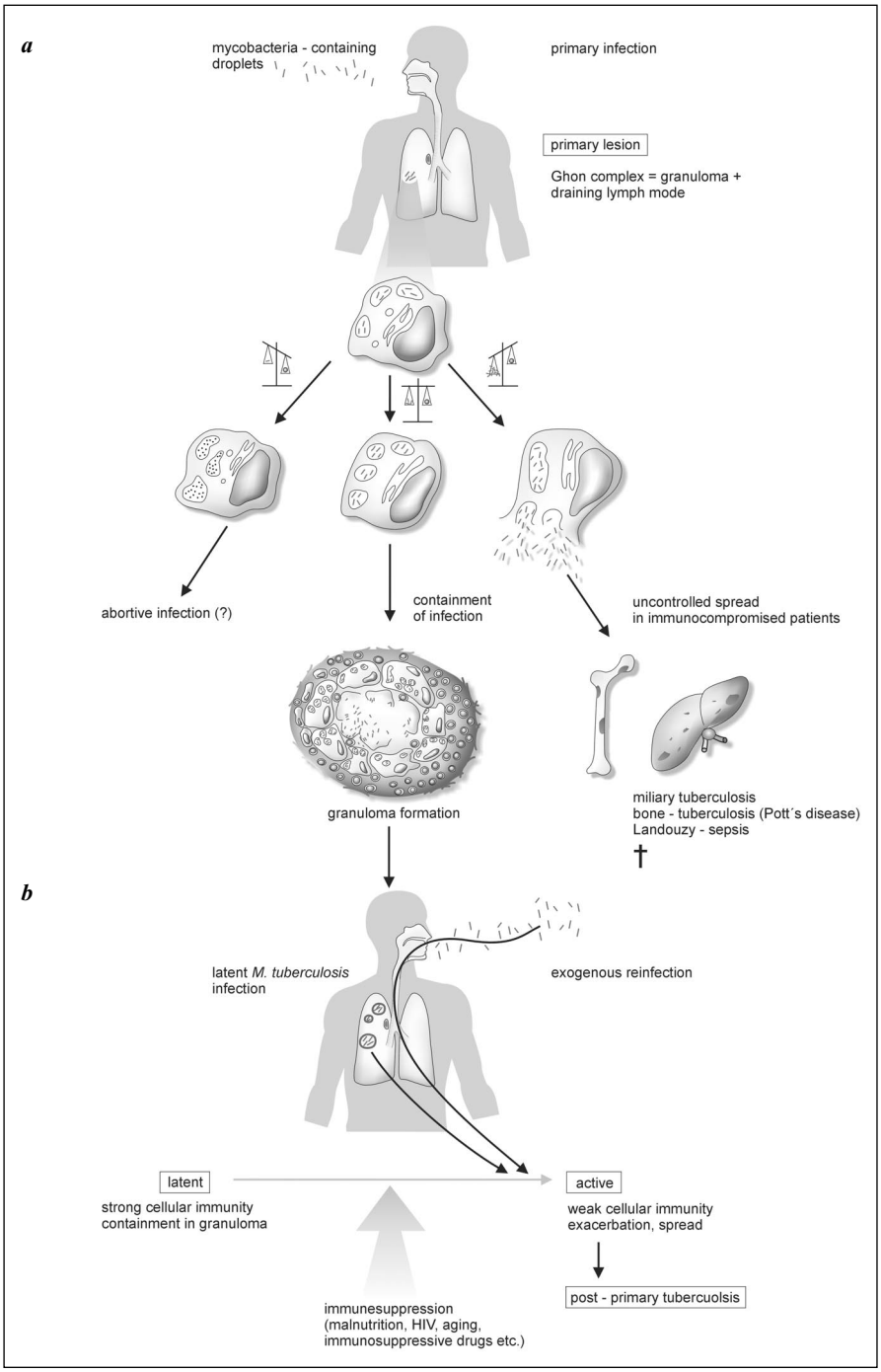
Tubercle bacilli are transmitted from person to person by aerosol infection (fig. 1a). The percentage of individuals becoming infected varies, mainly depending on environmental conditions, frequency of exposure and concentration of mycobacteria in the air, but is in general considered very low. Of the infected individuals, >90% successfully contain the infection by virtue of an efficient immune response. Replication and dissemination of the pathogen are

restricted by mononuclear phagocytes; T cells are recruited to the site of primary infection containing the bacilli. In an attempt to avoid direct confrontation with the host immune defense, *M. tuberculosis* successively retards its replication rate and transforms into a dormant state (dormant *M. tuberculosis* is defined as being in a state of low replication and strongly reduced metabolical activity). Thus, approximately one third of the world's population, i.e. 2 billion people, are latently infected with the pathogen. (Latency is a technical term originally derived from virology. Latent tuberculosis is used here to describe mycobacterial infection that does not present any clinical symptoms and is restricted to a contained primary site of infection harboring intact but dormant *M. tuberculosis*. The immune system is not able to clear the mycobacteria from the organism.) *M. tuberculosis* remains dormant until the balance between mycobacterial persistence and the immune response gets disturbed. (Because this chapter focuses on immune mechanisms underlying control of tuberculosis, persistence is defined from an immunologic standpoint as survival en face of an ongoing immune response. For persistence of *M. tuberculosis* in the presence of chemotherapeutic agents, see Niemann, S. and Rüsch-Gerdes, S., p. 84) An impaired host response due to various reasons including aging, malnutrition, steroids or HIV allows reactivation of the bacilli resulting in clinical manifestation of tuberculosis (fig. 1b).

Following inhalation of mycobacteria-loaded droplets, *M. tuberculosis* is engulfed by alveolar macrophages. A variety of host cell receptors, including Fc receptors (FcR), complement receptors (CR), the macrophage mannose receptor (MMR), surfactant protein receptors and CD14, bind mycobacterial surface molecules [reviewed in 1, 2]. Two members of the Toll-like receptor (TLR) family are also involved in interactions between macrophages and mycobacteria. The TLR-2 and TLR-4 interact with mycobacterial cell wall components, including lipoarabinomannan (LAM; see Saunders, G. and McFadden, J., p. 97) [3, 4] (fig. 2). It is proposed that the choice of the receptor used to enter the macrophage influences the cellular response: entry of IgG-opsonized mycobacteria via FcR results in activation of macrophage antimicrobial systems [5] (see below), whereas internalization via CR3 does not activate the host cell appropriately [6]. Cholesterol seems to support mycobacterial docking to macrophages. Membrane cholesterol is also involved in mediating the phagosomal association of tryptophane-aspartate-containing coat protein (TACO), which prevents phagosome maturation to the phagolysosome [7].

Persistence of *M. tuberculosis*

Following their entry into the macrophage, mycobacteria use this host cell as an intracellular niche, avoiding any extracellular exposure to the host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primary infection
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Ghon complex = granuloma + draining lymph mode
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containment of infection
uncontrolled spread in immunocompromised patients
granuloma formation
miliary tuberculosis
bone - tuberculosis (Pott´s disease)
Landouzy - sepsis
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b
latent M. tuberculosis infection
exogenous reinfection
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strong cellular immunity
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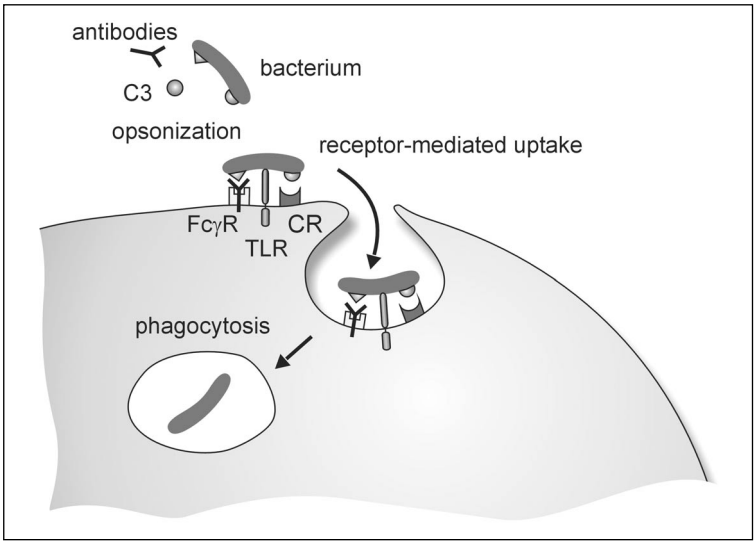

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Fig. 1. Balance between *M. tuberculosis* and the host immune system [adapted from 64]. ***a*** *M. tuberculosis* is inhaled in droplets. After an incubation period of 4–12 weeks, infected alveolar macrophages containing the pathogen either destroy their predators (a mechanism that has not yet been proven, but probably accounts for a small proportion, left) or they fail to contain the pathogen and die (right). In the first case, infection is abortive, in the second case, the pathogen spreads throughout the body and causes active disease. Given that the immune response and virulence of *M. tuberculosis* are balanced (middle), intracellular bacteria are contained by the macrophages, and the immune system isolates the primary site of infection by granuloma formation (primary lesion). In this third scenario, being the most frequent one, infection without clinical disease develops. ***b*** *M. tuberculosis* can persist in a dormant state for long periods of time. Any disturbance of the balance between host and pathogen after weakening of the cellular immune response (immunosuppression) causes endogenous exacerbation which leads to active (post primary) tuberculosis. Active tuberculosis can also be caused by exogenous reinfection.

Fig. 2. Macrophage receptors involved in the interaction with mycobacteria [adapted from 65]. Phagocytosis of mycobacteria is mediated by several receptors including pattern recognition receptors like toll-like receptors (TLR) and complement receptors (CR) and Fc-γ receptors. The way of uptake may influence the cellular response to intracellular mycobacteria.

immune system. *M. tuberculosis* has developed several strategies, ensuring its survival and persistence with the host cell. Early studies of infected macrophages in human and animal lung tissue revealed the presence of acid-fast bacilli. The bacilli proved to be not cultivable [8, 9], but it was unclear whether they entered an altered developmental state that rendered them unrecognizable by the immune system. Alternatively, it was possible that the bacilli

remained undetectable due to an alteration of their surface patterns and then no longer being acid-fast. Furthermore, chemotherapy appeared to reduce the risk of reactivation of remaining persistent bacilli [10].

Several models have been developed to facilitate investigations of the further persistence of *M. tuberculosis*. Up to date, however, none of them accurately describes mycobacterial infection of and persistence in man in satisfactory accuracy. McCune et al. [11, 12] developed a model of *M. tuberculosis* repression and reactivation in mice. In this model, mycobacteria in mice with active disease were initially repressed by chemotherapeutic treatment until they were no longer cultivable nor detectable by microscopic examination, then reactivated by immunosuppression so that the mice developed symptoms of endogenous reactivation of tuberculosis. However, since mice do not develop progressive cavitating granulomas typical for human active tuberculosis, and the occurrence of reactivation of *M. tuberculosis* in the mice in this model was highly variable, the value and implication of this and similar mouse models are limited.

Generally, reactivation progresses from the lung apices where latent bacilli are found [13]. Primary lesions become sterile after several years. However, it remains unclear why lung apices, the preferred site for persistent mycobacteria, do not always present typical granuloma formation [14].

Mycobacterial Enzymes Involved in Persistence

In an attempt to mimic the environmental conditions within a lung granuloma, Wayne [15, 16] established an in vitro model of mycobacterial persistence. He proposed that *M. tuberculosis*, an aerobic acid-fast bacillus, adapts to the low oxygen content within the granuloma and thus fails to grow under normal culture conditions. This could explain the failure to culture 'persistent' mycobacteria isolated from human lung granulomas. Wayne and Lin [17] noted a metabolic downshift in mycobacteria during gradual oxygen depletion. Oxygen-deprived *M. tuberculosis* upregulates enzymes involved in the glyoxylate shunt, a metabolic pathway that converts fatty acids into carbohydrates. Isocitrate lyase and malate synthase convert acetyl-CoA derived from fatty acids into malate (via glyoxylate and via succinate), which can then be used for glucose synthesis.

The glyoxylate shunt allows *M. tuberculosis* to generate glucose independently from oxygen-consuming steps of conventional carbohydrate synthesis [15]. A major advantage of the glyoxylate shunt is the usage of lipids, abundant in the caseous center of the granuloma, as energy and metabolic source. The key enzyme of the glyoxylate shunt is isocitrate lyase [18]. McKinney et al. [19] generated a *M. tuberculosis* mutant lacking this enzyme and observed declining numbers of mutant bacteria in infected mice in the later stage of infection.

These findings suggest that the glyoxylate shunt is essential for the pathogen to survive in the developing granuloma and therefore essential for the adaptation to this altered environment in order to persist.

Adaptation of mycobacteria to the low-oxygen pressure within the granuloma also seems to require an anaerobic nitrate reductase that allows using nitrate as electron acceptor instead of normal oxygen respiration [20]. *Mycobacterium bovis* BCG mutants lacking genes for the subunits of nitrate reductase express decreased virulence in SCID mice compared to wild-type *M. bovis* BCG [21]. Adaptation to low oxygen pressure requires induction of various gene products, and only a small proportion of these has been investigated thus far. Glickman et al. [22] generated a *M. tuberculosis* mutant deficient in cord formation due to malfunctioning cyclopropane synthase. This enzyme modifies mycolic acids by cyclopropanating the proximal end. The mycolic acids are components of trehalose-di-mycolate, the so-called cord factor and are a major component of the mycobacterial cell wall. This defect in mycolic acid synthesis could alter the outer surface of *M. tuberculosis* affecting membrane fluidity, permeability and antigenicity. In the mouse model, the mutant bacilli replicated rapidly, but were unable to kill the host and decreased numerically at later stages of infection. Mutants which are unable to synthesize or to transport the cell wall associated lipid phthiocerol dimycocerosate (PDIM) also fail to persist in the lung. PDIM is found only in cell walls of pathogenic mycobacteria and serves as virulence factor [23].

Mycobacterial Gene Regulators Involved in Persistence

The complete sequence of the *M. tuberculosis* genome provides us with a tool to elucidate gene regulation underlying mycobacterial persistence in the infected host [24]. Amongst the first genes to be discovered were the genes encoding the RNA polymerase σ factors which direct the transcription machinery to distinct genes required for bacterial survival under altered environmental conditions [25, 26]. One of the *M. tuberculosis* σ factors, SigF [27], shows homology to a σ factor of *Bacillus subtilis* which is involved in stress responses and in sporulation [28, 29]. SigF is highly expressed during persistence, when replication and growth are downregulated, and is undetectable in the exponential growth phase, with a relatively high division rate [26]. A *M. tuberculosis* mutant with an inactivated sigF gene failed to survive in later stages of infection when granuloma formation is induced (fig. 1). The failure of the sigF deletion mutant to adapt to the altered environment within the granuloma suggests its essential role for persistence of *M. tuberculosis* [30].

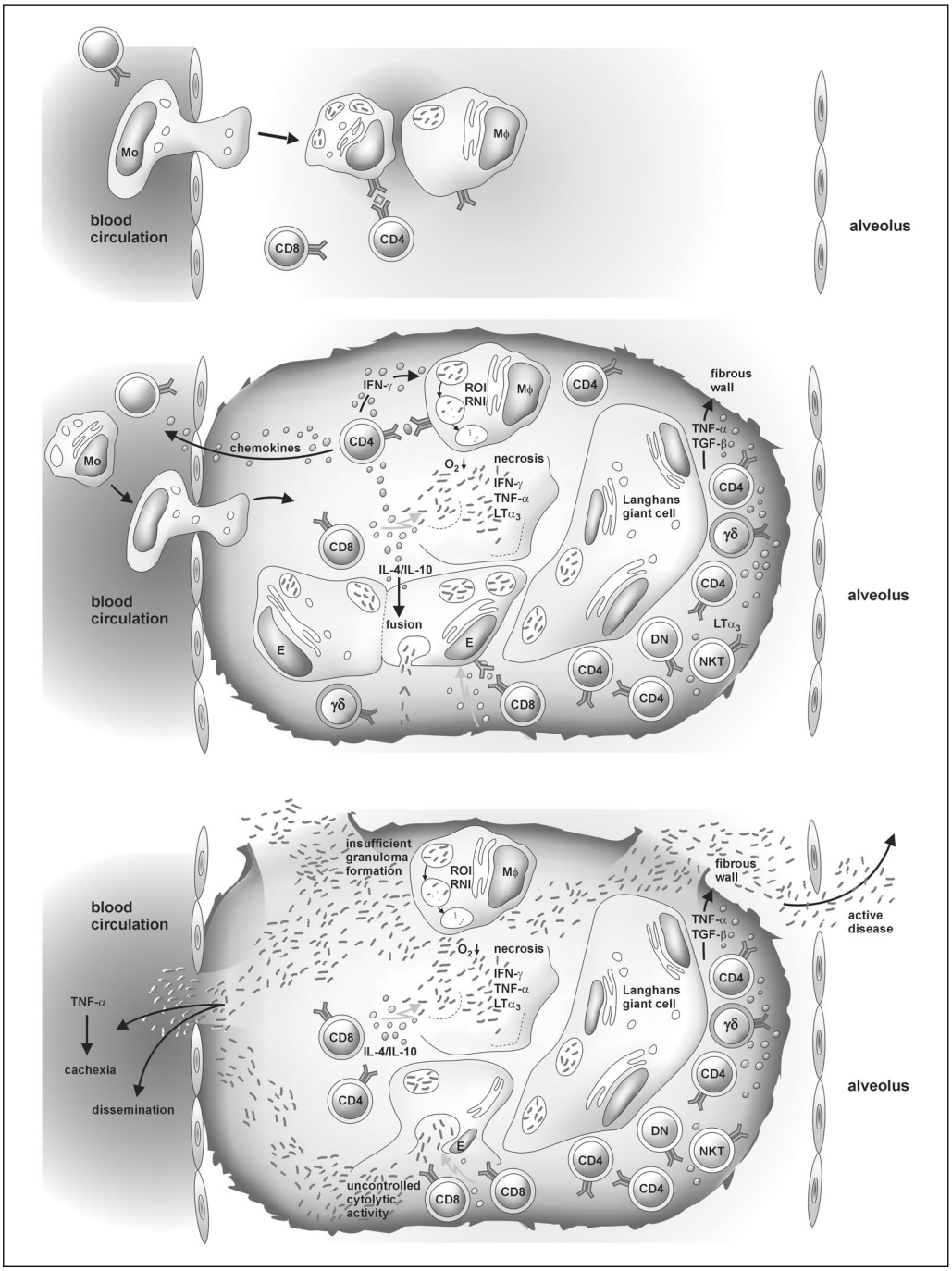
In the fast-growing *Mycobacterium marinum* which causes a tuberculosis-like disease in fish and amphibia, differential gene regulation in granulomas and during exponential growth has been identified [31]. These genes were identified

as two so-called PE-PGRS genes [32]. The repeat PE-PGRS is shared by approximately 60 genes of *M. tuberculosis* which encode glycine-rich proteins with a characteristic glutamate-, proline-containing motif. Although the functions of the PE-PGRS repeats remain unknown, directed mutations within the repeats impaired survival in granulomas [31]. This approach could provide a strategy towards identification of novel genes of *M. tuberculosis* involved in persistence. For example, it could be examined whether genes belonging to the PPE family (with a proline-, proline-, glutamate-rich motif) play a role in the survival of *M. tuberculosis* in lesions. PPE and PE-PGRS genes belong to a larger family of PE genes, which account for approximately 10% of all encoding genes in the mycobacterial genome. Some of them have been identified by mRNA differential display to be only expressed in the virulent H37Rv strain and not in the avirulent H37Ra strain [33, 34]. Additionally, PE genes are upregulated in mycobacteria residing in granulomas and therefore could contribute to persistence [31].

Immune Response against *M. tuberculosis* during Persistence

The appropriate immune response can control mycobacterial growth and establish a balance between host immune system and pathogen (fig. 1, 3). The cytokines IFN-γ and TNF-α play a key role during latency. They activate macrophages to produce inducible nitric oxide synthase (iNOS) and to sustain pathways generating reactive nitrogen intermediates (RNI) [reviewed in 35, 36]. In addition, oxidative effector molecules are generated. Although the oxidative and nitrosative stress reduces mycobacterial growth, it fails to eliminate the pathogen. Recent data suggest that *M. tuberculosis* resists host effector

Fig. 3. Host response and granuloma formation [adapted from 67]. The complex immune mechanisms that isolate the site of mycobacterial infection from the remaining part of the body still remain unknown. The following hypothetical scenario can be drawn: Alveolar macrophages (M⊖), epitheloid cells (E) or Langhans' giant cells (generated by fusion of epitheloid cells) harboring intracellular mycobacteria form the center of the granuloma. They present antigens to T cells and activate them to produce a variety of cytokines and chemokines or to kill the infected cells and intracellular mycobacteria. Chemokines recruit additional cells from the blood circulation to the site of primary infection. IFN-γ activates macrophages and other antigen-presenting cells to kill the intracellular bacteria via inducible nitric oxide synthase (iNOS) which generates reactive nitrogen intermediates. $CD4^+$ T cells produce TNF-α and lymphotoxin α3 (LTα3) which are required for the formation of the wall encapsulating the granuloma. In the center of the productive granuloma, cell detritus and low oxygen pressure form a hostile environment for released mycobacteria. Activated $CD8^+$ T cells kill mycobacteria by means of granulysin and perforin. Killing of infected cells, however, needs to be controlled, in order to retain the integrity of the granuloma.


blood circulation
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LTα3
IL-4/IL-10
fusion
E
Langhans giant cell
fibrous wall
TNF-α
TGF-β
γδ
DN
NKT
insufficient granuloma formation
active disease
TNF-α
cachexia
dissemination
uncontrolled cytolytic activity

3

mechanisms by means of a peroxidase/phosphonitrite reductase system which also participates in intermediary metabolism [37].

TNF-α plays a role in containing persistent *M. tuberculosis* organisms and in preventing them from reaching other regions of the lung or other organs [38, 39]. Encapsulation of the granuloma and formation of the fibrinous wall is primarily mediated by TNF-α [40]. TNF-α, administered to mice depleted of $CD4^+$ T cells and latently infected with *M. bovis* BCG, prevents recrudescence of infection [41, 42]. In mice, monoclonal antibodies against TNF-α caused reactivation of latent *M. tuberculosis* infection. IL-10 expression was augmented, whereas IFN-γ and IL-12p40 remained unchanged [43]. This finding suggests that TNF-α prevents endogenous reactivation by modulating cytokine levels and limiting histopathology. Elevated levels of TNF-α, TGF-β and IL-10 were also detected in sera and pleural fluids of patients with lung tuberculosis [44]. In general, the risk of endogenous reactivation of latent *M. tuberculosis* seems to increase when levels of TNF-α are decreased. This has been emphasized by the fact that reactivation of tuberculosis represents a major side effect of anti-TNF-α antibody therapy of severe rheumatoid arthritis [45, 46].

IFN-γ and iNOS [47] are also crucial for containing the infection and keeping the balance between replication of *M. tuberculosis* and immune defense [reviewed in 36]. Treatment with the iNOS inhibitor aminoguanidine impairs RNI production and causes reactivation of tuberculosis, leading to fatal disease in the mouse model [48]. Although $CD8^+$ T cells participate in control of latent infection [49], substantial evidence emphasizes the major role of $CD4^+$ T cells in containing the disease at later stages. $CD4^+$ T cells control persistent mycobacteria contained within a granuloma at least in part in an IFN-γ and RNI-independent way [50]. Depletion of $CD4^+$ T cells in the mouse model caused rapid reactivation of previously dormant *M. tuberculosis* organisms resulting in increased bacterial load and exacerbation to rapid progressive tuberculosis. Endogenous reactivation of latent tuberculosis in the mouse model occurred despite normal levels of IFN-γ and iNOS, suggesting that $CD4^+$ T cells regulate the balance between *M. tuberculosis* and activated immune cells. Both $CD4^+$ and $CD8^+$ T cells are found mainly in the periphery of intact granulomas, and their total numbers correlate with the structural integrity of the granuloma, underlining their key role in containing infection (fig. 3). That production of lymphotoxin α3 (LTα3) by $CD4^+$ T cells has a role in granuloma formation and maintenance is demonstrable by the failure of LTα3-deficient mice to form intact granulomas and consequent suffering from exacerbated tuberculosis [51]. Reduced numbers of $CD4^+$ T cells in HIV^+ patients have serious implications for reactivation of persistent *M. tuberculosis*, this phenomenon being largely responsible for the recent resurgence of *M. tuberculosis* and the increase of active disease especially in developing countries with high incidence of HIV infection.

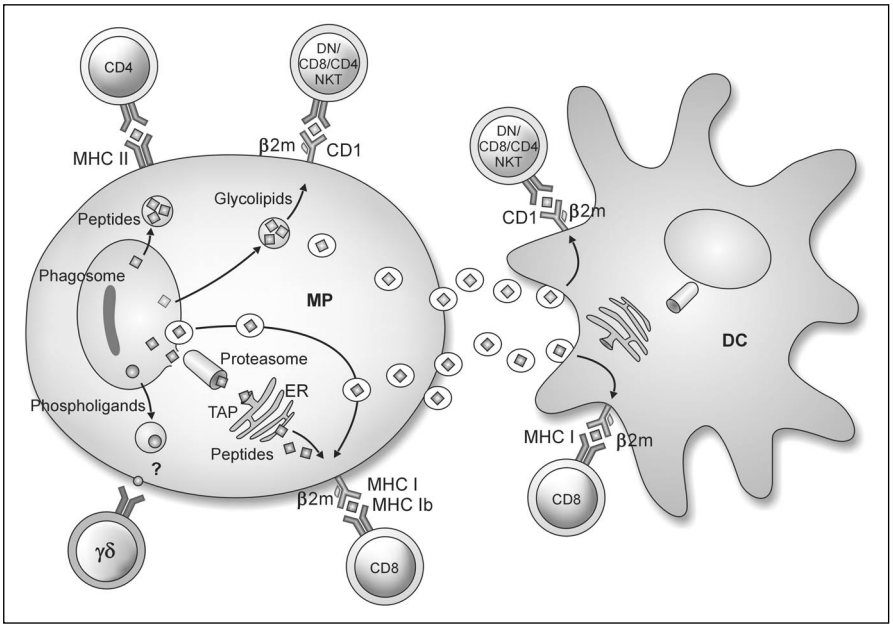


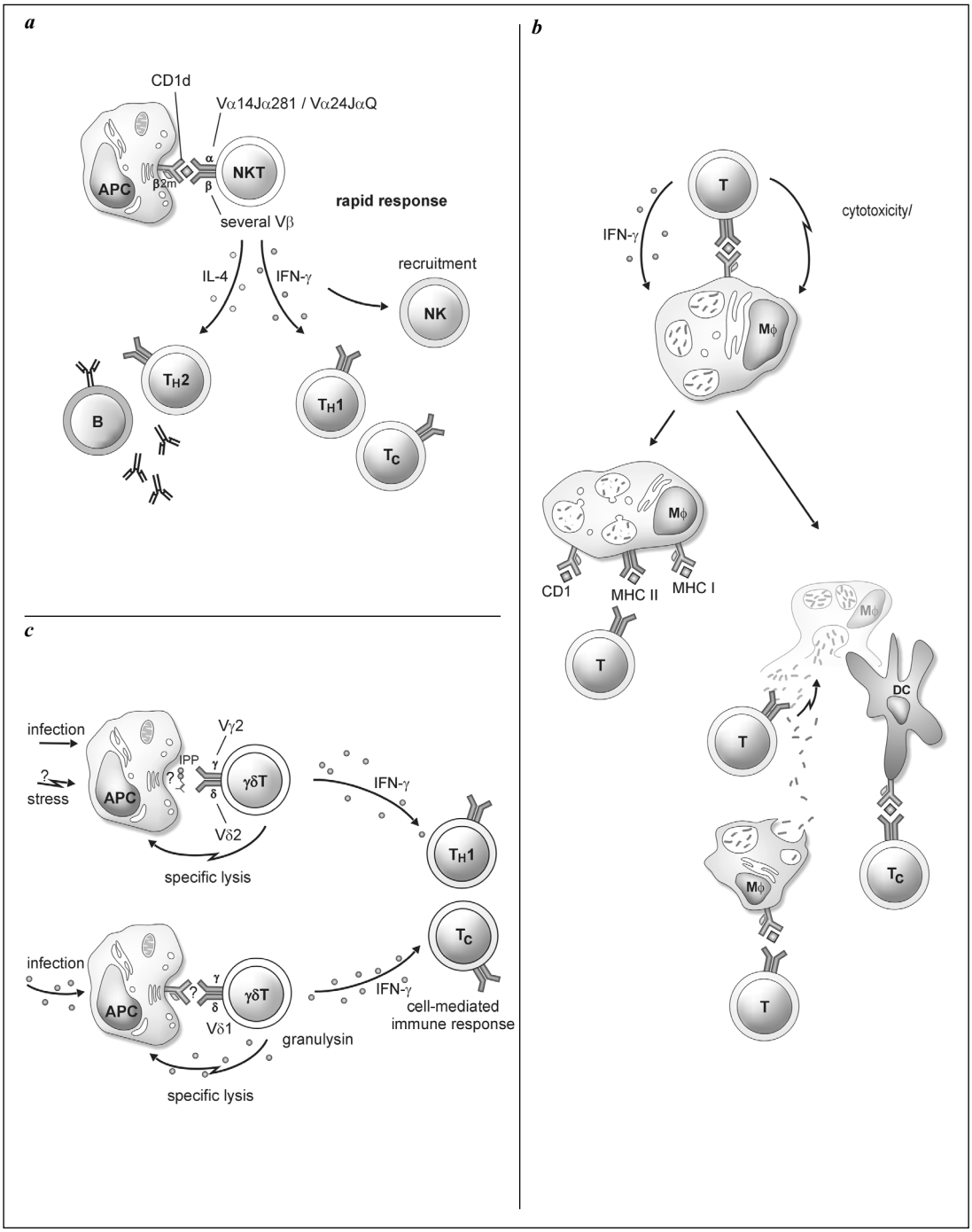
Fig. 4. Classical and alternative ways of antigen presentation in mycobacterial infection [adapted from 67]. Macrophages containing intracellular mycobacteria present mycobacterial antigens via MHC class I and II molecules and via CD1. An alternative pathway for antigen presentation to $CD8^+$ T cell can be initiated by induction of apoptosis by *M. tuberculosis* and formation of apoptotic blebs that can be internalized by bystander dendritic cells and presented to $CD8^+$ T cells.

$CD8^+$ T cells also contribute to the successful immune response against the pathogen (fig. 3). Experiments with mice deficient for β_2-microglobulin [52, 53], CD8α [54, 55] and the transporter-associated protein (TAP) [56] revealed that these mice were all more susceptible to *M. tuberculosis* infection than their wild-type counterparts. At first sight, the involvement of antigen-specific $CD8^+$ T cells in the immune response against *M. tuberculosis* is surprising since the pathogen resides within phagosomes (fig. 1) and has no direct contact to the MHC class I antigen processing machinery. Experiments with antigen model systems (like ovalbumin) suggest that there are alternative MHC class I pathways permitting the processing of phagosomal antigens [55]. Additionally, *M. tuberculosis* induces apoptosis of host cells and formation of apoptotic blebs that can be internalized by bystander dendritic cells and presented to $CD8^+$ T cells [Schaible et al., submitted], as it has recently be described for the *Salmonella* infection model [58] (fig. 4).

The presentation of mycobacterial protein antigens via the MHC presentation system is supplemented by the presentation of lipid and glycolipid antigens by CD1 molecules [reviewed in 59, 60] (fig. 5b). The CD1 family consists of antigen-presenting molecules encoded by genes located outside of the MHC. CD1 genes are conserved among mammalian species and are expressed on the surface of cells involved in antigen presentation, notably dendritic cells. The CD1 system is involved in activation of cell-mediated responses against mycobacterial infection: In mice, CD1d-restricted NKT cells are activated by mycobacterial cell wall components and involved in early granuloma formation [61] (fig. 5a). $CD4^+$ NKT cells produce IFN-γ in the early phase after infection of mice with BCG [62]. However, it is unclear whether CD1d-restricted T cells play any significant role in the protective immune response to mycobacterial infection, since CD1d-deficient mice do not have a higher susceptibility to TB.

Several mycobacterial lipid and glycolipid antigens have been identified that elicit a specific CD1-restricted T cell response [59]. T cells recognizing CD1-restricted antigens have a broad range of functional effector mechanisms

Fig. 5 Unconventional T cells involved in immune response to *M. tuberculosis* [adapted from 59]. ***a*** CD1-restricted NK T cells. NK T cells that recognize lipid antigens presented by CD1d molecules are shown to have a variety of different effector functions: They release high amounts of IFN-γ, directing the immune system to a T_H1-type response and recruiting circulating NK cells for a rapid immune response prior to the reaction of activated conventional T cells. The release of IL-4 induces T_H2 cells and causes antibody production by B cells. Activated NK T cells are also involved via IL-2 in tumor rejection. ***b*** Possible roles of CD1-restricted T cells in the immune response to *M. tuberculosis*. CD1-restricted T cells specific for mycobacterial lipid or glycolipid antigens have several effector functions in the immune response to *M. tuberculosis*: IFN-γ enables the mycobacteria-containing macrophage to activate antimicrobial activities that enable it to destroy the intracellular pathogens and present processed antigens via MHC class I and II and via CD1 molecules, recruiting and activating additional specific T cells. CD1-restricted T cells also possess cytotoxic activities. The destruction of the mycobacteria-containing macrophages decreases the reservoir of host cells for the pathogen and allows cytotoxic T cells to directly kill released mycobacteria. The antigens of the destroyed macrophage can be processed and presented by activated dendritic cells allowing an effective recruitment of additional cytotoxic T cells. ***c*** Roles of γδ T cells in early immune responses. $V\gamma2/V\delta2^+$ γδ T cells recognize pyrophosphate (e.g., isopentenyl-pyrophosphate, IPP) and alkylamine antigens derived from microbes and plants. The mechanism of antigen presentation remains unclear. Activated γδ T cells can lyse the antigen-presenting cells and elicit a T_H1 immune response. GM-CSF derived from activated cells during infection causes the expression of group I CD1 molecules on immature dendritic cells. $V\delta1^+$ γδ T cells recognize CD1c molecules on the surface of these cells; whether a presented antigen is necessary is not clear. After activation $V\delta1^+$ γδ T cells release IFN-γ, activating a T_H1-type immune response, the release of granulysin and specific killing of the $CD1c^+$ cell.


a
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rapid response
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TH1
TC
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T
IFN-γ
cytotoxicity/
Mϕ
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MHC II
MHC I
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infection
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granulysin
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cell-mediated
immune response
specific lysis

5

including cytotoxic activity. This suggests that the CD1 system is involved in both innate and adaptive immune responses against infection with *M. tuberculosis* in the early and late phases of infection (fig. 5b).

γδ T cells are involved in the rapid early phase of the immune response to mycobacteria [reviewed in 63]. γδ T cells usually recognize pyrophosphate and alkylamine antigens. The antigen-presenting molecule is still unknown (if it is required at all). Some of the γδ T cells recognize MICA and MICB on stressed cells, distantly related homologues of classical MHC class I molecules [63, 64], as well as CD1c on infected cells. These recognition mechanisms could play an important role in the early phase of the immune response following TB infection (fig. 5c).

Concluding Remarks

The capacity to persist for long periods of time in the presence of a competent host immune response and waiting for any weakness of the immune response, in order to exacerbate, are the main cause for the worldwide success of the tubercle bacillus. If we aim at efficacious control of tuberculosis, we first need to understand the survival strategies of the pathogen within the granuloma on the one hand, and the different stratagems of the immune system to control the infection on the other hand. The understanding of mycobacterial persistence and the host immune response will allow us to develop novel vaccine strategies [65] that are described in Gicquel, B., p. 128.

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New Strategies for the Design of Vaccines against Tuberculosis

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The identification of the etiologic agents of infectious diseases has led to the preparation of vaccines made of their constituents. Toxins inactivated by chemical agents provided very efficient vaccines against diphtheria and tetanus. The situation is more complicated for bacterial pathogens like tuberculosis (TB) for which no toxins have been identified. The discovery of the tubercle bacilli by Robert Koch as the etiologic agent of TB allowed the development of diagnostic tools. However, attempts to use extracts of this bacilli or heat-killed cultures as vaccines have failed. The isolation of an attenuated culture of *Mycobacterium bovis*, the etiologic agent of bovine TB, has led to the BCG, bacillus of Calmette and Guérin, being obtained [1]. After 231 in vitro passages, this culture was used to immunize children against TB. This BCG vaccine is ill defined, but is the only one currently used to protect against TB. As previously observed during retrospective studies performed by Calmette, this vaccine protects the infants against the severe forms of TB, i.e. tuberculous meningitis and miliary TB. However, its protective efficacy against the pulmonary forms of TB in the adult population is variable as illustrated by numerous trials in different regions of the world: protective efficacy was 85% in the schoolchild population in the UK but 0% in South India [2]. The occurrence of major side effects in immunocompromised individuals, despite being a rare event, encouraged the isolation of well-defined subunit vaccines.

TB is still an important public health problem. Immunization with vaccines more efficient than BCG would be the most cost-effective intervention. Recent studies in cell biology, molecular immunology and microbiology might provide valuable information for the preparation of new vaccines.

Protective Immune Responses

Only a small percentage of infected individuals, 5–10%, develop the TB disease during their lifetime. In immunocompromised individuals this percentage is much higher, about 5–10% each year after infection. The immune responses that effectively contain and eliminate the tubercle bacilli are cellular responses. Individuals with genetic defects involving the absence of synthesis of functional IFN-γ, IL-12 or STAT1, develop disseminated infection after vaccination with BCG and are susceptible to infections due to atypical mycobacteria from the environment [3]. Experiments with animal models show that passive transfer of sera from immunized animals after BCG vaccination does not induce protection, showing that antibodies are not sufficient to control infection. However, antibodies might be important in the uptake of bacilli by macrophages after opsonization. Opsonized bacilli are killed by macrophages whereas non-opsonized bacilli resist inside phagosomes that do not fuse with lysosomes [4]. T cells from BCG-immunized or TB-infected mice which are also treated with antibiotics have been transferred to naive mice. These T cells protect the recipients against challenge with *M. tuberculosis*. These findings were confirmed by experiments with knockout (KO) mice showing that $CD4^+$ T cells are essential for protection. $CD8^+$ T cells also contribute to protection at a chronic stage, although to a lesser extent. A $CD8^+$ T-cell proliferative response to *M. tuberculosis* is found in the peripheral blood of healthy BCG-vaccinated donors. The $CD8^+$ T-cell responses in TB patients are weaker than that in healthy BCG-vaccinated donors. Thus, $CD8^+$ T-cell reactivity is observed in patients controlling the infection [5].

More recent studies demonstrated the existence of T-cell responses to non-protein antigens. Lipids and lipoglycans are presented by CD1 molecules [6]. They are recognized by CD4/CD8 double negative T cells including NKT cells but also by $CD8^+$ and $CD4^+$ T cells. Two groups of CD1 genes are present in the human genome, one group including CD1 a, b and c, a second group comprising only CD1 d. Only one group including CD1 d1 and d2 is present in mice. These differences raise problems for the utilization of mice as a model. Although KO mice for CD1 are not more susceptible to TB, this finding does not exclude important roles for CD1 a, b or c. T cells reactive to CD1-presented antigens pre-exist adaptative responses. By their cytotoxic activity and secretion of IL-4 or IL-12 they may play a major role in the induction of the adaptive responses and also contribute to eliminating infected cells. Another class of T cells, γδ T cells are abundant after infection; they recognize phosphoantigens in humans [7]. T cells reactive to these small non-protein antigens are absent in mice, thus confirming the limitation of this animal model to study protection against TB.

The search for antigens that are recognized by TB patients and that constitute promising vaccine candidates is complicated by a heterogeneity of responses of humans and animals to TB antigens [8]. Recent studies in macaques gave unexpected results. Although BCG protects many animal models including the cynomolgus species, it does not protect rhesus macaques [9]. Both species induce high level of IFN-γ synthesis after vaccination. It will be extremely important to investigate this difference in controlling infection. Despite these difficulties, several vaccine candidates that show protection in several animal models have been isolated. Some of them will be tested in phase 1 clinical trials in a near future.

Subunit Antigens

The demonstration that culture filtrates protect mice led to the isolation of proteins from these filtrates [10, 11]. Separation by 2-D gels electrophoresis and testing antigens by their capacity to induce proliferative responses in PBMC of patients or memory responses of mice T cells allowed the isolation of a panel of antigens. In particular, a low molecular weight antigen, ESAT6, was recognized by a large proportion of TB patients [12]. When associated with potent adjuvants like DDA + MPL, ESAT6 induces protection in mice [13]. Its gene has been fused to a major secreted antigen gene, 85B, which belongs to the 85 antigen family and codes for a mycolyl transferase enzyme. The fusion protein confers protection in the mouse model [14]. The ESAT6 antigen gene is expressed together with another low molecular weight antigen, ESAT10, also recognized by a large number of TB patients [15]. The genetic region encoding these two antigens is absent from all BCG strains. The antigens could be used either as diagnostic reagent or as vaccine, but not for both purposes: immunization with these antigens would preclude their use for diagnosis. A number of antigens induce proliferation of PBMC isolated from TB patients. They will be studied as vaccine candidates in the near future. The complete sequence of the *M. tuberculosis* genome is now available and this facilitates the construction of a well-defined map of antigens separated by 2-D gels analysis and characterized after mass spectrometry studies [16].

One of the antigens, 85A, has been tested as a DNA vaccine [17]. Protection was observed in C57/BL6 mice. However, no protection was observed in the very sensitive guinea pig model [18]. Studies combining this gene with adjuvants are under way. Other antigens provide protection when used as DNA vaccines, for example PstS3. However, no protection was observed with PstS1, another member of the PstS family (encoding phosphate transport molecules) [19]. Immunizations with mycobacterial HSPs have given different results.

DNA vaccination with the *Mycobacterium leprae* gene encoding the stress protein HSP60 was reported to induce protection in outbred Parkes mice [20], but vaccination of C57BL/6 mice with the *M. tuberculosis* gene encoding HSP60 did not provide protection [21]. DNA encoding *M. leprae* HSP60 gave protection when used as a therapeutic vaccine [22]. Using the same DNA vector, similar protection was observed with the HSP60 and IL-12 genes suggesting that non-specific responses might have been responsible for the observed effect.

In guinea pigs, no significant protection with subunit vaccines (proteins or DNA) have been reported. Vaccine preparations are currently being optimized (by using new adjuvants).

Antigen 85A was used as a boost after DNA vaccination in mice. This protocol of immunization provided protection superior to BCG immunization alone [23]. These results suggest that immunization with mycobacterial antigens may improve protection in BCG-vaccinated populations which is the situation in many developing countries.

Non-protein antigens are a class of molecules which are potentially valuable. The T-cell populations recognizing phosphoantigens or CD1-presented lipids and lipoglycans make up about 20% of all circulating lymphocytes. They are of limited genetic diversity. Therefore, the use of non-protein antigens might offer a dual advantage of triggering T cells not restricted by the genetic diversity of MHC and that produce cytotoxic effects and secrete cytokines thereby regulating the outcome of T-helper responses. Because mice do not carry CD1 a, b, or c genes and do not possess T cells reactive to phosphoantigens, non-human primates are being used for work in this area. Non-protein antigens could be included in the composition of subunit antigen preparations to provide a strong innate immunity and induce appropriate adaptative Th1 responses.

New Attenuated *M. tuberculosis* or BCG Strains

The difficulty of finding *M. tuberculosis* antigens that are recognized by all individuals has encouraged other approaches. The utilization of attenuated pathogens would lead to vaccine candidates containing most antigens encoded by the pathogen. It is tempting to use BCG improved by genetic engineering or newly attenuated *M. tuberculosis* strains to immunize non-immunocompromised populations.

BCG was isolated after 231 in vitro passages and then distributed to various laboratories that propagated the BCG cultures in different conditions. This led to a series of BCG daughter strains differing both in the production of various antigens and in immune properties. Some of these strains induce side effects like adenitis more frequently than others after vaccination. Consequently, although

they might confer a higher degree of protection, they have been abandoned for vaccination purposes. Recent studies identified major genetic differences between BCG strains and between these strains and *M. tuberculosis*. Some of the differences correspond to polymorphism observed between *M. bovis* strains or between *M. tuberculosis* and *M. bovis*. It would be interesting to reintroduce into BCG some genetic regions coding for major immunogens like ESAT6 and ESAT10 and investigate whether the resulting strains conferred improved protection in animal models. This type of experiment is under way in a number of different laboratories. BCG strains expressing several copies of the major secreted antigens (antigen 85) were constructed. They induce slightly higher protection in guinea pigs in a high-dose aerosol model [24].

To increase its immunogenicity, cytokines, chemokines or co-stimulatory molecules could be associated with BCG by integrating the corresponding genes into the BCG genome. This approach has been tried with other vaccinial vectors like vaccinia. Increased immunogenicity was observed. A number of genes coding for cytokines have been inserted into BCG. For example, the team of R. Young inserted the genes for GM-CSF, IL-2, and IFN-γ. They observed a higher reactivity of spleen cells to mycobacterial antigens [25]. However, no studies of the protection conferred by such recombinant strains have been published.

To improve cytotoxic responses induced by BCG, the team of S. Kaufmann engineered BCG strains by inserting the listeriolysin gene. This enzyme is essential for disrupting phagosomal membranes in *Listeria*. It was suggested that its expression by BCG would increase the availability of antigens for class I presentation. Although no major changes were observed in the phagosomes, cytotoxicity responses were higher [26].

BCG was isolated from a bovine strain of TB: Calmette and Guérin were working on the calf animal model to study TB transmission through the intestinal barrier after oral contamination. The isolation of an attenuated strain after a series of in vitro passages during these investigations led to the discovery of the BCG vaccine. The isolation of an attenuated strain from the human bacilli might lead to a vaccine which immunizes against antigens that are more relevant to human TB. The development of genetic tools during the last decade allows the inactivation of genes in *M. tuberculosis* either by allelic replacement or random mutagenesis. Screening can be used to select attenuated strains. In the absence of screening, genes can be chosen for inactivation in light of previous studies with other pathogens.

The first attenuated strain which provided some protection in the very sensitive guinea pig model is a *purC M. tuberculosis* auxotroph [27]. This strain was obtained by allelic replacement using the very efficient ts/*sacB* vector. This strain is attenuated in mice, guinea pigs and in the in vitro cellular model based on bone marrow macrophages. The protection observed in guinea pigs is much

lower than that with another mutant, *erp M. tuberculosis*, which was obtained by inactivating a surface antigens required for virulence [28, and pers. unpubl. data]. It is interesting to note that higher protection is observed with the *erp* strain which persists than with the *pur* strain which is rapidly eliminated. This is consistent with results obtained with attenuated strains of *Salmonella* showing that rapidly eliminated strains do not induce substantial protection.

Other auxotrophic mutants have been isolated either from BCG or *M. tuberculosis* strains. BCG *leu* and *met* mutants are attenuated in BALB/c mice and provide protection similar to that conferred by BCG. They are also attenuated in SCID mice which can be considered to be a model for immunocompromised hosts [29]. *leu M. tuberculosis* mutants are attenuated in BALB/c mice and confer some protection against challenge with *M. tuberculosis* [30]. Mutants impaired in *trpD* or *proC* are avirulent in mice and provide some protection against challenge with *M. tuberculosis*. They are also attenuated in SCID mice. However, a *metB M. tuberculosis* mutant is still virulent in BALB/c mice [31].

To investigate a large number of different attenuated strains, libraries of insertion mutants of *M. tuberculosis* were constructed and screened for attenuated mutants. The STM technique was adapted to *M. tuberculosis*. Different classes of mutants were isolated, including mutants impaired in the synthesis of DIM, and mutants impaired in the synthesis of regulators or in the synthesis of other non-protein components of the cell wall [32]. Mutants impaired in the synthesis or in the transport of DIM are attenuated in mice and guinea pigs and provide protection similar to that conferred by the *erp* mutant in guinea pigs [pers. unpubl. data]. Animal models used to study attenuation and protection differ between laboratories. Different mouse lines are used, commonly either BALB/c, C57BL/6 or DBA/2. Only a limited number of attenuated strains have been tested in the sensitive guinea pig models. Although technically trivial, inoculates containing different numbers of CFU have been used so that it is difficult to compare findings between studies. Furthermore, many results remain to be reproduced.

Recombinant Live Vaccines: *Salmonella*, MVA

Live vaccines could be used to express and present mycobacterial antigens that are promising as vaccine candidates. *Salmonella* have been used to induce mucosal responses that are important in some pathologies. Recombinant *Salmonella* expressing ESAT6 were constructed. They induce a slight level of protection in mice [33]. A combination of recombinant *Salmonella* expressing ESAT6 and DNA vaccination with ESAT6 gene did not increase the level of protection.

To induce cytotoxic responses, poxvirus was used. Recombinant MVA vaccines were used in protocols including DNA vaccination, and protection was observed [34]. However, although MVA can be used for human trials, DNA vaccination still raises some concern. A MVA recombinant vaccine expressing 85A in combination with BCG is currently undergoing phase 1 clinical trials.

Global Analysis

The genome sequences of many pathogens, including *M. tuberculosis*, are now known. However, existing software is unable to predict which antigens will be protective. Some epitopes can be predicted from isolated proteins, thus facilitating studies when antigens are already isolated and proved to be immunogenic [35]. Knowledge of human and mouse genome sequences allows the construction of microarrays containing a large number of sequences including key players of the innate and acquired immune responses. They can be used to analyze mRNA produced by the host in response to an infection or after immunization with antigens in different vaccine preparations. It will be important to define immune responses correlated with protection and investigate their presence in different individuals to adapt vaccination so as to maximize effectiveness for those individuals. The cytokines induced after infection with *M. tuberculosis* in the mouse models have been identified. The kinetics of induction differs according to the cytokine [36]. Analysis of cytokines, chemokines and their receptors induced in macrophages after infection with different pathogens revealed common patterns of induction but also repression, for example of the synthesis of MHC molecules [37]. This lends weight to the idea that adding cytokines to vaccine preparations could improve effectiveness.

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Modern Drug Development

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Current recommended drug therapy for tuberculosis (TB) consists of 2 months of daily treatment with isoniazid, rifampicin, and pyrazinamide, often with the inclusion of ethambutol in areas where drug resistance is common. This 2-month course of therapy is usually sufficient to render all patients sputum culture negative but an additional 4 months of therapy is required to guarantee that patients will not relapse with disease following completion of their regimen [1]. This optimum 'short-course' chemotherapy was determined to be effective through an extraordinary series of field trials conducted in TB patients globally from 1946 to 1976 and it is highly effective – if the entire 6-month course is completed, 95% or more of patients can be cured of their disease [2].

The reality of the situation is that TB control programs now focus their resources on increasingly complex logistical solutions to the problem of ensuring that patients complete this extensive chemotherapy rather than on drugs per se or ancillary activities such as surveillance and detection of new cases. Direct supervision of therapy by a health care provider (originally implemented by the British Medical Research Council in 1958 in Hong Kong and Madras but then later re-marketed as 'DOT' or Directly Observed Therapy [3]) is now considered standard despite that this requires the vast majority of the resources for TB 'chemotherapy' go not to pay for the drugs, but for the support infrastructure necessary to deliver them effectively [4]. Despite heroic efforts by the international community, in many regions of the world TB control programs either do not exist or are loosely organized coalitions with little understanding of the principles of TB therapy and with little access to high-quality drugs or a stable system through which to supervise their administration for such an extensive time period.

A major contributing factor, therefore, to the difficulty of achieving worldwide success with TB eradication stems from the fundamental limitation

that current chemotherapy does not result in the rapid sterilization of TB infections. The current arsenal of front-line therapeutics was for the most part developed half a century ago. Many of the individual antimycobacterial agents have low potency, and resistance to these is increasingly common [5]. The pharmaceutical industry has developed many innovative new approaches, both with respect to the science of drug discovery and optimization, and with respect to formulation and safety, that could be creatively applied to TB [6]. The industry, however, has not perceived significant financial incentives to engage in TB drug development, but this situation may be changing [4]. This review will therefore highlight some of the innovations most likely to impact the development of new TB drugs. Technological solutions will not, however, be sufficient to radically change the duration of anti-TB therapy, they will only shorten and refine the process of developing drug-like molecules for certain well-characterized targets. Only by integrating these solutions with a solid appreciation of the biology of the organism *Mycobacterium tuberculosis* and its interaction with *Homo sapiens* will a true revolution in TB therapy come to pass. This review will therefore attempt to highlight some of the issues and approaches to target selection that will result in modern drugs for this ancient disease.

Development of Current TB Chemotherapeutics

Before examining the impact of modern technology on future TB drug development, it is important to have a sense of how the current TB therapeutic agents were first discovered and developed. The golden age of TB drug discovery was clearly the period from 1945 to 1960 when every front-line agent currently utilized was discovered and introduced into the clinic. Millions of patients suffering from TB were spared as a result of the tremendous activity in pharmaceutical laboratories in Europe and North America. Many of the contemporary paradigms of drug discovery were established in the process of searching for new cures for TB including the identification and synthetic modification of natural products (streptomycin and rifampicin), screening and lead optimization (ethambutol), and application of knowledge of the basic physiology of the tubercle bacillus (isoniazid, *p*-aminosalicylate and pyrazinamide). This section will review the 'discovery' phase of each of these agents and will highlight the pathway that led to their clinical development.

Streptomycin

The beginning of chemotherapy for TB was the discovery of streptomycin by Albert Schatz and Selman Waksman at Rutgers. This discovery

emerged from a systematic, brute-force screen of soil-dwelling Actinomycetes for broad-spectrum antibacterial activity. Streptomycin was isolated from *Streptomyces griseus* based initially on agar plate cross-streaking assays with candidate microbes. The organism itself came from heavily-manured soil (and was apparently independently isolated from throat swabs of a sick chicken) [7]. Although the organism was eventually shown to produce a substance with activity against TB, it was initially selected for based on broad-spectrum activity. Streptomycin was identified as the component responsible for the antimycobacterial activity by fractionation and purification of broths from fermentation of *S. griseus*. It is important to note that even before the active component had been identified, indeed within weeks of its discovery, streptomycin was evaluated in a chicken embryo model of infection with *Salmonella gallinarum* and within 6 months of its discovery streptomycin was being evaluated for efficacy in guinea pigs infected with *M. tuberculosis* [8]. Even more amazingly, within 1 year of its discovery, streptomycin was used for the first time to treat a pulmonary TB patient [9]. Eight years later, in 1952, Selman Waksman was awarded the Nobel Prize for medicine for discovering the first highly effective anti-TB agent.

p-*Aminosalicylic Acid*

p-Aminosalicylic acid, or PAS, was the second major anti-TB medication discovered. Its utility was predicted based on the observation that aspirin, acetylsalicylic acid, induced respiration of the bacillus. Jorgen Lehmann [10] therefore hypothesized that the *p*-amino analog of salicylate would act as a competitive inhibitor of respiration that would block replication of the bacteria through oxidation of the anilino nitrogen. Although he was likely not correct about the details of the actual mechanism, which still remain unclear, Lehmann predicted the efficacy of the compound long before it had been synthesized and the identification of the drug relied completely on an understanding of the basic physiology of the bacterium [11]. Like modern-day computer-generated lead molecules, the difficulty with PAS lie in actually synthesizing the molecule, which despite its apparent chemical simplicity, took longer to synthesize than the 3 months that separated delivery of the drug and the first human clinical usage [12].

Isoniazid

The discovery of isoniazid was made possible by a combination of rational design and elements of high-throughput evaluation of analogs. Gerhard Domagk, who won the Nobel Prize for his efforts in developing sulfonamides, had synthesized and tested a large series of compounds called thiosemicarbazones which ultimately resulted in the discovery of thiacetazone, a highly

active agent still used clinically in some parts of the world [13]. Domagk, and his contemporaries, were aware that nicotinamide had an unexplained but potent effect on TB [14–16]. Because of this activity, amides, hydrazides, and even semithiocarbazones were made with a pyridine nucleus similar to nicotinamide and tested against TB. Amazingly, three different groups arrived at isoniazid from these observations virtually simultaneously [17–19]. To their disappointment the compound was subsequently discovered not to be new (and therefore not patentable) as it had been synthesized previously in 1912 as a simple hydrazine analog of a pyridine carboxylic acid [20].

Pyrazinamide

The same fundamental observation that nicotinamide had a potent effect on the course of experimental TB infections led to the discovery in 1952 of another antitubercular, pyrazinamide [21]. Pyrazinamide is an example of a compound that would be entirely missed by modern methods of high-throughput screening. This is because there is no consistent in vitro effect of treatment of the bacteria with pyrazinamide unless the pH is low and the inoculum size is small. Nonetheless pyrazinamide (like nicotinamide) has a substantial impact in mouse models of TB infection and appears to have a unique impact on the sterilization of murine lesions that other TB drugs lack [22]. Pyrazinamide appears to have been first used in animals in an attempt to circumvent the rapid metabolism of the vitamin nicotinamide while preserving the activity of this compound in animals that was first noted in lepratomous rats by Chorine [16] in 1945. Although there was a slight in vitro effect of nicotinamide (and nicotinic acid), what possessed Chorine to attempt leprosy therapy in animals with nicotinamide remains unclear. The same phenomenon was independently observed and reported several years later by Kushner et al. [15] and McKenzie et al. [23] of Lederle Laboratories, the team that would ultimately produce the ‘analog’ pyrazinamide.

Rifampicin

Rifamycin was discovered by the Italian company Lepetit SPA in Milan in 1959 emerging from a screening program of fermentation broth of soil bacteria [24–26]. Originally isolated as Rifamycin ‘B’ from the culture broth of *Streptomyces mediterranei*, the antibiotic activity actually came from a degradation product later designated Rifamycin ‘S’ [27]. Rifamycin S by itself was not suitable for therapeutic use but could be transformed by reduction into Rifamycin SV which retained the antibacterial activity, showed low toxicity, and had both good stability and acceptable water solubility [28]. The modern history of the rifamycins revolves around the many semisynthetic derivatives of the parent natural compound, some of which have vastly different antibacterial

spectra, pharmacokinetic parameters, and toxicity profiles [27]. The derivative used currently (known as rifampicin) is a hydrazone derived from 3-formyl Rifamycin SV that was selected based upon good oral bioavailability, Rifamycin SV being only useful with parenteral administration [26]. Many derivatives with various properties have been produced recently including such notable additions as Rifabutin [29–31], Rifapentine [32, 33], and Rifalazil [34]. The future potential of semisynthetic rifamycins continues to generate interest particularly with an eye to developing intermittent dosing formulations with low toxicity and few interactions with agents used to treat other infections, particularly HIV.

Ethambutol

Ethambutol was developed by Ray Wilkinson and colleagues [35] at the Lederle Laboratories in the 1950s from a hit obtained in a screen against TB of a subset of their compound library. The original lead molecule was *N*,*N*-diisopropylethylenediamine, which had modest activity against the bacteria in vitro. A lead optimization program was conducted at the time that resulted in selection of ethambutol from about 2,000 mostly symmetrical diamines [36]. It was apparent during this program that the size and nature of the alkyl groups on the ethylenediamine nitrogens were critical for determining activity. Small, α-branched alkyl groups were much more active than either longer aliphatic chains, or chains branched at positions other than α [37]. Removal or significant alteration of the basicity of either of the amino groups also resulted in loss of potency with the exception that conversion of the amine to an amide by introduction of a carbonyl in one position of the ethylene linker resulted in partial activity for some analogs [36]. Alteration of the linker region of the molecule was similarly unsuccessful and even lengthening the ethylene unit by a single methylene or introducing a variety of heteroatoms into the chain resulted in loss of activity. Because of this absolute requirement for the 1,2-substituted ethylenediamine pharmacophore and the known propensity of similar diamines to form stable chelates with divalent metal ions such as copper, a series of derivatives were made that incorporated hydroxy groups in positions that might be available to stabilize a potential metal complex [38]. This insight led to the synthesis and evaluation of ethambutol, a 1,2-aminoalcohol that possessed four times the in vivo activity of the lead molecule when prepared chirally pure as the *dextro* isomer from (*S*)-2-amino-1-butanol. Strikingly, the opposite *levo* isomer has less than 1/500th the activity. The active molecule therefore has the same absolute configuration as that of the naturally occurring amino acids. EMB was a useful addition to TB chemotherapy despite a relatively modest MIC of 10 μ*M* in part because of very low toxicity and relatively few side effects.

Current Status of TB Drug Development

Although the basic goals of drug discovery have remained the same since the 1950s, many of the tools available have changed. The solution of the complete genome sequence of TB in 1998 [39] has opened the door to many genome-scale solutions to the problems of target identification and validation, and the introduction of combinatorial techniques in chemistry has improved the efficiency with which highly potent molecules can be identified and brought to preclinical development. Finally, a better understanding of the disease dynamic and improvements in animal models and methods of screening mean more compounds can be evaluated in more relevant assays and should decrease the rate of compound failure.

Genome-Scale Target Selection Strategies

One of the many impacts of the elucidation of the genome sequence of *M. tuberculosis* has been an increased emphasis on the identification and validation of new drug targets [39]. One of the suppositions in such work is that by comparing genome sequences of various organisms drug targets that are especially useful could be easily identified and these could be as narrow-spectrum or as broad-spectrum as desired based upon their phylogenetic distribution [40]. Identification of such targets also relies on the knowledge that interruption of the function of the encoded proteins would represent a lethal event for the organism. There are many methods for identifying the subset of genes within a bacterial genome that are 'essential'. These include identification of conditionally lethal mutations through the isolation of temperature-sensitive mutants [41], targeted gene disruptions of highly conserved genes [42], and large-scale transposon mutagenesis and sequencing strategies to identify genes that cannot be interrupted by transposition [43, 44]. Such strategies have been applied to *M. tuberculosis* and rough maps of the essential genes have been produced [K. Duncan and R. McAdam, pers. commun.]. More sophisticated strategies that rely on replacing the native promoters of genes with a regulatable promoter through random transposition followed by screening for promoter-dependent growth have also been successfully applied to map essential genes in, for example, *Vibrio cholerae* [45].

Although such information is useful for establishing whether interruption of gene function would block the growth of actively replicating organisms, in practice it does not limit the gene list to those targets whose inactivation would eliminate organisms that are not replicating. TB patients do not typically present at the beginning of their illness when bacilli would be actively replicating but rather they seek care at an advanced stage when well-established lesions in their lungs are often filled with replicating and non-replicating bacteria in unknown

microenvironments and in unknown physiological states. Because the infection is already established, the chemotherapeutic problem is not so simple as inhibiting replication but instead involves eliminating all the bacilli regardless of their physiologic state. Identifying 'essential' targets may be a starting point but understanding 'lethal' targets will ultimately prove to be much more important. Such targets would have irreversible consequences for both replicating and non-replicating bacilli or would commit the cell to an irreversible course of action, for example a non-repairable cell wall lesion or programmed cell death [46].

Target Validation and Identification Using Microarrays

Arrays of DNA samples spotted on glass microscope slides that correspond to all 4,000 genes encoded by the chromosome of *M. tuberculosis* are available and are increasingly coming into use as tools for drug discovery. Such arrays can be used to interrogate the global transcriptional response of the organism following treatment with antibiotics or other substances [47–49]. Such information can provide critical insight into the mechanism of action of old and new substances and has been applied successfully to the mechanism of isoniazid action in TB [50]. Importantly, these studies were in good agreement with previous proteomic and biochemical analyses of isoniazid treatment, thereby validating both techniques [51, 52]. This technology has also been applied in fungal infections with success. For example, microarrays have been used in *Candida albicans* to study the mechanism of itraconazole treatment [53]. Other antifungals that target the ergosterol biosynthetic pathway have also been examined in *Saccharomyces cerevisiae* [54]. In this study it was possible to conclude that an unknown novel imidazole derivative had the same mechanism of action by virtue of a concordant transcriptional response with other azoles. We have similarly used microarrays to derive SAR and predict mechanism of action of a series of diamines related to ethambutol and microarray-derived information formed the basis for a high-throughput screen of a combinatorial library of ethambutol analogs [R.E. Lee, M. Protopopova and C.E. Barry, III, unpubl. data].

Microarrays can also provide additional fundamental insights into important metabolic processes that may suggest non-obvious drug targets as well [55]. By examining conditions in vitro that are thought to mirror conditions within human lung lesions, for example hypoxia and nutrient starvation, novel biochemical pathways and targets have been identified in *M. tuberculosis* [56, 57]. The caveat to such studies is the same as that indicated previously with defining 'essential' genes, although the genes may be expressed and necessary for growth under altered physiologic conditions, interruption of their function may not be sufficient to result in the elimination of pre-existing populations already adapted to such conditions.

High-Throughput Screening (HTS) Strategies

Advances in miniaturization and automation have made possible the automated screening of numbers of compounds that were once unthinkable. 96-well plates, formerly the industry standard for screening libraries of compounds, have been replaced by 386-, 1,536- or even higher density plates and typical high-throughput screens in the pharmaceutical industry now screen on average 10,000 compounds per day with some companies aiming at ultra-high-throughput assays (uHTS) that run 100,000 compounds per day [58]. How many compounds are enough? It has been estimated that 10^{12} assays would be required to completely map the SAR of all known therapeutic targets in the human genome [59].

Antibacterials in general, and antituberculars in particular, have not been at the forefront of uHTS programs which have tended to focus on highly reproducible assays of purified proteins for commercially important targets. Although there are some purified enzymes with suitable assays that have been subjected to HTS screens, these have been relatively uncommon for TB [60]. Screening campaigns for new TB agents have focused primarily on whole-cell based assays using indicators of cell viability such as alamar blue reduction or green fluorescent protein [61–63]. Genetically modified reporter strains of TB expressing firefly luciferase have been applied profitably to drug-screening programs [64]. Such genetically modified strains have typically been linked to constitutively expressing promoters such as p*hsp65*, but by selecting for genes that are specifically upregulated in microarrays in response to ethambutol, we have created fusions that respond with high specificity to certain classes of compounds [R.E. Lee and C.E. Barry, III, unpubl. data]. We have used such fusions in 96-well plate assays to run moderate-throughput screens of ethambutol related compounds.

Combinatorial Approaches to Lead Optimization

The advent of split-and-pool combinatorial methods in chemical synthesis has allowed the creation of the raw materials necessary to fuel highly intensive screening efforts such as those described above. Automated synthesis, robotic manipulations and resin-based technologies have come together to produce an environment where medicinal chemists now measure output in terms of numbers of libraries of compounds produced, rather than in numbers of compounds produced. A ‘library’ represents anywhere from two to a million variations around a single chemical scaffold that may have originated from an HTS-like assay, or may be derived from a natural product or an existing (or computationally predicted [65]) molecule with known activity. This is distinct from a ‘collection’ which represents a series of compounds unrelated in their core structure. Focussed libraries are often the second step in the discovery process

following the identification of a lead molecule. Increasingly, concerns regarding the ultimate 'drug likeness' of a molecule have permeated the earliest steps in library construction and library synthesis is often preceded by a filtering out of molecules with characteristics incompatible with oral absorption or rapid metabolism [66, 67].

Although the techniques involved are relatively new, combinatorially driven lead optimization strategies have already been proven to be useful in the design and optimization of antimicrobials. β-Lactams, quinolones and azoles have all been optimized through the use of such techniques, and one of the newest classes of antibacterial agents to be introduced into clinical practice (the oxazolidinones) has likewise been optimized using combinatorial strategies [68, 69]. Against *M. tuberculosis*, combinatorial strategies have been applied to the development of second-generation ethambutol analogs with enhanced potency [R.E. Lee, M. Protopopova and C.E. Barry, III, unpubl. data] [6]. In addition, combinatorial and parallel techniques are currently being applied to the development of derivatives of thiolactomycin to optimize the antitubercular activity of this promising compound class [C. Dowd and C.E. Barry, III, unpubl. data].

High-Information Content Biological Screening

Despite the advances in the ability to predict essential targets, pre-select compounds for favorable indication of activity and bioavailability, and screen compounds *en masse* against a target or a whole organism in HTS or uHTS format, the most substantial limitation to developing a truly active antimycobacterial agent lies in understanding the disease pathophysiology sufficiently to produce a highly active substance that would genuinely shorten and simplify the course of therapy. The linkage of advanced disease models with the new innovations in technology described above represents one of the most important areas being explored in the modern development of antimycobacterial agents.

In vitro models that explore bacterial adaptation to altered environments may provide important clues to guide target selection and provide advanced screening tools. One such model that has been explored by a number of authors revolves around the hypothesis that hypoxic conditions dominate the environment wherein non-replicating bacilli persist in the face of drug therapy [70]. This has led to the development of oxygen-limited in vitro culture as a model for these persistent organisms. There have been dramatic advances in our understanding of the physiology of the organism grown under oxygen limitation with the application of genomic tools such as proteomics and microarrays to organisms cultivated in this way [56, 57]. Such hypoxia-adapted organisms represent a potentially very useful screening resource for the evaluation of agents active against persistent organisms. It has also been hypothesized, based on the

abundance of lipid-degradative enzyme encoded by TB, that the primary carbon source of bacteria within human lung tissue is lipid rather than carbohydrate [39]. An implication of this observation is that a certain enzyme involved in maintaining metabolic balance in carbon flow (an enzyme of the glyoxylate shunt) would be critical to the ability of the organism to persist. This prediction was verified experimentally with one enzyme in the pathway, isocitrate lyase, whose deletion from the chromosome resulted in a TB strain that was unable to persist in infected mice [71]. The X-ray crystal structure of this target has been solved and an in vitro assay of enzymatic activity is available making this a prime candidate for HTS against a target whose function may be critical to persistence [72, 73]. The same caveats apply as above of course, lack of isocitrate lyase activity during the transition to the persistent state may not translate to interruption of the function of this enzyme in organisms that are already adapted.

Screening compounds on the whole organism level has considerable appeal given the uncertainty surrounding the precise environmental conditions to which these difficult to eradicate organisms are subjected. One potentially useful whole-cell model of persistent organisms mimics therapy to some extent by examining bacilli that survive rifampicin killing in stationary-phase, microaerophilic cultures [74]. Although intuitively appealing, this model requires substantial validation to demonstrate that the bacilli produced are both phenotypically tolerant of other drugs and resemble those harbored by chronically infected patients. Other models for whole-cell screening assays might additionally involve simply screening for activity against stationary-phase organisms as enzymes involved in adaptation to this condition have been shown to be essential for long-term survival of the organism [75]. The most relevant screen would of course be to examine persistently infected animals that have achieved control of the infection but will ultimately relapse. Such animal models are available and in fact were developed nearly half a century ago [76–78]. These experiments involve drug treating infected mice to apparent sterilization and then reactivating the ‘persistent’ bacilli by immunosuppression of the animal. Unfortunately this screening system requires a significant investment in time and effort and is limited to an extremely small handful of compounds. Improvements in such relevant animal models are desperately needed to facilitate the examination of the effects of large numbers of compounds on such bacteria.

Conclusions

The development of existing therapeutics for the treatment of TB was responsible for the establishment of many of the principles and techniques currently utilized for drug development in general. Although the current ‘short-course’

chemotherapy is far from short, it has saved millions of TB patients from an otherwise uncertain fate. The most critical goal for improving the cure rate for TB and eradicating this disease is to shorten the course of therapy and thereby simplify the logistical problems associated with enforcing completion of a six month drug regime. There are powerful new tools accessible to scientists working in TB drug development because of the completion of the genome sequence, the revolution in genomic sciences, combinatorial chemistry, and HTS technologies. The choice of which targets to apply these technologies to is absolutely critical if persistent organisms are to be eradicated more quickly. Increasing our understanding of the physiology of those organisms that contribute to relapse is also absolutely critical to facilitate target selection and the development of high-throughput assays that accurately reflect the complexity of the host-pathogen dynamic and can measure the effect of new chemical entities on this dynamic is essential for success.

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